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CHARACTERISATION OF THE UNUSUAL EXPRESSION OF CROSS RESISTANCE TO CISPLATIN IN A SERIES OF ETOPOSIDE-SELECTED RESISTANT SUBLINES OF THE SuSa TESTICULAR TERATOMA CELL LINE

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Abstract—Three etoposide-selected resistant sublines of the SuSa testicular teratoma cell line expressing 9-, 21- and 33-fold levels of resistance, proved increasingly cross resistant to cisplatin with levels approximating to 3-, 4- and 6-fold in sublines VPC2, VPC3 and VPC4, respectively. Cisplatin resistance was not associated with any significant modifications in levels of total glutathione or associated enzyme activities. Decreased platinum (Pt) accumulation was detected, although this did not correlate either with total platination levels judged immunochemically or with peak induction of interstrand crosslinks (ISC) determined by alkaline elution. Following exposure to cisplatin in the least resistant subline, VPC2, total platination levels were markedly decreased (3-fold) relative to those of the parental cells, whilst peak ISC levels were markedly increased (4-fold). In the most highly resistant subline, VPC4, peak levels of ISCs were even higher (9-fold), although total platination levels remained comparable with those in parental cells. Both VPC2 and VPC4 cells appeared highly proficient in removing ISCs, unlike the parental cells. However, whilst VPC2 cells appeared to share deficient removal of the intrastrand platinated lesions with parental cells, VPC4 cells proved proficient in removing specific adducts in the sequence pApG. This unusual expression of cross resistance to cisplatin in a series of etoposide-selected resistant sublines derived from an inherently repair deficient parental cell line, SuSa, therefore appears to be associated with enhanced removal of the specific intrastrand crosslinks in the sequence pApG and/or of DNA–DNA ISCs. Similar mechanisms have been implicated in two other cisplatin resistant SuSa sublines selected following *in vitro* exposure to the drug itself or to fractionated X-irradiation (Hill BT, *Cancer Treat Rev* **18**: 149–190, 1991).

Key words: etoposide resistance; cisplatin cross resistance; intrastrand platinum-DNA adducts; DNA–DNA interstrand crosslinks

Resistance to specific antitumour drugs has been identified in tumour cells used as experimental model systems following their exposure not only to the drug itself, but also to other selection agents including different antitumour drugs or fractionated X-irradiation, as reviewed recently [1–3]. These observations raised the question as to whether different resistance mechanisms might operate or predominate depending on the selection pressure applied. In a recent study [4] a series of etoposide-resistant sublines were selected by continuous exposure of a human testicular teratoma cell line, SuSa, to increasing concentrations of etoposide. These were all characterized by decreased topoisomerase II content, but significant overexpression of P-glycoprotein was only identified in the two most highly resistant sublines [4,‡]. Unusually, these sublines expressed increasing cross resistance to cisplatin. We have therefore investigated the mechanisms of resistance to cisplatin operating in these sublines and compared them with published data on sublines, established earlier from this SuSa parental cell line, which also expressed resistance to cisplatin, but as a result of *in vitro* exposure either to cisplatin itself (SuSa/CP+)§ or to fractionated X-irradiation (SuSa/DXR$_{10}$) [5]. The following parameters have been studied: Pt† accumulation, total GSH levels and associated enzyme activities and cisplatin-mediated DNA damage, namely Pt-

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† Abbreviations: ELISA, enzyme-linked immunosorbent assay; GP, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione: GST, glutathione *S*-transferase: ISC, interstrand crosslinks; Pt, platinum; Pt-AG, *cis*-Pt(NH_3)$_2$d(pApG); Pt-GG, *cis*-Pt(NH_3)$_2$d(pGpG); Pt-GMP, Pt-(HN_3)$_3$dGMP; Pt-(GMP)2, Pt-(NH_3)$_2$d(GMP)$_2$; AAS, atomic absorption spectrophotometry.

‡ Hosking LK, Whelan RDH, Shellard SA, Davies SL, Hickson ID, Danks MK and Hill BT, Multiple mechanisms of resistance in a series of human testicular teratoma cell lines selected for increasing resistance to etoposide. *Int J Cancer*, in press.

§ Hill BT, Shellard SA, Fichtinger-Schepman AMJ, Schmoll HJ and Harstrick A, Differential formation and enhanced removal of specific cisplatin-DNA adducts in two cisplatin-selected resistant human testicular teratoma sublines. *Anticancer Drugs* submitted for publication.

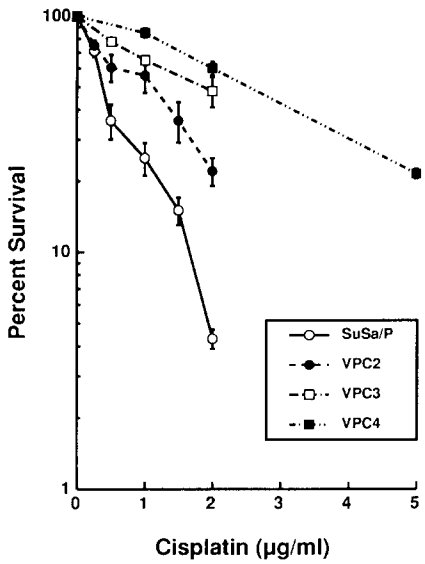

Fig. 1. Survival curves for SuSa/P, SuSa/VPC2, SuSa/VPC3 and SuSa/VPC4 cells exposed to a range of cisplatin concentrations for 1 hr, as judged by colony forming assays. Points, mean of two to three experiments in which duplicate cultures were tested. Bars, SE.

DNA intrastrand crosslinks as judged by immunochemical quantitation and DNA–DNA ISC as estimated by the technique of alkaline elution.

MATERIALS AND METHODS

Cell liners and drug sensitivity assays. The SuSa cell line was established from a human testicular teratoma obtained from a patient who had not received chemotherapy [6]. Sublines VPC2, VPC3 and VPC4 were derived by continuous exposure to increasing concentrations of etoposide commencing with 50 ng/mL (concentration resulting in 10% cell survival) and reaching final concentrations of 80 ng/mL (VPC2), 200 ng/mL (VPC3) or 300 ng/mL (VPC4). All cell lines were maintained in RPMI 1640 medium supplemented with 10% foetal calf serum (Gibco Biocult, Renfrewshire, U.K.) at 37°. No significant differences were noted between all four lines in terms of population doubling times in logarithmic growth, Cellular volumes, protein and DNA content and colony forming efficiencies. Survival of the cells following exposure to a range of cisplatin concentrations for 1 hr was determined by cloning in semi-solid agar [7]. Cisplatin was purchased from the Sigma Chemical Co. (Poole, U.K.).

GSH and associated enzyme activities. Logarithmically-growing cells were harvested 3 days after plating and the assay procedures detailed earlier were used [8]. Enzyme values and total GSH contents were normalized for cellular protein according to Lowry *et al.* [9].

Drug uptake studies. Cells (2×10^6) were exposed to 0, 25 or 50 μg/mL cisplatin for 1 hr. Then the procedure described earlier [10] was adopted with Pt uptake being quantitated by AAS (Perkin Elmer Model 4000).

Quantitation of Pt-DNA damage. Cells were treated for 1 hr with 5 μg/mL (16.7 μM) cisplatin solubilized in 0.9% saline immediately prior to use and then washed with PBS and harvested immediately or after an 18-hr incubation in drug-free medium to permit 'repair' or adduct removal to occur. Cells were lysed in the presence of 100 mM ammonium bicarbonate to inactivate monofunctionally-bound drug and DNA was isolated using phenol/chloroform prior to enzymatic digestion to nucleotides and platinated nucleotides. Separation of platinated products was carried out using an anion exchange chromatography column and then a competitive ELISA procedure using specific antibodies to quantitate the major adducts including the monofunctional Pt-GMP, the two intrastrand lesions Pt-AG and Pt-GG and the bifunctional lesion Pt-(GMP)2, as detailed in early publications [5, 10]. DNA–DNA ISC were measured by the alkaline elution technique of Kohn *et al.* [11] as a proteinase-resistant reduction in the rate of elution of DNA containing X-ray induced single-strand breaks (600 rad, 500 rad/min; Pantak Ltd., Slough, U.K.), as detailed previously [12].

RESULTS

Figure 1 depicts the effects on cell survival following exposure to a range of cisplatin concentrations for 1 hr. Interpolating IC_{50} values (drug concentration required to reduce survival by 50%) from repeated experiments and comparing those of each resistant subline with the parental cells, indicates that sublines VPC2, VPC3 and VPC4 expressed 3-, 4- and 6-fold levels of cisplatin resistance, respectively. These data indicate that this series of etoposide-selected sublines with increasing levels of etoposide-resistance, namely 9-, 21- and 33-fold for the VPC2, VPC3 and VPC4 cells, respectively [4], also proved increasingly cross resistant to cisplatin.

We reported earlier that total GSH levels and the activities of associated enzymes, namely GR, GP and GST were not significantly modified in the VPC2 and VPC3 sublines, relative to parental cell values [8]. Similarly, no significant alterations in any of these parameters were noted in the VPC4 cells, with the following values relative to the parental cells expressed per mg protein: GSH, 21.4 ± 0.35 vs 18.8 ± 3.0 nmol; GR, 16.8 ± 4.6 vs 23.3 ± 0.7 units (one unit conjugates 1 nmol of 1-chloro-2,4-dinitrobenzene/min at 25°); GP, 5.5 ± 2.1 vs 4.5 ± 0.7 units (one unit oxidizes 1 nmol NADPH/min at 25°); GST, 106.6 ± 4.6 vs 160.0 ± 4.0 units (one unit oxidizes 1 nmol NADPH/min at 25°). These data suggest that there is no differential inactivation of cisplatin via GSH-mediated pathways in these resistant sublines.

Pt accumulation following *in vitro* exposure for 1 hr to 0, 25 or 50 μg/mL cisplatin was monitored in the parental cells and the VPC2 and VPC4 cell lines by AAS. Uptake was concentration dependent (data not shown) and when normalized for extracellular drug concentration, proved significantly lower in

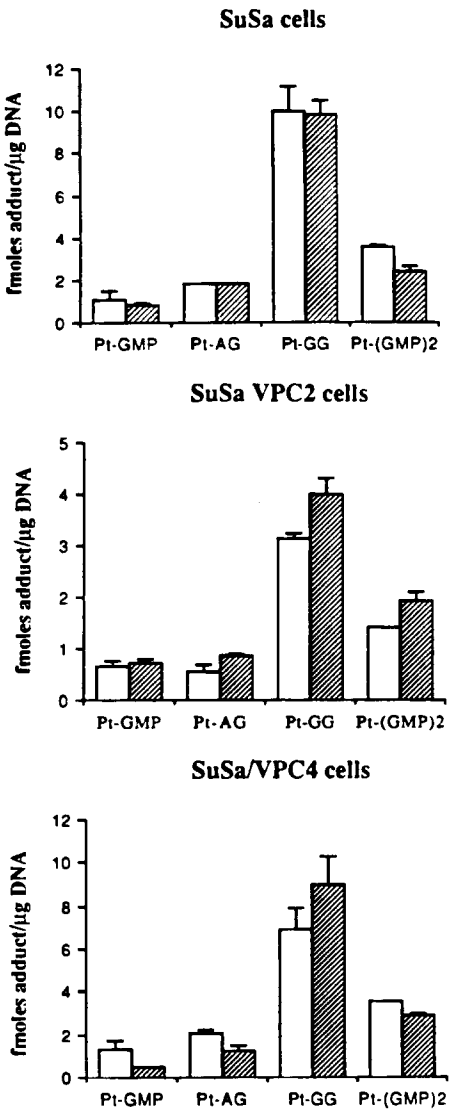

Fig. 2. Induction and removal of Pt-GMP, Pt-AG, Pt-GG and Pt-(GMP)2 in the human testicular teratoma cell lines SuSa/P, SuSa/VPC2 and SuSa/VPC4. The number of adducts was determined immediately after a 1 hr exposure to 5 μg/mL cisplatin (open boxes) and following an 18 hr post-treatment incubation period (hatched boxes). Columns, (± range) represent the mean of two different competitive ELISAs, each performed in four dilutions on duplicate cell samples. Using the *t*-test as a measure of statistical significance, the number of adducts remaining after the 18 hr post-treatment period was significantly lower ($P < 0.05$) than the number initially formed immediately after drug treatment in the following: for parental SuSa cells, Pt(GMP)2; for SuSa/VPC4 cells, Pt-GMP, Pt-AG and Pt-(GMP)2.

both the resistant sublines by factors of 1.6 ($P < 0.05$) and 4.2 ($P < 0.001$). The results expressed as pmol Pt per 10^6 cells/μg/mL cisplatin were as follows: SuSa/P, 3.05 ± 0.38; VPC2, 1.88 ± 0.8; VPC4, 0.73 ± 0.09.

The level of total platination, as judged immunochemically, immediately after a 1 hr exposure to 5 μg/mL cisplatin was markedly lower (2.8-fold) in the VPC2 subline than that identified in the parental cells or in the VPC4 subline, with values expressed in fmol Pt/μg DNA being: SuSa/P, 16.5 ± 2.3; VPC2, 5.8 ± 0.35; VPC4, 13.7 ± 1.6. These levels of initial platination therefore reflect neither cisplatin sensitivity nor the extent of Pt accumulation in these three cell lines.

Table 1. DNA–DNA ISC in SuSa cell lines following a 1-hr exposure to 10 μg/mL cisplatin

Cell line	ISC (radiation equivalents)* 14 hr	24 hr	% Removal†
SuSa/P	18.7 ± 4.9	20.8 ± 13.0	0
SuSa/VPC2	69.1 ± 4.5	19.4 ± 5.6	72
SuSa/VPC4	165.3 ± 49.6	58.1 ± 10.9	65

* Values are the means ± SE of duplicate experiments in which duplicate or triplicate filters were evaluated.

† Percentage decrease in ISC at 24 hr compared with values at 14 hr following a 1-hr drug exposure.

Examination of specific adduct formation and removal (see Fig. 2) shows that: (i) the percentage distribution of the four adducts is comparable in all three cell lines, with the major adduct formed being Pt-GG; (ii) both SuSa/P and VPC2 cells were deficient in removing the two intrastrand adducts Pt-AG and Pt-GG; (iii) VPC2 cells additionally appeared not to repair the bifunctional lesion Pt-(GMP)2 and (iv) VPC4 cells, whilst failing to remove the major Pt-GG lesions, proficiently removed both Pt-Ag and Pt-(GMP)2 adducts.

The technique of alkaline elution was used to quantitate ISC and the results are listed in Table 1. A higher frequency of ISC formation was noted in the two resistant sublines, relative to parental cells, which appeared to correlate with their increasing cisplatin resistance. Again, no correlation with peak induction of ISCs at 14 hr after drug exposure and extent of drug accumulation was noted, as exemplified by VPC4 cells with their 4-fold decrease in Pt accumulation, but a 9-fold increase in ISCs, relative to the parental line. In contrast to the parental cells, both resistant sublines tested appeared highly proficient in repairing ISC during a further 10-hr incubation period with approximately 70% of lesions being removed by 24 hr. As a result, the levels of ISC were similar at this 24-hr time point in both the VPC2 and parental cells, but still remained 2-fold elevated in the more highly resistant VPC4 cells.

DISCUSSION

This expression of increasing levels of cisplatin cross resistance in these sequentially derived etoposide-selected resistant sublines of the human testicular teratoma cell line, SuSa, was unexpected since generally it has been reported that epipodophyllotoxin-selected resistant sublines show

Table 2. Overall summary of data on the variously-derived SuSa sublines expressing resistance to cisplatin

Parameter measured	SuSa/P cells	Resistant sublines			
		DXR_{10}	CP+	VPC2	VPC4
Selection agent	None	X-rays	Cisplatin	Etoposide	Etoposide
Order of cisplatin resistance*	1.0	2.5	3.0	3.0	6.0
GSH and related enzyme activities	NC†	NC	NC	NC	NC
Fold decrease in Pt accumulation	1.0	2.0	1.4	1.6	4.2
Relative total platination at 0 hr‡	1.0	2.3	0.7	0.4	0.8
Removal of specific adducts:					
Pt-AG	−	+	+	−	+
Pt-GG	−	−	−	−	−
Pt-(GMP)2	+	+	+	−	+
Relative peak induction of ISC at 14 hr	1.0	2.1	1.0	3.7	8.8
Removal of ISC by 24 hr	0%	62%	53%	72%	65%

* Judged by comparing IC_{50} values from colony forming assay data involving 1-hr drug exposures.
† NC, not changed.
‡ Judged immunochemically immediately after a 1-hr drug exposure.

either unaltered responses or some collateral sensitivity to cisplatin [13–15].

In these sublines cisplatin resistance was not associated with any significant modifications in levels of total; GSH or associated enzyme activities, consistent with similar findings reported earlier in the SuSa/CP+ subline established by continuous exposure to cisplatin*. In contrast, a significant reduction, but only in total GSH levels, was identified in the irradiated cisplatin resistant $SuSa/DXR_{10}$ subline [5]. However, as reviewed by Andrews and Howell [16], although differences in GSH metabolism and synthesis have been identified in a range of cisplatin resistant sublines, this is by no means an invariable finding. The role of metallothioneins in cisplatin resistance also remains controversial [16, 17], but future studies are planned to evaluate metallothionein gene expression in these cell lines.

Reduced Pt accumulation appears a significant mechanism associated with the most highly resistant VPC4 cells and was also detectable in the VPC2 subline. Decreased uptake had been reported earlier in both the DXR_{10} and CP+ sublines [5, *], although in these latter cells the difference failed to reach statistical significance. These modification in Pt accumulation, however, were not reflected either in reduced total platination levels, nor in decreased peak levels of ISC formed, consistent with earlier reports studying other cisplatin resistant cell lines [5, 10, 18, 19].

In these sequentially developed resistant sublines, therefore, initial resistance to cisplatin following etoposide selection does not appear to result in any significant modification in the extent of formation and removal of the major Pt-DNA formed as judged by examination of VPC2 cells. With the expression of increasing resistance to cisplatin, however, enhanced removal of Pt-AG adducts was noted in the VPC4 cells, a feature already identified in the CP+ and DXR_{10} sublines [5, *], coupled with proficient removal of the bifunctional Pt(GMP)2 lesions. The marked proficiency in removing ISC shown by these resistant VPC2 and VPC4 cells was also identified in the CP+ and DXR_{10} sublines [5, *]. Unexpectedly, these resistant cells had a higher frequency of ISC formation after cisplatin treatment, since more generally enhanced crosslinking has been associated with comparative sensitivity to the drug [12, 20]. However, this is by no means a universal finding and we reported earlier that increased ISC formation was detected in the $SuSa/DXR_{10}$ cisplatin resistant subline [5] and Teicher *et al.* [21] made a similar observation in a human squamous carcinoma cisplatin resistant subline. A possible explanation for these observations is that the resistant cells can tolerate higher levels of DNA damage.

A summary of the various results obtained with the four independently-derived sublines all expressing cisplatin resistance following different resistance selection protocols, is provided in Table 2. Overall these results suggest that irrespective of whether cisplatin resistance is selected for by exposure either to the drug itself or to X-irradiation or is expressed following etoposide selection, some decreased Pt accumulation commonly results, but this is not associated with any decreased extent of total cellular platination or ISC formation. More significantly, however, removal of cisplatin-induced DNA damage can be enhanced by all these resistant selection procedures in cells inherently repair deficient by increasing removal of the specific Pt-AG intrastrand crosslinks and/or DNA–DNA ISC.

The establishment of this series of human tumour cell lines, all expressing cisplatin resistance but after exposure to various different selection pressures, represents a novel approach to investigating drug resistance mechanisms. It is of particular interest, therefore, that this study provides evidence that similar mechanisms of resistance to cisplatin can be

* Hill BT, Shellard SA, Fichtinger-Schepman AMJ, Schmoll HJ and Harstrick A, Differential formation and enhanced removal of specific cisplatin-DNA adducts in two cisplatin-selected resistant human testicular teratoma sublines. *Anticancer Drugs* submitted for publication.

"induced" or "selected for" irrespective of the *in vitro* selection conditions applied. Although, it remains to be established whether or not this is a general phenomenon. Similarly the significance, if any, of the fact that these resistant sublines were derived from an inherently repair deficient parental cell line requires clarification. However, these SuSa sublines with demonstrated altered repair capacities provide useful additional model systems for elucidating mechanisms associated with the repair of cisplatin-induced DNA damage.

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