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# *De novo* Cisplatinum Resistance Does Not Influence Cellular Radiosensitivity

**Richard A. Britten and Hilmar M. Warenius**

**The intrinsic sensitivity to 4 MeV photons, and 62.5 MeV (p→Be$^+$) neutrons has been examined in a panel of 11 cultured human cell lines exhibiting a wide spectrum of inherent cisplatinum sensitivity. Irrespective of whether cellular sensitivities to these therapeutic agents were compared at the 10% survival level, relative to the initial portion of the cell survival curves, or to their relative rank order of response, there were no significant correlations between inherent cisplatinum sensitivity and sensitivity to either 4 MeV photon, or 62.5 MeV neutron irradiation. This data raises the possibility that the previously reported decreased radiosensitivity of human tumour cell lines with acquired cisplatinum resistance may be due to the induction of cellular processes which confer resistance to both cisplatinum and ionising radiation, rather than the selection of innately cisplatinum-resistant cells, which are collaterally radioresistant.**
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## INTRODUCTION

THE DEVELOPMENT of cisplatinum resistance in some human tumour cell lines (acquired resistance) is associated with a concomitant reduction in photon sensitivity [1–7]. It is currently unclear how the development of acquired cisplatinum resistance results in reduced radiosensitivity, although analysis of the radiation survival curves of these cisplatinum-resistant lines, using the linear quadratic equation suggests that in comparison to the more chemosensitive parental line, the reduced radiosensitivity of cells which have developed acquired cisplatinum resistance is primarily due to a reduction in the magnitude of the initial slope (α) [5, 6].

There is currently insufficient data to demonstrate whether the reduced radiosensitivity of these platinum-resistant cells is the direct consequence of the altered expression of a single common component in the cellular response pathways to both cisplatinum and radiation, or due to multiple genetic alterations arising from exposure to cisplatinum.

The hypothesis that the reduced photon sensitivity of human cells with acquired cisplatinum resistance is attributable to the increased expression of genes responsible for intrinsic sensitivity to both therapeutic agents would seem to be supported by the close correlation between intrinsic cisplatinum and photon resistance in early passage human tumour cell lines [4, 8].

Genetic manipulation experiments have, however, shown that the cellular responses to photon radiation, and cisplatinum can be differentially modified in radiosensitive mutant CHO cells [9, 10], suggesting that innate cellular sensitivity to cisplatinum and photon irradiation may be independently encoded. Our previous studies [7], have also failed to demonstrate a consistent association between decreased photon sensitivity and the development of acquired cisplatinum resistance in five human tumour cell lines, suggesting that photon and cisplatinum sensitivity in human tumour cells with acquired cisplatinum resistance may also be independently coded. However, the same study did demonstrate a consistent reduction in sensitivity to 62.5 MeV (p→Be) neutrons, suggesting that the reduction in neutron sensitivity in cells with acquired cisplatinum resistance could be due to the induction of cellular processes which confer resistance to cisplatinum, and collateral resistance to neutron radiation.

The differential induction of collateral resistance to 62.5 MeV (p→Be$^+$) neutrons and 4 MeV photons in cells with acquired cisplatinum resistance, also implies that the mechanisms of collateral resistance to these radiations are independent of each other, and may be subject to different regulatory processes. There is, however, no evidence as to whether the reduced neutron sensitivity of these cisplatinum-resistant cells is directly

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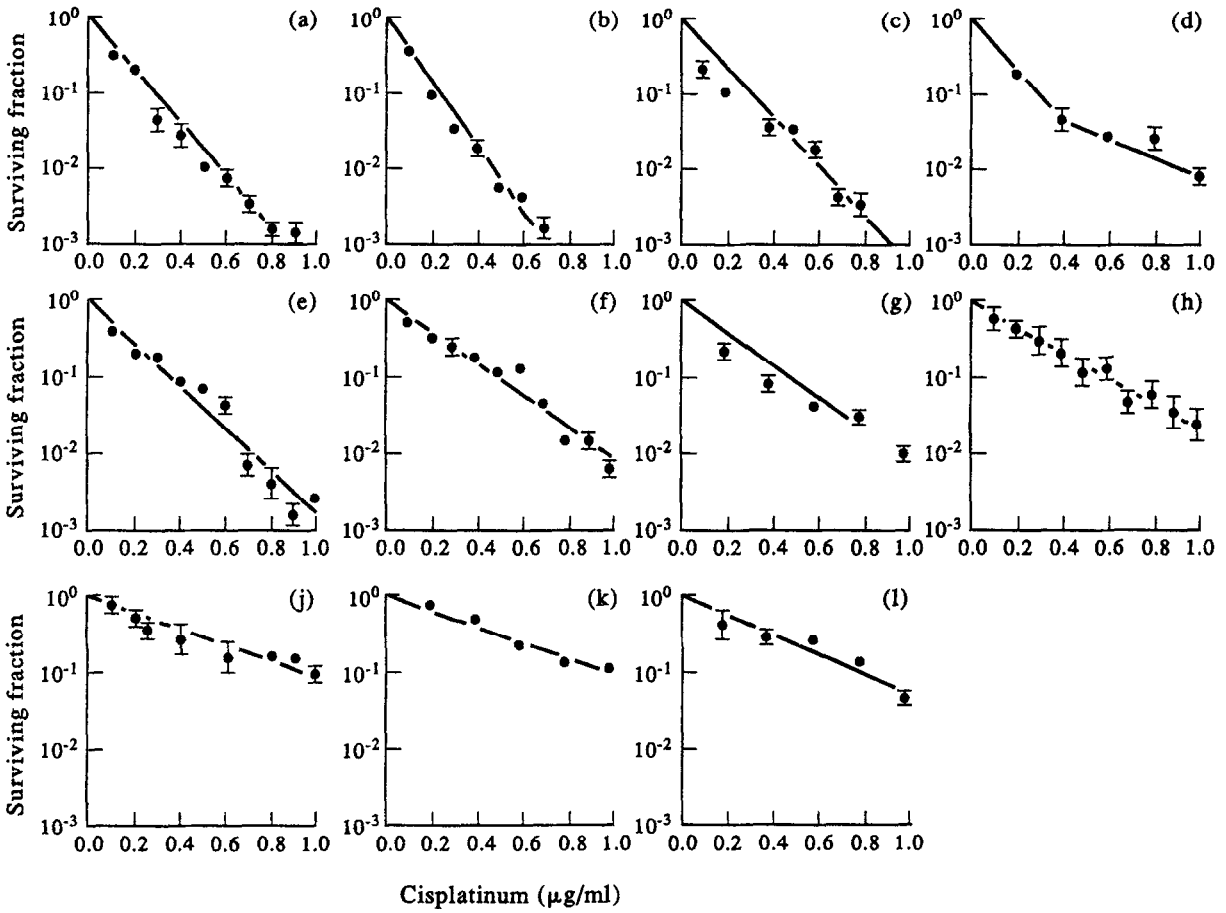


**Fig. 1. The response of (a) KB; (b) NCTC; (c) Hep2; (d) L23-COR; (e) MRC5; (f) HT29/5; (g) 2780; (h) RT112; (j) OAW42; (k) H322 and (l) MOR; cells following cisplatinum exposure. Each point represents the mean and 1 S.E. of at least three experimental data sets, each of three replicates.**

attributable to (a) the development of acquired resistance to both cisplatinum and 62.5 MeV ($p \rightarrow Be^+$) neutron irradiation, or (b) due to the selection of cell sub-populations which are innately resistant to both cisplatinum and 62.5 MeV neutrons. This study has, therefore, established the relative intrinsic sensitivities to cisplatinum, 4 MeV photons and 62.5 MeV ($p \rightarrow Be^+$) neutrons of 11 established human cell lines, five of which constitute the parent cells from which the cisplatinum-resistant cells used in our previous studied [5–7] were developed.

*Table 1. Cisplatinum dose–response curve parameters of previously untreated human cultured cell lines ranked in increasing resistance*

| Cell line | Histological type | $D_{0.1}$(μg/ml) | Figure panel |
|---|---|---|---|
| KB | Oral epidermoid | 0.289 | a |
| NCTC | Normal skin epithelium | 0.242 | b |
| Hep2 | Laryngeal epidermoid carcinoma | 0.312 | c |
| L23-COR | Non-small cell lung cancer | 0.303 | d |
| MRC5 | Transformed fibroblast | 0.364 | e |
| HT29/5 | Colorectal adenocarcinoma | 0.502 | f |
| 2780 | Ovary | 0.484 | g |
| RT112 | Bladder | 0.625 | h |
| OAW42 | Ovary | 0.932 | j |
| H322 | Small cell lung cancer | 0.981 | k |
| MOR | Small cell lung cancer | 1.069 | l |

## MATERIALS AND METHODS

*Cell lines and cultures*

Eleven human cell lines were used in this study. Nine of these were derived from tumours, whilst the other two lines consisted of transformed fibroblast (MRC5) and normal epithelial (NCTC 2544) cells. All these cell lines were routinely maintained as monolayer cultures, in RPMI 1640 medium, with the exception of OAW42, KB, Hep2, HT29/5 and MRC5, which were grown in Dulbecco's modified Eagles medium (DMEM), and NCTC 2544, which was maintained in Ham's F-12 medium. All lines were grown in the presence of 10% heat-inactivated fetal calf serum. Cell cultures were passaged every 2–3 days to ensure exponential growth.

*Chemosensitivity testing*

Cells from stock cultures were detached using trypsin–versene, centrifuged, resuspended in the appropriate complete medium (previously equilibrated to 37°C), and diluted to the final required density. Cells were plated at densities ranging from $10^2$ to $10^5$ cells in 60 mm petri dishes, and incubated at 37°C (5% $CO_2$) for at least 4 h, before cisplatinum was added, during which time there was less than 1% multiplicity. Cell cultures were then incubated in the dark at 37°C, in the presence of cisplatinum for 2 h, after which the medium was removed, the cultures washed twice in complete medium, and fresh medium added. Cultures were then incubated for 13 days in humidified 5% $CO_2$ at 37°C, colonies fixed in 70% ethanol,

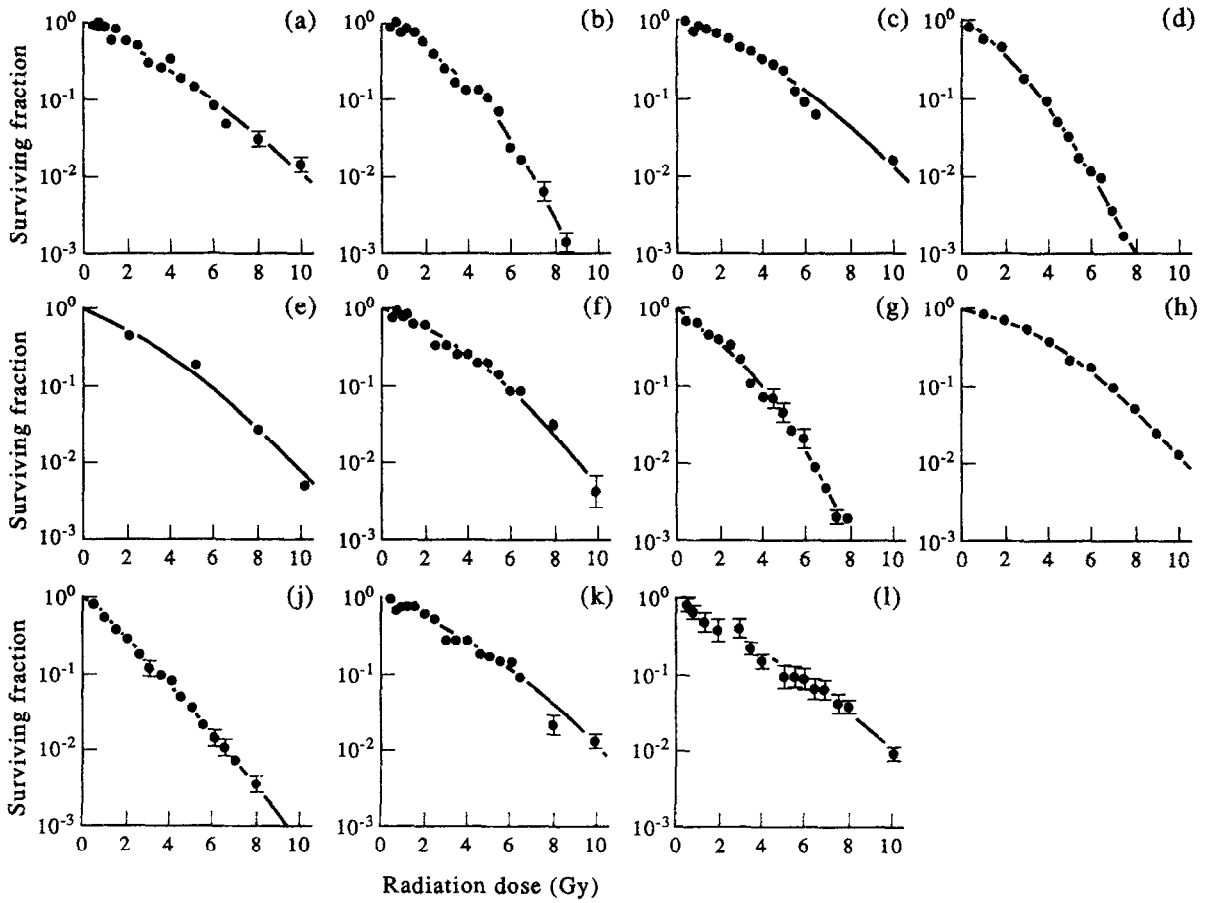


**Fig. 2. The survival curves of (a) KB; (b) NCTC; (c) Hep2; (d) L23-COR; (e) MRC5; (f) HT29/5; (g) 2780; (h) RT112; (j) OAW42; (k) H322 and (l) MOR; cells following acute doses of 4 MeV photon irradiation (dose rate = 2 Gy/min). Each point represents the mean and 1 S.E. of at least three experimental data sets, each of three replicates. Error bars are shown only where they exceed the dimensions of the symbols.**

*Table 2. Acute 4 MeV photon survival curve parameters of previously untreated human cultured cell lines ranked in increasing resistance*

| Cell line | Alpha (Gy-1) | Beta (Gy-2) | $D_{0.1}$ (Gy) |
|---|---|---|---|
| OAW42 | 0.608 (0.025) | 0.012 (0.004) | 3.55 |
| 2780 | 0.363 (0.043) | 0.056 (0.006) | 3.95 |
| L23-COR | 0.366 (0.047) | 0.063 (0.007) | 3.79 |
| NCTC | 0.179 (0.058) | 0.066 (0.011) | 4.66 |
| MOR | 0.394 (0.028) | 0.005 (0.004) | 5.49 |
| MRC5 | 0.283 (0.069) | 0.021 (0.007) | 5.72 |
| KB | 0.313 (0.035) | 0.014 (0.005) | 5.86 |
| HT29/5 | 0.189 (0.028) | 0.034 (0.004) | 5.94 |
| H322 | 0.253 (0.034) | 0.019 (0.004) | 6.15 |
| Hep2 | 0.233 (0.031) | 0.021 (0.004) | 6.36 |
| RT112 | 0.111 (0.023) | 0.033 (0.003) | 6.78 |

and stained with 10% Giemsa, and those with greater than 50 cells counted.

*Irradiation procedure*

Following trypsinisation, cells were suspended at $5 \times 10^4$ ml, in the appropriate culture medium (previously equilibrated to 37°C) supplemented with 10 mmol/l Hepes, aliquoted into 1.8 ml cryotubes (Nunc), and incubated for at least 30 min at 37°C in a 5% $CO_2$ atmosphere before being irradiated by either photons from a 4 MeV linear accelerator at does rate of 2.0 Gy min, or neutrons from a beryllium target bombarded with 62.5 MeV cyclotron-accelerated protons, at a dose rate of 0.5 Gy min. The same experimental set-up was used to irradiate the cells with both photon and neutron irradiation, with the exception that the thickness of dose build-up material facing the beam was 1 cm for photons, and 2.0 cm for neutrons. Following irradiation, exponentially growing cells were seeded at densities of between $10^2$ and $5 \times 10^4$ in 60 mm petri dishes, and incubated in Ham's F12 medium supplemented with 10% heat-inactivated serum, and incubated for 12–14 days at 37°C (5% $CO_2$). Colonies were fixed, stained and counted in the same manner as described for chemosensitivity assays.

*Data handling and presentation*

The experimental data were analysed using the linear-quadratic equation:

$$S = e^{-\alpha D - \beta D^2}$$

where $S$ is equivalent to the surviving fraction at a dose $D$ and $\alpha$

and β are constants. The data were fitted to a linear-quadratic function using the non-linear regression program of the AGPAD package. The derived parameter $D_{0.1}$ was obtained from the fitted curve. In addition to assessing the intrinsic sensitivity of the human cells to cisplatinum and ionizing radiation at the isoeffect doses producing 10% cell survival ($D_{0.1}$), the relative sensitivity to these therapeutic agents has been assessed as a function of the initial slope (α) of the survival curve. Such a comparison has two advantages, the first being clinical relevance, the second avoiding any artefacts introduced from comparing curvi-linear radiation survival curves with the straight exponential cisplatinum curves, and from possible multiplicity.

## RESULTS

The survival curves of the 11 human cell lines following cisplatinum exposure are depicted in Fig. 1(a–l). With the exception of the L23-COR line, the responses of all the lines were characterised by single exponential survival curves. Linear regression analysis of the data yielded $D_{0.1}$ isoeffect doses ranging from 0.24 to 1.07 μg/ml (Table 1). The L23-COR line exhibited a biphasic response to cisplatinum. Linear regression analysis over the dose range from 0 to 0.4 μg/ml (L23-COR) yielded $D_{0.1}$ values of 0.303 μg/ml, whilst similar analysis of dose points above 0.4 μg/ml, resulted in a $D_{0.1}$ value of 0.924 μg/ml; these were not comparable to $D_{0.1}$ values obtained in the cisplatinum-resistant L23-COR/CPR cell line [7], which suggests that either adaptive cisplatinum resistance was induced in the innately cisplatinum-resistant subpopulation, or the selection of an even smaller innately resistant subpopulation.

The cell survival curves of the 11 human cell lines following irradiation from a 4 MeV linear accelerator (Fig. 2) were analysed using the linear quadratic equation; the derived parameters of these acute survival curves are presented in Table 2. The magnitude of the initial slope (α) varied by 5-fold, whilst there was a 2-fold variation in the $D_{0.1}$ isoeffect dose between the most radiosensitive and resistant cell lines.

The majority of the survival curves following 62.5 MeV neutron irradiation were curvi-linear (Fig. 3), and characterised by a significant β component (Table 3), which ranged from 0.001 to 0.073 $Gy^{-2}$. The inherent cellular sensitivity to 62.5 MeV neutrons varied by 1.5-fold when assessed at the 10% survival level ($D_{0.1}$), whilst the magnitude of the α varied by 1.9-fold.

The innate sensitivities of the human cell lines to cisplatinum, photon and neutron irradiation were compared at the 10% survival level ($D_{0.1}$) (Fig. 4a, b), and relative to the exponential, linear portion of the survival curves (α) (Fig. 4c, d). Irrespective of the end-points chosen, there were no significant correlations between innate cisplatinum sensitivity, and either innate photon or neutron sensitivity. In these cell lines there was also no significant correlation between the intrinsic sensitivities to photons and neutrons, when assessed relative to $D_{0.1}$ doses or the magnitudes of α.

Similarly, if the cells were ranked in order of their innate

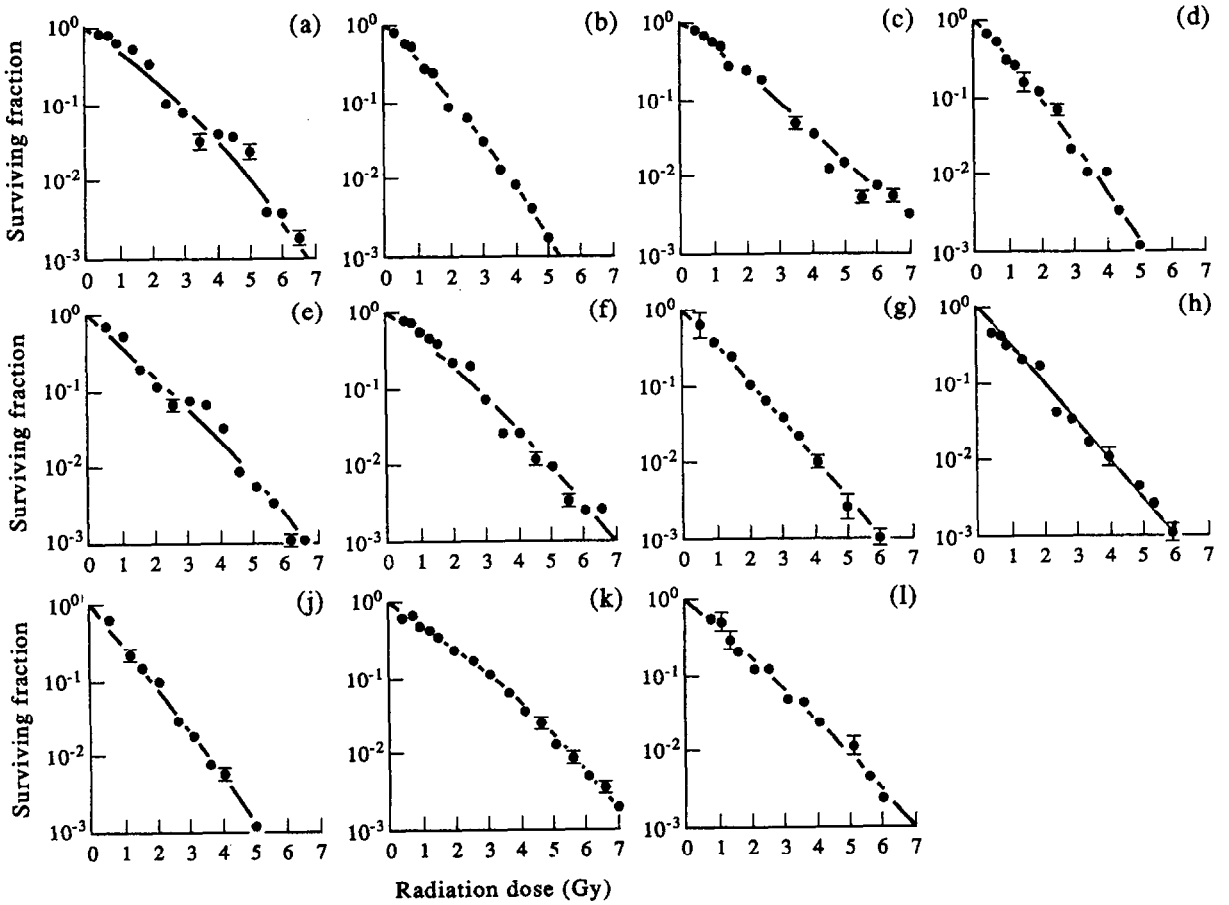


**Fig. 3. The survival curves of (a) KB; (b) NCTC; (c) Hep2; (d) L23-COR; (e) MRC5; (f) HT29/5; (g) 2870; (h) RT112; (j) OAW42; (k) H322 and (l) MOR; cells following acute doses of 62.5 MeV (p→$Be^+$) neutron irradiation (dose rate = 0.5 Gy/min). Each point represents the mean and 1 S.E. of at least three experimental data sets, each of three replicates. Error bars are shown only where they exceed the dimensions of the symbols.**

*Table 3. Acute 62.5 MeV (p→Be⁺) neutron survival curve parameters of previously untreated human cultured cell lines ranked in increasing resistance*

| Cell line | Alpha (Gy-1) | Beta (Gy-2) | $D_{0.1}$ (Gy) |
|---|---|---|---|
| L23-COR | 1.216 (0.085) | 0.018 (0.015) | 1.84 |
| NCTC | 0.929 (0.073) | 0.073 (0.018) | 2.12 |
| 2780 | 1.096 (0.054) | 0.014 (0.011) | 2.06 |
| OAW42 | 1.276 (0.106) | 0.013 (0.027) | 1.77 |
| RT112 | 1.189 (0.077) | <0.001 | 2.00 |
| MRC5 | 0.836 (0.144) | 0.036 (0.027) | 2.47 |
| HT29/5 | 0.744 (0.088) | 0.042 (0.017) | 2.69 |
| MOR | 0.944 (0.067) | 0.009 (0.014) | 2.36 |
| Hep2 | 0.867 (0.037) | <0.001 | 2.65 |
| H322 | 0.689 (0.027) | 0.032 (0.005) | 2.94 |
| KB | 0.594 (0.116) | 0.066 (0.022) | 3.02 |

sensitivity to each modality, and compared using the Mann–Whitney test, there was no significant correlation between cisplatinum sensitivity and radiosensitivity, or between photon and neutron sensitivity.

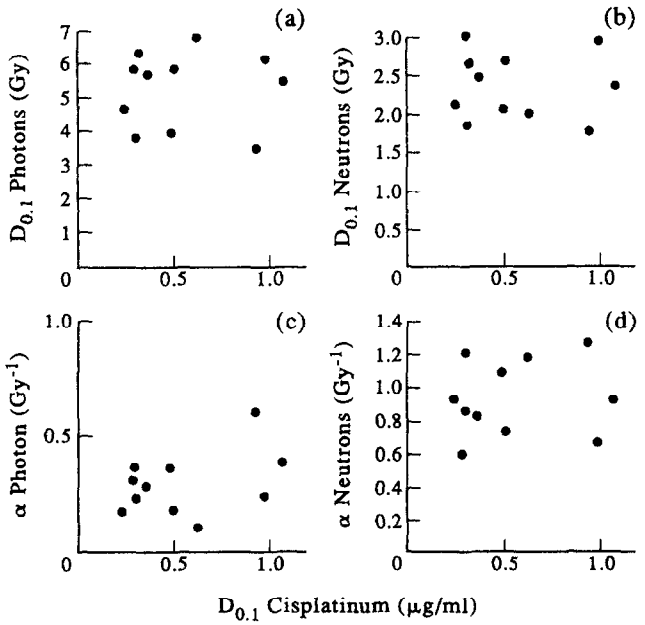


**Fig. 4. The relationship between the intrinsic sensitivities of the 11 human cell lines to cisplatinum and ionising radiation, when amended at the 10% ($D_{0.1}$) survival level: (a) 4 MeV photons and (b) 62.5 MeV (p→Be⁺) neutrons; or relative to the initial slope (α): (c) 4 MeV photons and (d) 62.5 MeV (p→Be⁺) neutrons.**

## DISCUSSION

In the cell lines studied in this paper, there appears to be no significant correlation between innate cisplatinum resistance and inherent sensitivity to either 4 MeV photons or 62.5 MeV (p→Be⁺) neutrons. The lack of correlation between the inherent sensitivities to these therapeutic agents suggests that the reduced radiosensitivity of clinical tumours [11–13] and cultured human tumour cells with acquired cisplatinum resistance [1–7], is probably not due to the selection of a subpopulation of innately cisplatinum-resistant cells, which are collaterally resistant to radiation; rather, acquired radiation cross-resistance may be due to a concomitant induction of radioprotective mechanisms during the development of acquired cisplatinum resistance.

However, in view of the lack of correlation between innate cisplatinum sensitivity, and either 4 MeV photon or 62.5 MeV neutron sensitivity, our previous finding that the development of acquired cisplatinum resistance differentially conferred collateral resistance to 62.5 MeV neutrons and 4 MeV photons [7] is extremely interesting. It seems reasonable to conclude that during the development of acquired cisplatinum resistance, at least two radioprotective pathways may be induced, which independently confer protection to 62.5 MeV neutrons and 4 MeV photons. Whether the induction of these radioresistant phenotypes is the result of transcriptional activation of two or more methylated silent genes, or due to multiple genetic alterations is not currently known.

Combined modality therapy has been advocated as a possible means of improving survival rates, and assumes that there is no interaction between temporally separated courses of the diverse cytotoxic agents. In such circumstances, the sequence in which the chemotherapy and radiotherapy are administered should not be important, although some therapeutic advantage may be gained by the use of induction chemotherapy, prior to radiotherapy, due to the reduced hypoxic fraction and treatment field size following regression. However, since it appears that the development of acquired chemoresistance is accompanied by a marked decrease in radiosensitivity [1–7] it may be advisable to administer radiotherapy first, at least in historically radioresponsive tumours. However, it should be noted that there are some reports whereby prior exposure of human tumour cells to radiotherapy has resulted in a decreased sensivitity to a wide number of chemotherapeutic agents [14, 15].

Clearly, the effective use of combined cisplatinum/XRT therapy will depend upon a better clinical knowledge of the occurrence of innate and acquired cisplatinum resistance in human tumours, and the characterisation of the processes responsible for cisplatinum resistance in these different situations.

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# Vinca Alkaloids: Anti-vascular Effects in a Murine Tumour

**S.A. Hill, S. J. Lonergan, J. Denekamp and D. J. Chaplin**

**We have investigated the blood flow modifying effects of the vinca alkaloids, vincristine and vinblastine in the murine carcinoma CaNT. Vinblastine at doses of 7.5 or 10 mg/kg induced profound and chronic reductions in tumour blood flow as measured by $^{86}$RbCl extraction. Following the maximum tolerated dose of 10 mg/kg, blood flow was reduced to 10% of pretreatment values after 2 h and remained below 20% of pretreatment values 24 h after drug administration. These findings are consistent with the early induction of necrosis by vinblastine and suggest that vascular-mediated cell death may account for a large part of the 11 day growth delay induced by this drug dose. In contrast to the large reductions in tumour blood flow, in skin, kidney, liver and muscle, blood flow reductions did not, at any time examined, exceed 40%. In all the normal tissues studied, blood flow had fully recovered by 6 h after vinblastine administration. Similar results, albeit less pronounced, have been obtained with vincristine at the maximum tolerated dose of 3 mg/kg. The results clearly show that both vinblastine and vincristine can induce, with some selectivity, a dramatic and prolonged reduction in tumour blood flow and that this may contribute to the anti-tumour effects against the CaNT tumour.**
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## INTRODUCTION

THE CONCEPT of attacking tumours indirectly via their vascular supply is receiving increasing attention as experimental studies reveal that many therapies can produce transient or permanent changes in tumour vascular function [1]. A prolonged blood flow reduction can lead to substantial tumour cell death as a result of induced ischaemia [2]. A reduction in the supply of oxygen and other nutrients will also result from a disrupted vascular supply. The ensuing increase in the levels of hypoxia and acidity within the tumour might be exploitable by the addition of bioreductive or chemotherapeutic drugs.

Therapies already identified as mediating their action, at least in part, via damage to the tumour vasculature include hyperthermia, photodynamic therapy and the cytokines TNFα (tumour necrosis factor), and interleukin 1 [3–6]. Flavone acetic acid (FAA) has also been found to have pronounced effects on vascular function in many experimental tumours [7–10], although this agent has not been shown to have clinical activity [11]. In view of the potential benefits of selectivity damaging tumour vasculature, there is a need to identify other agents which might mediate their effects in this manner.

To date, little attention has been paid to the possibility of the conventional chemotherapeutic drugs having a vascular

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