Anti-Cancer Drugs 2000, **11,** pp. 193–200

Preclinical report

Low-dose twice-daily fractionated X-irradiation of ovarian tumor cells *in vitro* generates drug-resistant cells overexpressing two multidrug resistance-associated proteins, P-glycoprotein and MRP1

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Failure of chemotherapy is frequently observed in patients previously treated with radiotherapy. To establish a cellular model for examining this resistance phenotype a series of mammalian tumor cell lines were exposed *in vitro* to fractionated X-irradiation and were then shown to express resistance to multiple antitumor drugs, including vincristine, etoposide and cisplatin. In these experiments the radiation was delivered as 10 fractions of 5 Gy (dose resulting in 1 log cell kill) given intermittently over several months. We now report that a comparable multidrug-resistance profile is expressed by human SK-OV-3 human ovarian tumor cells exposed *in vitro* to low dose (2 Gy) twice-daily fractions of X-rays given for 5 days on two consecutive weeks, essentially mimicking clinical practice, involving an overexpression of two MDR-associated proteins, P-glycoprotein and the multidrug resistance protein 1 (MRP1), with the latter being readily detectable by immunocytochemistry. [© 2000 Lippincott Williams & Wilkins.]

Key words: Fractionated X-irradiation, drug resistance, MRP1, P-glycoprotein, ovarian cancer.

Introduction

Clinical drug resistance has been identified in certain subsets of patients following their treatment not only with chemotherapy, but also after radiotherapy, as reviewed earlier.[1] These observations led to our original proposal that exposure to radiation could 'induce' or 'select' for drug resistance and thus subsequent chemotherapy would provide a positive selection pressure for these resistant tumor cells resulting in the outgrowth of a drug-resistant cell population.

Establishing an *in vitro* model system to investigate this hypothesis we observed that exposure of two rodent and four human tumor cell lines to fractionated X-irradiation resulted in the expression of resistance to multiple drugs in the surviving populations.[2–5] Subsequently in irradiated Chinese hamster ovary[5–7] and SK-OV-3 human ovarian carcinoma[8] sublines this resistance was associated with increased expression of a functional P-glycoprotein (P-gp), thus providing a biological basis for the clinical problem of drug resistance that can occur in previously irradiated tumors. Furthermore, this multidrug-resistant (MDR) phenotype of the human ovarian tumor cells was retained *in vivo* on their xenografting into nude mice.[9]

Further support came from a concurrent study by Mattern *et al.*,[10] who pre-exposed human lung cancer xenografts to fractionated X-irradiation *in vivo* and reported their resistance to Vinca alkaloids and overexpression of P-gp. Then, more recently, Harvie *et al.*[11] reported increased MRP expression associated with resistance to anthracyclines and etoposide in a human leukemic subline treated *in vitro* with fractionated γ-radiation.

It was proposed that these cellular models, although few in number, could be useful in studying further the resistance phenotypes developed in response to radiation exposure. However, a criticism leveled at our initial

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studies was that although the total radiation dose administered *in vitro* was within the same range as clinical radiation therapy protocols, the fractionation procedure used was completely different and thus might influence the results obtained. To counter this criticism the present study was initiated using SK-OV-3 ovarian cells and employing a low-dose (2 Gy) twice-daily fractionated X-irradiation schedule on five consecutive days for two consecutive weeks, as adopted in various clinical programmes. We report here that the resultant SK-OV-3/DXR-20x2 subline showed marked resistance to multiple standard anticancer agents, including vinorelbine, etoposide and cisplatin, associated with the overexpression of two MDR-associated proteins, P-gp and multidrug resistance protein 1 (MRP1).

Materials and methods

Cell lines

The human ovarian carcinoma parental (P) cell line, SK-OV-3, and X-ray-pretreated sublines, designated SK-OV-3/DXR-10 and SK-OV-3/DXR-20x2, were used. The DXR-10 subline was derived[4] by exposing logarithmically growing cultures intermittently over 1–2 week periods to 10 fractions of 5 Gy (dose reducing survival by 1 log). The newly derived DXR-20x2 subline was established following twice-daily (morning and afternoon) exposures to 2 Gy fractions, Monday to Friday on two consecutive weeks. This protocol attempted to more closely mimic widely used clinical radiotherapy scheduling. Between each X-ray dose fresh culture medium was added, with the surviving population, which was not cloned, being allowed to repopulate and resume logarithmic growth, generating the subline DXR-20x2. The total radiation doses administered, i.e. 50 and 40 Gy, were within the range of clinical radiation protocols for ovarian cancer.[4] These cell lines were maintained in Dulbecco's modification of Eagle's medium plus 10% fetal calf serum (FCS) (Gibco-Biocult, Glasgow, UK or Gibco/BRL, Gergy Poutoise, France).

The human small cell lung cancer cell line GLC_4 and its doxorubicin (Adriamycin)-resistant subline termed GLC_4/ADR[12], gifts from Dr EGE de Vries (Division of Medical Oncology, University Hospital, Groningen, The Netherlands), were cultured in RPMI 1640 medium (Gibco/BRL) supplemented with 10% FCS.

Cytotoxicity assays

Etoposide and vinorelbine were provided by Pierre Fabre Medicament (Castres, France); cisplatin, doxorubicin and 5-fluorouracil were purchased from Sigma (St-Quentin Fallavier, France); methotrexate was obtained from Janssen Chemica (Geel, Belgium). Etoposide was solubilized in dimethylsulfoxide (DMSO), cisplatin in 0.9% NaCl, but water was used as solvent for all the other test compounds.

Cytotoxicity was determined using a standard 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT)-based colorimetric assay, with minor modifications, as detailed earlier.[13] Logarithmically growing cells were exposed to a range of drug concentrations for a 72 h incubation period before MTT (Sigma) addition. IC_{50} values, i.e. concentration of test compound required to reduce absorbance to 50% of that of controls, were determined from replicates of independent experiments, based on linear interpolation between data points.

Glutathione (GSH) levels and total activities of glutathione-S-transferase (GST)

Total GSH contents and GST activities were measured in cell lysates of logarithmically growing cells using the glutathione reductase recycling method of Griffith[14] or the procedure of Habig and Jackoby,[15] with 1-chloro-2,4-dinitrobenzene (Sigma) as substrate. Values normalized for cellular protein content[16] are expressed as the mean $\pm$ SE from two to five separate experiments.

Immunohistochemical detection of P-gp

Immunocytochemical studies were carried out on cytospins of each of the three ovarian tumor cell lines using two monoclonal antibodies which specifically recognize the *mdr-1*-encoded protein, P-gp, i.e. Mab 6/1C (BioResearch Ireland, Enterprise Ireland, Dublin, Ireland) and JSB-1 (Serotec, Oxford, UK).

The method of Hsu *et al.*[17] using an avidin–biotin horseradish peroxidase (HRP)-conjugated kit plus an appropriate secondary antibody (Dako, Cambridge, UK) was followed. Briefly, using room temperature conditions throughout, cytospin preparations were fixed for 1 (for mAb 6/1C) or 10 (for mAb JSB-1) min in ice-cold acetone and allowed to air-dry for at least 15 min prior to immunostaining. Endogenous peroxidase activity was quenched by placing cytospins in 0.6% (v/v) H_2O_2/methanol for 5 min. Cytospins were then blocked for non-specific staining with 20% (v/v) normal rabbit serum/TBS (0.05 M Tris–HCl, 0.15 M NaCl, pH 7.6) for 20 min. Antibody 6/1C was applied to each sample (1/40 dilution in TBS/0.1% Tween 20) and JSB-1 (10 μg/ml in TBS/0.1% Tween 20) for 2 h. This was followed by a 30 min incubation with biotinylated rabbit anti-mouse IgG (1/300 dilution in TBS/0.1% Tween 20). Finally, avidin–biotin HRP-

conjugated was applied for 25 min. Peroxidase substrate was then applied, i.e. 3,3-diaminobenzidine tetrahydrochloride (Dako) containing 0.02% H_2O_2 for 10–15 min at room temperature. All slides were washed after each incubation in three changes of TBS/0.05% Tween 20 within 15 min. Cytospins were lightly conterstained with Coles hematoxylin, differentiated in 1% acid alcohol and blued in Scott's tap water (Clintech, Clacton-on-Sea, UK). Following dehydration in graded alcohols, slides were cleared in xylene and mounted in DPX (BDH, Lutterworth, UK). Negative control slides were included in which primary antibodies were omitted and replaced by control mouse ascites (Sigma), which was used at the same dilution as mAb 6/1C or irrelevant supernatant used at the same concentration as JSB-1.

Detection of MRP1 by Western blotting

Cellular levels of MRP were quantified by Western blot analyses. Briefly, approximately 20×10^6 logarithmically growing cells were collected using trypsin–EDTA, washed twice in 10 ml cold phosphate-buffered saline containing protease inhibitors (Cocktail Inhibitor Tablets, Boehringer Mannheim, Strasbourg, France), pelleted and then frozen immediately at -80°C. Prior to use, pellets were thawed at room temperature and then refrozen at -80°C for two more cycles, before resuspension in sodium dodecyl sulfate (SDS) sample buffer and boiling. SDS–PAGE was carried out according to Laemmli-style analysis in 7% acrylamide gels. The resolved proteins were transferred onto nitrocellulose membranes (Amersham, Les Ulis, France) and stained with the primary antibody after blocking (5% non-fat milk). The anti-MRP1 antibody MRPr1 (ID Labs, Glasgow, UK) was used at 0.4 mg/ml. HRP-conjugated antibodies (Jackson IR, Immunotech, Marseilles, France) were used as secondary antibodies (diluted 1/5000) proir to an enhanced chemoluminescence detection procedure (Pierce, Interchim, Montlucon, France).

Detection of topoisomerase IIα and IIβ by Western blotting

Logarithmically growing cells were trypsinized, washed twice in PBS containing 0.1 mg/ml aminoethylbenzenesulfonylfluoride (Sigma), pelleted and then frozen immediately at -80°C. Details of the subsequent processing of these samples, including SDS–PAGE, transfers onto nitrocellulose membranes and subsequent probing with antibodies, have been published recently.[13] The anti-topoisomerase IIα (Topogen, Columbus, OH) and the anti-topoisomerase IIβ (BioTrend, Koln, Germany) were used at dilutions of 1/5000 and 1/7000, respectively, for 1 h.

Results

In vitro cytotoxicity assays

Logarithmically growing cultures of the three sublines had approximate doubling times in hours of 26 ± 3, 27 ± 5 and 35 ± 4 for the SK-OV-3/P cells and their DXR-10 and DXR-20x2 sublines, respectively. Values for the former two lines are comparable with earlier data,[4] providing evidence of their stability in culture, whilst DXR-20x2 cells had a slower rate, but not significantly so. Dose–response curves following a 72 h exposure to a range of concentrations of six

Table 1. Cytotoxicity data on SK-OV-3/P cells and their DXR-10 and DXR-20x2 sublines

Test compound IC_{50} values	Cell lines		
	SK-OV-3/P	SK-OV-3/DXR-10 [ratio[a]]	SK-OV-3/DXR-20x2 [ratio[a]]
Etoposide	3.2 μM	6.3 μM [$\times 2.0$]	10.6 μM [$\times 3.3$]
Vinorelbine	8.9 nM (plateau at 10–15%)	16.8 nM [$\times 2.0$] (plateau at 35–40%)	320 nM [$\times 36$] (plateau at 50–60%)
Cisplatin	2.6 μM	10.4 μM [$\times 4.0$]	14.5 μM [$\times 5.5$]
Doxorubicin	0.19 μM	0.38 μM [$\times 2.0$]	0.62 μM [$\times 3.2$]
5-Fu	200 μM	NE[b]	200 μM [$\times 1$]
Methotrexate	>200 μM (plateau at 55–60%)	NE	0.72 μM [$< \times 0.003$] (plateau at 30–35%)

[a]Ratio of IC_{50} value of subline relative to the IC_{50} value of the parental line=resistant index.
[b]Not evaluated.

clinically useful antitumor agents were derived, and IC_{50} values interpolated and listed in Table 1. Under these conditions, evidence of low levels (2- to 4-fold) of resistance was obtained in the DXR-10 cells to etoposide, vinorelbine, cisplatin and doxorubicin. This resistance profile is similar to that described earlier using a 24 h exposure and monitoring cytotoxicity by clonogenic assay,[4] with the exception of doxorubicin and the testing of vincristine, rather than vinorelbine. In the newly derived DXR-20x2 subline overall higher levels of resistance were identified to etoposide, vinorelbine, cisplatin and doxorubicin, ranging from 3.2- to 36-fold (*cf.* also Figure 1). This effect was particularly marked with vinorelbine, with evidence not only of a higher IC_{50} value, but also a markedly different level at which the dose–response curves plateaued (Figure 1A), a phenomenon associated with a significant refractory population. These DXR-20x2 cells though showed no resistance to 5-fluorouracil (5-FU), yet proved collaterally sensitive to methotrexate, with a markedly lower IC_{50} value and a lower plateau level of the dose–response curve relative to the parental cells (data not shown). In earlier studies responses to methotrexate and to 5-FU were unaltered in these DXR-10 cells relative to the parental cells.[4]

Therefore *in vitro* exposure of a human ovarian tumor cell line to twice-daily fractionated X-irradiation over 2 weeks resulted in the emergence of a subline expressing significant resistance to etoposide, vinorelbine, doxorubicin and cisplatin.

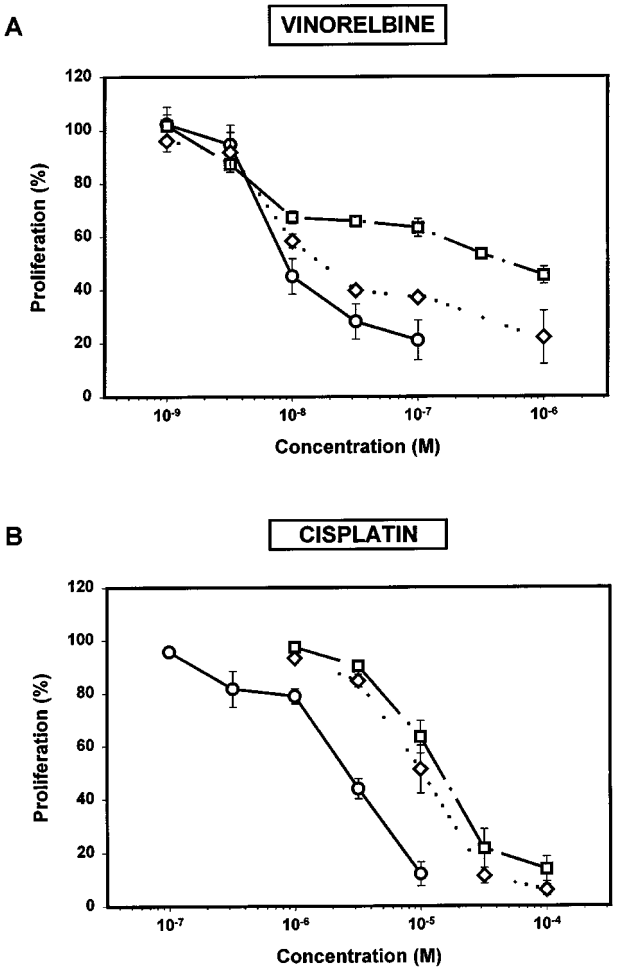

Figure 1. Dose–response curves of SK-OV-3/P (solid line), DXR-10 (dotted line) and DXR-20x2 (dotted and dashed line) cells following a 72 h *in vitro* incubation with a range of concentrations of either vinorelbine (A) or cisplatin (B), assessed by a standard MTT assay. Values are the mean ± SE (bars) of two to four experiments.

Investigations of associated drug-resistance mechanisms

In the DXR-10 subline resistance to cisplatin was associated with an elevation in GST activity and a reduction in cellular GSH levels.[4] Comparable data are reported here when assaying total GST activities with values of 118 ± 7, 280 ± 29 and 288 ± 12 nmol CDNB/min/mg cellular protein for the SK-OV-3/P cells and the DXR-10 and DXR-20x2 sublines, respectively. Some reduction in total GSH levels were also recorded in the irradiated sublines, relative to the parental cell value of 125 ± 22 nmol/mg cellular protein, but these were only marginal (i.e. less than 2-fold).

Resistance to etoposide and to vincristine in the DXR-10 subline has been associated with P-gp overexpression.[8] Immunocytochemical studies were carried out on cytospins of each of the three lines using two monoclonals which specifically recognize the *mdr-1*-encoded protein, P-gp, i.e. the 6/1C anti-P-170-specific antibody and the JSB-1 antibody. Data relating to use of the mAb 6/1C antibody are shown in Figure 2. Figure 2(A) illustrates the fact that the parental SK-OV-3 cell line does not express any detectable P-gp, essentially confirming our earlier findings using Western blotting.[8] Figure 2(B) shows intense membrane staining for P-gp, with some associated cytoplasmic staining, consistent with the strong inner plasma membrane/cytoplasmic staining reported earlier with this 6/1C antibody.[18,19] The pattern of staining is remarkably uniform throughout this DXR-10 cell population. In Figure 2(C), there is a noticeably higher extent of predominantly membrane staining and the more apparent cytoplasmic staining may be associated with the more punctate nature of the cell membranes of this population of DXR-20x2 cells. The JSB-1 antibody identifies intracellular P-gp[20] and has proved useful in its detection in cells with low levels of drug resistance.[21] The results obtained with this

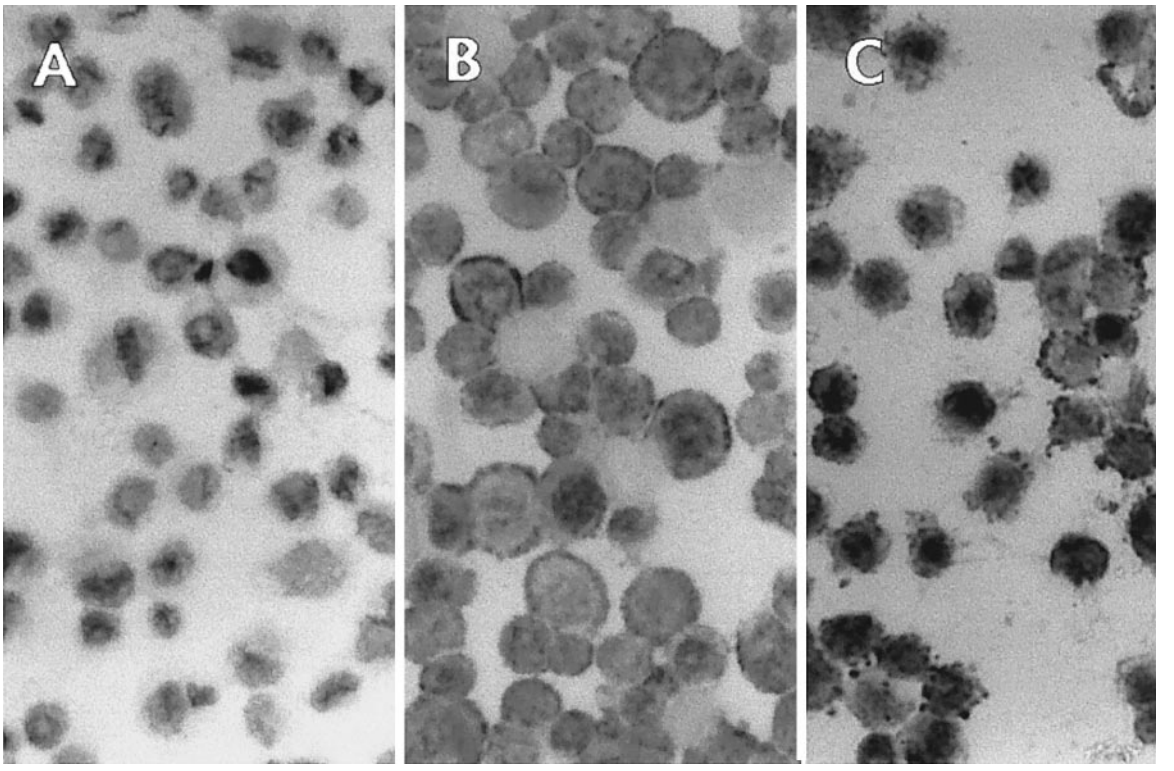

Figure 2. Cytospin preparations of SK-OV-3/P (A), DXR-10 (B) and DXR-20x2 (C) cells stained with the anti-P170-specific antibody 6/1C, showing both cytoplasmic and plasma membrane staining in (B) and (C).

antibody (data not shown) confirmed the parental cells as P-gp-negative and provided evidence of definite positivity in a uniform manner again amongst cells of the DXR-10 subline, whilst more heterogeneity was apparent amongst the DXR-20x2 cells, with some cells though proving strongly positive. This heterogeneity though may merely reflect the fact that the DXR-20x2 subline was more recently developed, whilst the DXR-10 subline has been maintained in culture over several years. Overall these immunocytochemical data clearly illustrate definite overexpression of P-gp in both irradiated sublines. Earlier studies of the DXR-10 sublines had confirmed that the P-gp overexpressed was functional[7] and a comparable susceptibility to verapamil modulation of vinorelbine resistance, as judged by cytotoxicity, was confirmed in these DXR-20x2 cells (data not shown).

More recently another member of the ATP-binding cassette family of transporter proteins, MRP1 (190 kDa), has been implicated in MDR,[22,23] particularly, but by no means exclusively, in human tumor cell lines not overexpressing P-gp.[12,18,24] Western blotting revealed definite overexpression of MRP1 in both the DXR-10 (about $\times 6$) and DXR-20x2 (about $\times 10$) irradiated sublines (Figure 3), thus implicating fractionated X-irradiation as an inducer of MRP1.

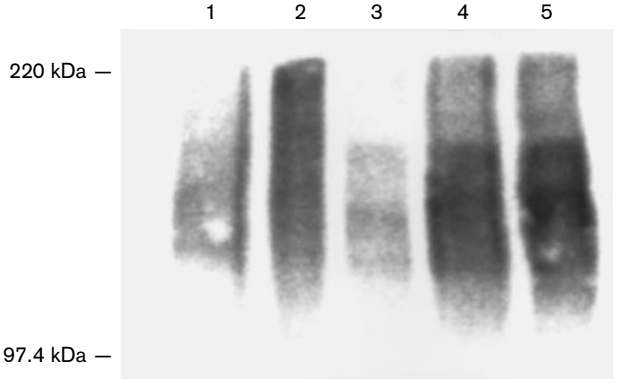

Figure 3. Detection of the 190 kDa protein, MRP1, in membrane preparations of SK-OV-3/P (lane 3), DXR-10 (lane 4), DXR0-20x2 (lane 5) cells, together with GLC4 (lane 1) and GLC4/ADR (lane 2) cells included as respective negative and positive controls. Western blot analysis was carried out using the MRPr1 antibody, as detailed in Materials and methods. Immunoblots were semi-quantiated using a BioRad (Ivry sur Seine, France) Molecular Imager.

Resistance to etoposide has been associated with reduced expression of topoisomerase II (*cf.* review by Beck *et al.*[25]). Western blot analyses of whole cell extracts prepared from the SK-OV-3/P, DXR-10 and

DXR-20x2 cell lines revealed comparable levels of both topoisomerase IIα and IIβ (data not shown).

Discussion

The expression of an MDR phenotype results following exposure of human ovarian carcinoma cells to a series of 2 Gy dose fractions repeated twice daily, for five consecutive days over 2 weeks. This study not only confirms earlier reports[2–4,8–11] that drug resistance can be induced or selected for in experimental systems exposed *in vitro* or *in vivo* to X- or γ-radiation, but also clearly shows that use of a fractionated protocol, essentially mimicking that adopted clinically, also results in the expression of this resistant phenotype in the surviving tumor cell population.

This recently derived SK-OV-3/DXR-20x2 subline proved resistant to etoposide, vinorelbine and doxorubicin, drugs implicated in the so-called 'classic' MDR phenotype. The levels of resistance expressed though relatively low were clearly associated with very definite and readily detectable expression of P-gp, as demonstrated here using two different antibodies and cytospin preparations. These procedures for P-gp detection could therefore be readily applied to tumor tissue from patients.

The MDR-associated protein, MRP1, was originally identified as being overexpressed in a number of MDR human tumor cell lines which did not overexpress P-gp.[22,26–28] However, an extensive study by Izquierdo *et al.*[24] provided overwhelming evidence of overlapping phenotypes of MDR drugs in the National Cancer Institute panels of human tumor cell lines. Indeed co-expression of these MDR-associated markers including P-gp, MRP1 and the more recent lung resistance protein (LRP) has now been reported in a series of publications relating to drug-resistant tumor cell lines, for example.[18,29–31] Such co-expression of different resistance markers also now appears common to clinical cancer specimens.[32,33] In both these irradiated SK-OV-3 sublines there was clear overexpression of MRP1, consistent with their resistance to the anthracyclines and to etoposide, considered by Davey *et al.*[34] as characteristic of cells overexpressing MRP. Furthermore, MRP expression has been correlated with resistance to vincristine and this most probably will also apply to vinorelbine, in agreement with the preferential resistance to this drug observed in MRP-transfected cells.[23]

However, several reports[35,36] have shown that MRP1 can act as a GS-X pump involved in detoxification of heavy metals, so implicating cisplatin resistance, although this is disputed.[36–38] These two irradiated SK-OV-3 sublines showed definite resistance to cisplatin (Figure 1B), but whether their overexpression of MRP1 resulted from their resistance to etoposide, doxorubicin and the Vinca alkaloids or to cisplatin, remains unclear. Sharp *et al.*[38] recently claimed a lack of any role for MRP1 in platinum drug resistance in these SK-OV-3 cells, since an MRP1-transfected line proved resistant to doxorubicin, vincristine and etoposide, but not to cisplatin. Furthermore, Harvie *et al.*[11] using the CCRF-CEM human leukemic cell line and fractionated γ-irradiation identified MRP1 expression in the absence of any associated cisplatin resistance.

A decrease in the level of topoisomerase II has been identified in many non-P-gp MDR cell lines,[12] although again this finding is not invariable. In these low level resistant irradiated SK-OV-3 sublines, no reduction in the expression of either the α or the β isoforms of topoisomerase II were detected by Western blotting.

Finally, an unexpected characteristic of the newly derived DXR-20x2 subline was their marked collateral sensitivity to methotrexate, a finding of definite value if shown to have clinical relevance. However, the underlying biochemical mechanism involved remains to be identified.

In conclusion, these data confirm that low-dose twice-daily fractionated X-irradiation of ovarian SK-OV-3 tumor cells *in vitro* generates a cell population expressing detectable levels of resistance to etoposide, vinorelbine, doxorubicin and cisplatin, which overexpresses significant levels of two multidrug resistance-associated proteins, P-gp and MRP1. This experimental model system may be useful for further investigating the optimal usage of radiotherapy and chemotherapy in the clinic.

Acknowledgments

The technical assistance of Valerie Cassabois is gratefully acknowledged and Marain Cabailh is thanked for her secretarial expertise.

References

1. Hill BT. Interactions between antitumour agents and radiation and the expression of resistance. *Cancer Treat Rev* 1991; **18**: 149–90.
2. Hill BT, Deuchars K, Hosking LK, Ling V, Whelan RDH. Overexpression of P-glycoprotein in mammalian tumor cell lines after fractionated X irradiation *in vitro*. *J Natl Cancer Inst* 1990; **82**: 607–12.
3. Hill BT. Differing patterns of cross-resistance resulting from exposures to specific antitumourdrugs or to radiation *in vitro*. *Cytotechnology* 1993; **12**: 265–88.

4. Dempke WCM, Shellard SA, Hosking LK, Fichtinger-Schepman AMJ, Hill BT. Mechanisms associated with the expression of cisplatin resistance in a human ovarian tumor cell line following exposure to fractionated X-irradiation *in vitro*. *Carcinogenesis* 1992; **13**: 1209–15.
5. McClean S, Whelan RDH, Hosking LK, *et al*. Characterisation of the P-glycoprotein overexpressing drug resistance phenotype exhibited by Chinese hamster ovary cells following their *in vitro* exposure to fractionated X-irradiation. *Biochim Biophys Acta* 1993; **1177**: 117–26.
6. McClean S, Hosking LK, Hill BT. Dominant expression of multiple drug resistance after *in vitro* X-irradiation exposure of intraspecific Chinese hamster ovary hybrids. *J Natl Cancer Inst* 1992; **85**: 48–53.
7. Hill BT. Evidence of differential regulation of P-glycoprotein overexpression in drug-resistant tumour cells selected by exposure to X-irradiation as opposed to antitumour drugs. *Onkologie* 1996; **19** (suppl 1): 6–9.
8. Hill BT, Whelan RDH, Hurst HC, McClean S. Identification of a distinctive P-glycoprotein-mediated resistance phenotype in human ovarian carcinoma cells after their *in vitro* exposure to fractionated X-irradiation. *Cancer* 1994; **73**: 2990–9.
9. Dempke WCM, Whelan RDH, Hill BT. Expression of resistance to etoposide and vincristine *in vitro* and *in vivo* after X-irradiation of ovarian tumor cells. *Anti-Cancer Drugs* 1992; **3**: 395–9.
10. Mattern J, Efferth T, Volm M. Overexpression of P-glycoprotein in human lung carcinoma xenografts after franctionated irradiation *in vivo*. *Radiat Res* 1991; **127**: 335–8.
11. Harvie RM, Davey MW, Davey RA. Increased MRP expression is associated with resistance to radiation, anthracyclines and etoposide in cells treated with fractionated γ-radiation. *Int J Cancer* 1997; **73**: 164–7.
12. Versantvoort CHM, Withoff S, Broxterman HJ, *et al*. Resistance-associated factors in human small-cell lung-carcinoma GLC4 sub-lines with increasing adriamycin resistance. *Int J Cancer* 1995; **61**: 375–80.
13. Etievant C, Barret J-M, Kruczynski A, Perrin D, Hill Bt. Vinflunine (20′,20′-difluoro-3′,4′-dihydrovinorelbine), a novel *Vinca* alkaloid, which participates in P-glycoprotein (Pgp)-mediated multidrug resistance *in vivo* and *in vitro*. *Invest New Drugs* 1998; **16**: 3–17.
14. Griffith OW. Determination of glutathione and glutathione disulfide using glutathione and 2-vinyl pyridine. *Anal Biochem* 1978; **106**: 207–12.
15. Habig WH, Jackoby WB. Assays for differentiation of glutathione S-transferases. *Methods Enzymol* 1981; **77**: 398–405.
16. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; **72**: 248–54.
17. Hsu SM, Raine L, Fanger H. Use of avidin–biotin–peroxidase complex (ABC) in immunoperoxidase techniques. *J Histochem Cytochem* 1981; **29**: 577–80.
18. Moran E, Cleary I, Larkin AM, *et al*. Co-expression of MDR-associated markers, including P-170, MRP and LRP and cytoskeletal proteins, in three resistant variants of the human ovarian carcinoma cell line, OAW42. *Eur J Cancer* 1997; **33**: 652–60.
19. Moran E, Larkin AM, Doherty G, Kelehan P, Kennedy SM, Clynes M. A new mdr-1 encoded P-170 specific monocolonal antibody: (6/1C) on paraffin wax embedded tissue without pre treatment of sections. *J Clin Pathol* 1997; **50**: 465–71.
20. Scheper RJ, Bulte JW, Brakkee JG, *et al*. Monoclonal antibody JSB-1 detects a highly conserved epitope on the P-glycoprotein associated with mult-drug-resistance. *Int J Cancer* 1988; **42**: 389–94.
21. Broxterman HJ, Pinedo HM, Kuiper CM, van der Hoeven JJM, de La P. Immunohistochemical detection of P-glycoprotein in human tumor cells with a low degree of drug resistance. *Int J Cancer* 1989; **43**: 340–3.
22. Cole SP, Bhardwaj G, Gerlach JH, *et al*. Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. *Science* 1992; **258**: 1650–4.
23. Zaman GJR, Flens MJ, van Leusden MR, *et al*. The human multidrug resistance-associated protein MRP is a plasma membrane drug-efflux pump. *Proc Natl Acad Sci USA* 1994; **91**: 8822–6.
24. Izquierdo MA, Shoemaker RH, Flens MJ, *et al*. Overlapping phenotypes of multidrug resistance among panels of human cancer-cell lines. *Int J Cancer* 1996; **65**: 230–7.
25. Beck WT, Danks MK, Wolverton JS, Kim R, Chen M. Drug resistance associated with altered DNA topoisomerase II. *Adv Enz Reg* 1993; **33**: 113–27.
26. Krishnamachary N, Center MS. The MRP gene associated with a non-P-glycoprotein multidrug resistance encodes a 190-kDa membrane-bound glycoprotein. *Cancer Res* 1993; **53**: 3658–61.
27. Slovak ML, Pelkey Ho J, Bhardwal G, Kurz EU, Deeley R, Cole SPC. Localisation of a novel multidrug-resistance-associated gene in the HT-1080/DR4 and H69AR human tumor cell lines. *Cancer Res* 1993; **53**: 3221–5.
28. Zaman GJR, Versantvoort CHM, Smit JJM, *et al*. Analysis of the expression of MRP, the gene for a new putative transmembrane drug transporter in human multidrug-resistant lung-cancer cell lines. *Cancer Res* 1993; **53**: 1747–50.
29. Feller N, Kuiper CM, Lankelma J, *et al*. Functional detection of MDR1/P170 and MRP/P190-mediated multidrug resistance in tumour cells by flow cytometry. *Br J Cancer* 1995; **72**: 543–9.
30. Hasegwa S, Abe T, Naito S, *et al*. Expression of multidrug resistance-associated protein (MRP), MRD1 and DNA topoisomerase II in human multidrug-resistamt bladder cancer cell lines. *Br J Cancer* 1995; **71**: 907–13.
31. Wyler B, Schneider E, Cianfriglia M, *et al*. Intermittent exposure to doxorubicin *in vitro* selectes for multifactorial non-P-glycoprotein-associated multidrug resistance in RPMI8266 human myeloma cells. *Br J Haematol* 1997; **97**: 65–75.
32. Schuurhuis GJ, Broxterman HJ, Ossenkoppele GJ, *et al*. Functional multidrug resistance phenotype associated with combined overexpression of Pgp/MDR1 and MRP together with 1-β-D-arabinofuranosylcytosine sensitivity may predict clinical response in acute myeloid leukemia. *Clin Cancer Res* 1995; **1**: 81–93.
33. Campling BG, Young LC, Baer KA,. Expression of the *MRP* and *MDR1* multidrug resistance genes in small cell lung cancer. *Clin Cancer Res* 1997; **3**: 115–22.

34. Davey R, Su G, Hargrave R, Harvie R, Baguley B, Davey M. The potential of *N*-[2-(dimethylamino)]acridine-4-carboxamide (DACA) to circumvent three multidrug resistance phenotypes *in vitro*. *Cancer Chemother Pharmacol* 1997; **39**: 424–30.
35. Zaman GJR, Lankelma J, van Tellingen O, *et al*. Role of glutathione in the export of compounds from cells by the multidrug resistance-associated protein. *Proc Natl Acad Sci USA* 1995; **92**: 7690–4.
36. Ishikawa T, Bao JJ, Yamane Y, *et al*. Coordinated indicution of MRP1/GS-X pump and gamma glutamylcysteine synthase by heavy metals in human leukemia cells. *J Biol Chem* 1996; **271**: 14981–8.
37. Moscow JA, Schneider E, Ivy SP, Cowan KH. Multidrug resistance. In: Pinedo HM, Longo DL, Chabner BA, eds. *Cancer chemotherapy and biological response modifiers annual 17*. Amsterdam: Elsevier Science 1997: 139–77.
38. Sharp SY, Smith V, Hobbs S, Kelland LR. Lack of a role for MRP1 in platinum drug resistance in human ovarian cancer cell lines. *Br J Cancer* 1998; **78**: 175–80.

(Received 5 October 1999; revised form accepted 11 January 2000)