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Establishment of Vincristine-Resistant and Vindesine-Resistant Lines of Murine Lymphoblasts in vitro and Characterisation of their Patterns of Cross-Resistance and Drug Sensitivities

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Summary. *Clinical evidence suggests some lack of cross resistance between vincristine (VCR) and vindesine (VDS). To investigate this phenomenon experimentally, drug-resistant L5178Y lymphoblast cell lines have been derived in vitro. These lines, under conditions of continuous drug exposure, exhibit a 50-fold order of resistance. Resistance appears due, at least in part, to impaired cellular drug accumulation and retention. Exposure of these resistant cells to VCR or VDS for 24 h showed that the presence or absence of cross resistance was dose-dependent, being most noticeable at low concentrations (< 0.5 ng/ml) and absent at higher drug levels. Cross resistance also showed some dose-dependency for vinblastine and formyl-leurosine, but this was not seen with other drugs. Marked and complete cross resistance at all concentrations tested was noted with adriamycin and 4'-epiadriamycin in both resistant lines, which, however, retained the same sensitivities as the parent line to VM26, VP-16-213, 5-fluorouracil, and methotrexate. Responses to actinomycin D and mAMSA differed in these two resistant lines. VDS-resistant cells exhibited cross resistance to both drugs, whilst VCR-resistant cells showed only slight resistance to actinomycin D whilst retaining full sensitivity mAMSA.*

This observation that cross resistance between VCR and VDS is not invariable in vitro appears to reflect clinical experience.

Introduction

Vindesine (VDS), the new semi-synthetic vinca alkaloid, is showing value in phase II clinical studies in the treatment of cancers of the breast and oesophagus, non-small cell lung cancers, melanomas, and various haematological malignancies [14, 15, 18, 19, 21]. The promising results it has yielded in the treatment of malignant melanoma unresponsive to other forms of therapy [21], acute lymphoblastic leukaemia resistant to other drugs [16], and blastic crises of chronic myeloid leukaemia [3] suggest that it may have a different spectrum of activity from vincristine (VCR). Preliminary clinical evidence also indicates that prior therapy with VCR should not preclude subsequent treatment with VDS, since further responses have been documented [3, 16, 18, 25, 28]. This had led to the proposal that there is some lack of cross resistance between these drugs. To investigate this phenomenon experimentally drug-resistant cell lines have been developed in vitro from L5178Y lymphoblasts.

Material and Methods

Drugs and Chemicals. The following drugs were kindly donated for our studies: VCR, VDS and vinblastine (VLB) by Eli Lilly & Co. Ltd, Basingstoke, Hants., Great Britain; formyl-leurosine (F-LEU) by Dr I. Palyi, The Research Institute of Oncopathology, Budapest, Hungary; adriamycin (ADR) and 4'-epiadriamycin (EPI-ADR) by Farmitalia Carlo Erba Ltd, Barnet, Herts, Great Britain; methotrexate (MTX) by Lederle Laboratories, Gosport, Hants., Great Britain; and 4'-(9-acridinylamino)-methanesulfon-m-anisidide (mAMSA) by Warner-Lambert Co., Morris Plains, NJ, USA. Other drugs were purchased: VM26 and VP-16-213 from Sandoz Ltd, Basle, Switzerland; 5-fluorouracil (5-FU) from Roche Products, Welwyn Garden City, Herts., Great Britain and actinomycin D (ACT D) from Merck, Sharp and Dohme Ltd, Hoddesdon, Herts., Great Britain. [^{3}H]VCR sulphate (3.45 Ci/mmol) was supplied by the Radiochemical Centre, Amersham, Bucks., Great Britain. Media and sera were obtained from Gibco Bio-Cult, Renfrewshire, Scotland. All drug dilutions were made in phosphate-buffered saline or media, except for mAMSA, for which the diluent was 0.0353 *M* L-lactic acid.

Cell Culture. Details of the origin and maintenance of L5178Y cells have been provided earlier [11]. Drug-resistant lines were derived from the parent line by exposure of logarithmically growing cells to 200 ng VCR/ml or VDS/ml for 24 h and then cloning in soft agar as

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described previously [11], except that RPMI 1640 medium containing 10% heat-activated horse serum was used. Cell lines established from individual colonies had their drug responses checked and were then maintained in the continuous presence of 100 pg VCR or VDS/ml. Experiments described in this paper were carried out with cells cultured under these conditions for not more than 30 passages. At least 1 week prior to experimentation maintenance drug was removed.

Cell counts and volumes were determined by means of a Coulter Counter Model ZBI. Cellular DNA, RNA, and protein contents were measured after extraction by the Scott procedure [23] as described earlier [11]. Cell cycle distributions were estimated by flow microfluorimetric analyses of mithramycin-stained whole cells with the aid of an FACS-1 machine (Becton-Dickinson, California, USA) as described elsewhere [8]. DNA histogram evaluation was performed according to the procedure recommended by Barfod [1]. Modal chromosome numbers were estimated from metaphase spreads. Chromosomes were stained with Giemsa.

Cellular Uptake and Retention of [^{3}H]VCR. Logarithmically growing L5178Y cells removed from culture medium by centrifugation were resuspended in Hank's balanced salt solution plus glucose at 2×10^6 cells/ml. [^{3}H]VCR was then added to give a final concentration of 1 μM and 0.375 μCi/ml, and incubation with shaking was carried out at 37° C. A 1-h exposure to this drug concentration was without effect on cell survival. At various time intervals aliquots were removed and processed for scintillation counting as described earlier [10]. The extent of VCR efflux was determined by pre-incubating cells with drug for 30 min, followed by rapid centrifugation at room temperature and resuspension to the same volume in fresh incubation medium without drug at 37° C. Aliquots were then removed at various time intervals. Radioactivity was determined by means of a Packard Tricarb Scintillation Counter with a 40% counting efficiency for ^{3}H.

Results

Characterisation of VCR-Resistant and VDS-Resistant Lines of L5178Y Cells

Data in Table 1 show that both drug-resistant lines have similar growth characteristics, cell volumes, modal chromosome numbers, and cellular macromolecular contents to the parent line from which they were derived. Resistance to VCR or VDS was established by (i) determining the effects of continuous exposure of cells to either VCR or VDS over 2 weeks, where it was shown that the concentrations allowing 100% survival were 2.5 pg/ml for the parent line and 100 pg/ml for each of the resistant lines; and (ii) comparing the effects of a 24-h exposure of the cells to a range of VCR or VDS concentrations (results are shown in Fig. 1). Under these conditions the LD_{50} values in the resistant lines are increased by a factor of 3.5 for VCR or 7 for VDS.

A comparison of patterns of cellular uptake and retention of [^{3}H]VCR is provided in Fig. 2. Both resistant lines demonstrated significantly reduced uptake and binding compared with the parent line. However, drug uptake by resistant cells can be increased by the addition of Tween 80 (see Tables 2 and 3). This enhancement appears sufficient to overcome drug resistance, since joint exposure to VCR or VDS plus Tween 80 resulted in enhanced cytotoxicity against the drug-resistant cells, and cell survival, assessed by colony formation, is comparable in the three lines.

Cross Resistance Between VCR and VDS

The results shown in Fig. 3 suggest that the presence or absence of cross resistance between VCR and VDS is dose-dependent. At drug concentrations of less than 0.5 ng/ml for 24 h both resistant lines exhibited cross resistance to either VCR or VDS. However, at higher drug levels (1−2 ng/ml) cross resistance was not expressed.

Table 1. Characteristics of L5178Y cell lines

	Cell lines		
	Parent	VCR-resistant	VDS-resistant
Doubling time (h)	21.5 ± 0.5	22.0 ± 0.5	23.6 ± 0.3
Cell cycle distribution (%)[a]			
G_1	46.9	47.4	47.4
S	32.9	34.7	34.6
$G_2 + M$	20.2	17.9	18.6
Cell volume (μm^3)	884 ± 21	844 ± 12	858 ± 14
DNA content ($\mu g/10^6$ cells)	8.8 ± 0.5	8.8 ± 0.3	9.3 ± 0.2
RNA content ($\mu g/10^6$ cells)	23 ± 2	28 ± 3	20 ± 1
Protein content ($\mu g/10^6$ cells)	130 ± 10	137 ± 8	139 ± 6
Modal chromosome number	40 ± 1	40 ± 2	40 ± 2

[a] As judged by flow microfluorimetry and calculated by the method of Barfod [1]. The overall scatter never exceeded 5%. Each value represents the mean of at least four estimations plus or minus the standard error of the mean

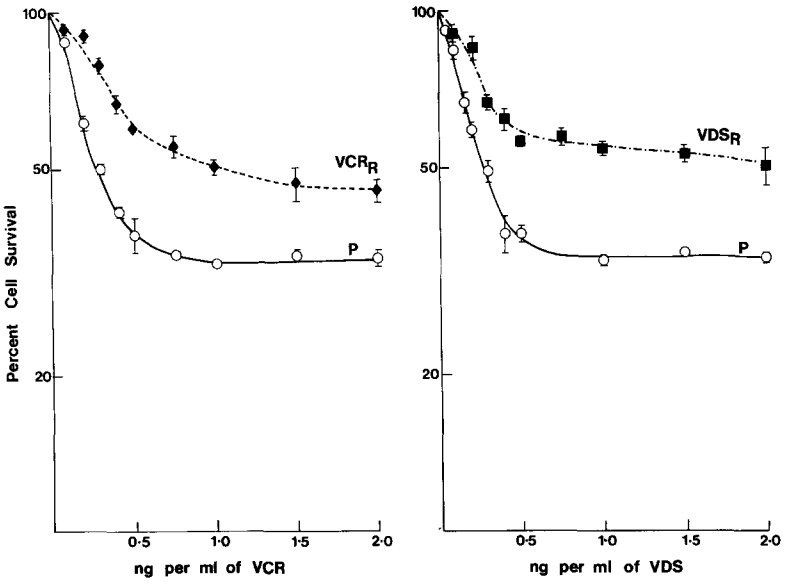

Fig. 1. The lethal effects of drug treatment for 24 h on the colony-forming ability of logarithmically growing L5178Y lymphoma cells. *P* parent line; VCR_R VCR-resistant line; VDS_R VDS-resistant line. Each *point* represents the mean and each *bar* the SEM of four assays

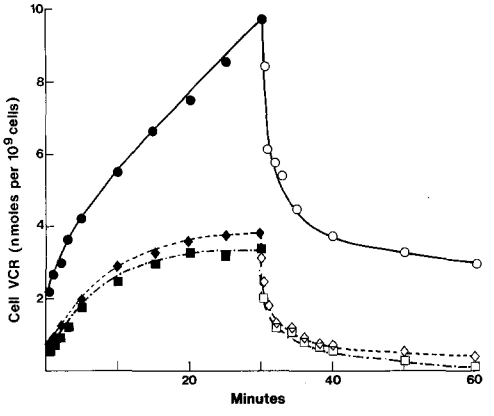

Fig. 2. A comparison of uptake and efflux of [^{3}H]VCR at 37° C by L5178Y cell lines. (○———●) parent line; (◆---◆) VCR-resistant line; (■—·—■) VDS-resistant line. The cells were exposed initially to VCR at 1 μ*M* and 0.375 μCi/ml. After 30 min at 37° C the cells were removed from drug-containing medium and resuspended in fresh drug-free medium. Each *point* represents the mean of six values; the overall scatter did not exceed 10%

Table 2. Influence of Tween 80 addition on [^{3}H] VCR uptake[a]

Cell line	VCR alone	VCR + Tween 80
	nmoles per 10^9 cells	
Parent	9.5	8.7
VCR_R	3.7	6.9
VDS_R	3.3	6.7

[a] 2×10^6 cells/ml were incubated at 37° C for 30 min with 1 μ*M* [^{3}H]VCR + 0.02% Tween 80

Table 3. Influence of Tween 80 addition on drug lethality estimated by colony formation

Cell line	% Survival			
	VCR alone	VCR + Tween 80	VDS alone	VDS + Tween 80
Parent	38 ± 2.4	30 ± 4.2	34 ± 3.2	26 ± 1.8
VCR_R	65 ± 5	25 ± 2.0	57 ± 4.3	29 ± 0.8
VDS_R	89 ± 6	28 ± 2.8	86 ± 4.5	27 ± 2.7

[a] Cells were exposed for 24 h to 0.5 ng drug/ml + 0.002% Tween 80. This concentration of Tween 80 had no effect on cell survival

Cross Resistance with Other Antitumour Drugs

In these experiments drug exposure was for 24 h and survival was assessed by colony-forming assays. Figure 4 shows (i) that VCR-resistant and VDS-resistant lines were both cross resistant to VLB and F-LEU, although again the extent of cross resistance showed some dose-dependency, tending to be negligible at the highest doses tested; and (ii) there was marked cross resistance by both resistant lines and particularly for VCR-resistant cells, to ADR and the newer derivative EPI-ADR, which was apparent irrespective of dose. Figure 5 shows that both resistant lines retained the same sensitivity as the parent line to VM26, VP-16-213, 5-FU, and MTX, whilst in Figure 6 it should be noted that these two resistant lines differ in their responses to treatment with ACT D and mAMSA. The VDS-resistant line exhibited cross resistance to both drugs, but the VCR-resistant cells showed only slight resistance to ACT D and retained full sensitivity to mAMSA.

A summary of these cross resistance studies is provided in Table 4.

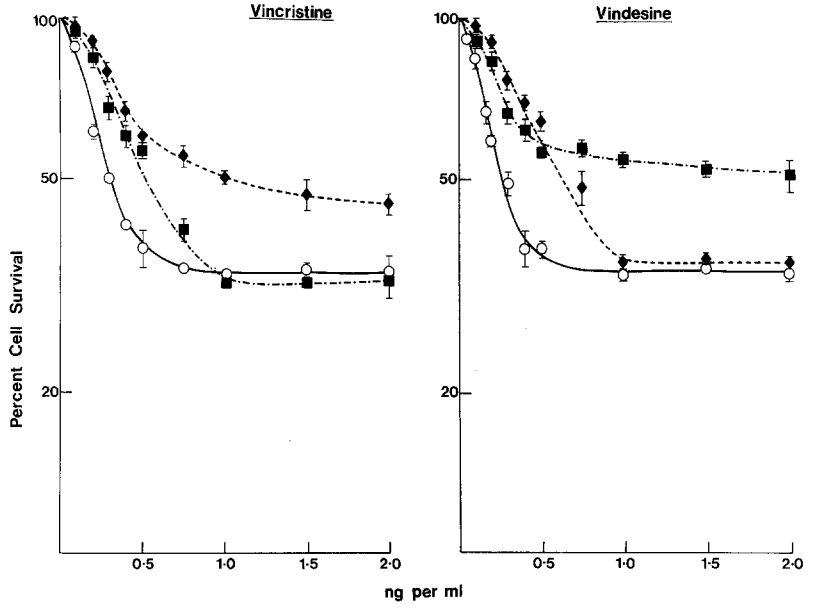

Fig. 3. A comparison of the lethal effects of a 24-h exposure to VCR (left-hand graph) or VDS (right-hand graph) on the colony-forming ability of logarithmically growing L5178Y cell lines. Each *point* represents the mean and each *bar* the SEM of four assays (see also Fig. 1, above) (○– – –○) *P*; (◆– – –◆) VCR_R. (■–·–■) VDS_R

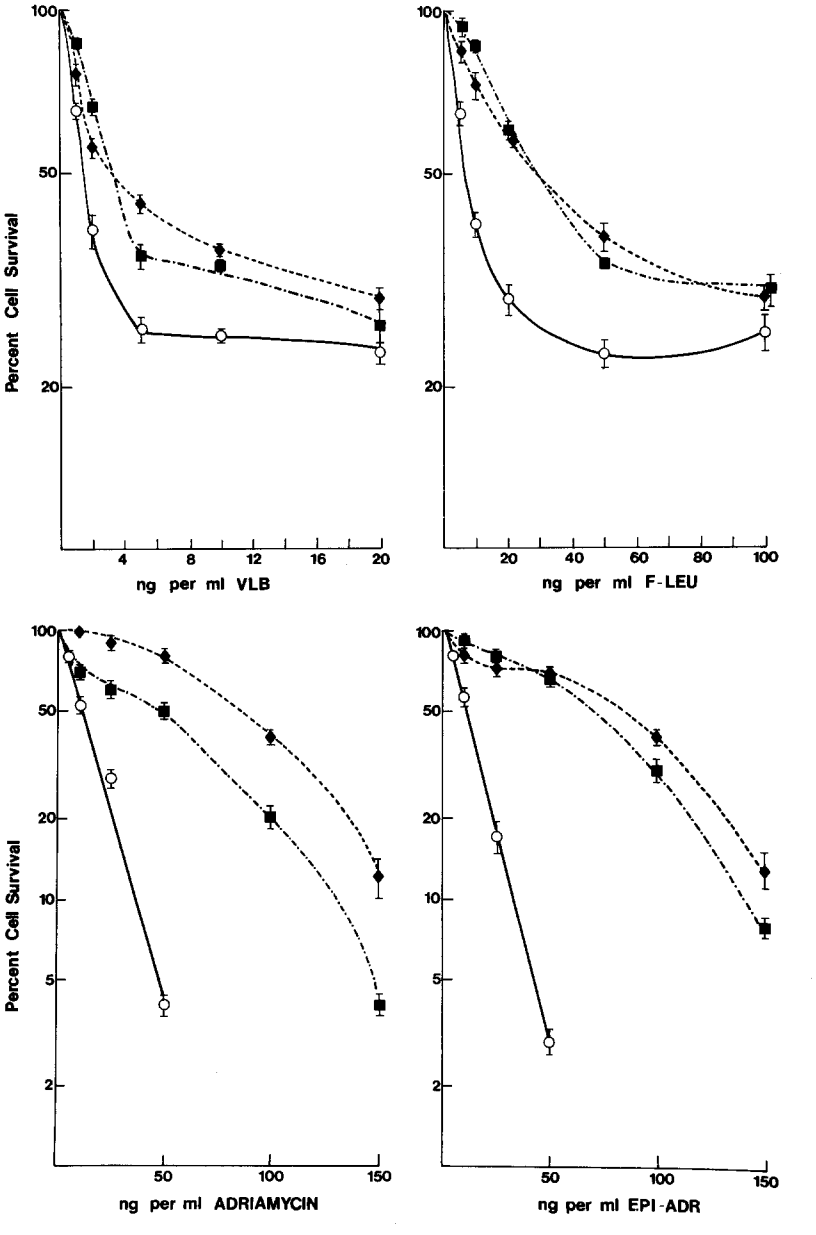

Fig. 4. The lethal effects of a 24-h exposure to VLB, F-LEU, ADR, or EPI-ADR on the parent and drug-resistant L5178Y cell lines, which all exhibit some cross resistance with VCR and VDS. Each *point* represents the mean and each *bar* the SEM of four assays. (○– – –○) *P*; (◆– – –◆) VCR_R; (■– · –■) VDS_R

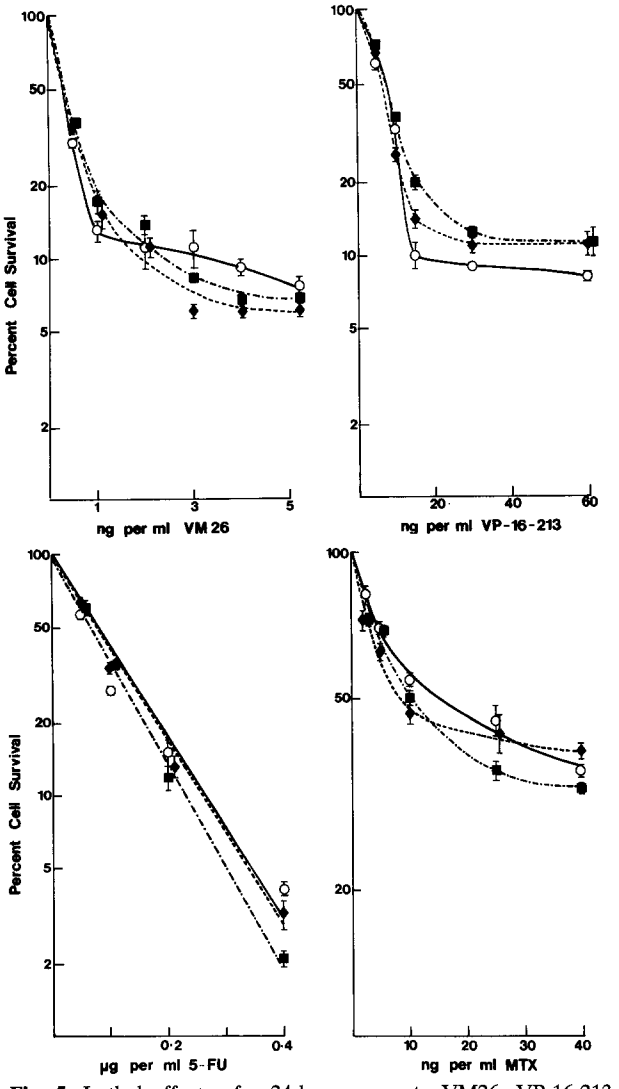

Fig. 5. Lethal effects of a 24-h exposure to VM26, VP-16-213, 5-FU, or MTX on the parent and drug-resistant L5178Y cell lines. No cross resistance is noted with these drugs. Each *point* represents the mean and each *bar* the SEM of four assays. (○---○) *P*; (◆---◆) VCR_R; (■-·■) VDS_R

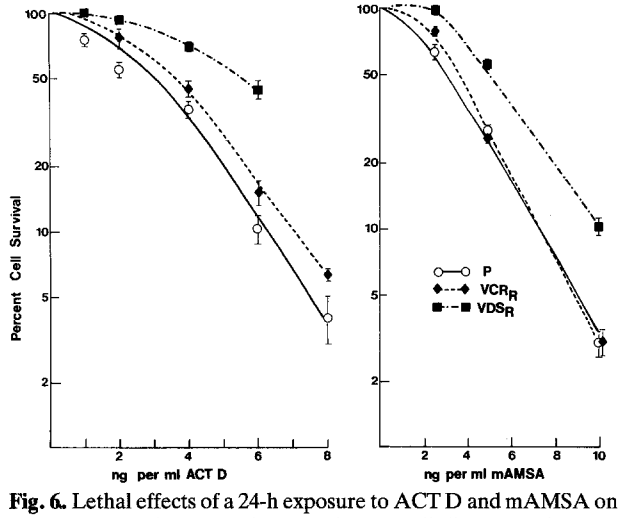

Fig. 6. Lethal effects of a 24-h exposure to ACT D and mAMSA on the parent and drug-resistant L5178Y cell lines. Each *point* represents the mean and each *bar* the SEM of four assays. (○---○) *P*; (◆---◆) VCR_R; (■-·-■) VDS_R

Table 4. A summary of the cross resistance studies

Drugs tested	Cell lines		
	Parent	VCR-resistant	VDS-resistant
Vinblastine	S[a]	R+	R+
Formyl-leurosine	S	R++	R++
Adriamycin	S	R+++	R+++
4′-Epiadriamycin	S	R+++	R+++
VM26	S	S	S
VP-16-213	S	S	S
5-Fluorouracil	S	S	S
Methotrexate	S	S	S
Actinomycin D	S	R	R++
mAMSA	S	S	R+

[a] S, sensitive; order of resistance: R+++ > R++ > R+ > R

Discussion

This study has shown that cell lines resistant to VCR or VDS may be readily established following a limited exposure to 'high' drug concentrations of 200 ng/ml. When first established, these lines exhibited approximately a 50-fold order of resistance under conditions of continuous drug exposure, which was stable over 50 in vitro passages (approximately 200 population doublings) even in the absence of maintenance drug. The two resistant lines were similar to the parent in terms of doubling time, cell cycle distribution, cell volume, modal chromosome number, and cellular DNA, RNA, and protein contents. Resistance in both cell lines was associated with impaired VCR accumulation and binding within the cell. These results are similar to data obtained with a subline of P388 leukaemia resistant to VCR [4, 13]. These P388 cells also exhibited a reduced uptake of F-LEU, a newer vinca derivative developed in Hungary [6]. Preliminary data with [^{3}H]VDS in these L5178Y cell lines also indicate impaired drug influx in the resistant lines (B. T. Hill, unpublished data). However, drug resistance can be overcome by the concurrent addition of the detergent Tween 80, which enhances the level of VCR achieved in VCR-resistant cells. Recently it has also been shown that VCR-resistance in P388 leukaemia may be overcome by co-administration of verapamil, a coronary vasodilator known to inhibit Ca^{2+} transport across membranes, which appears to inhibit VCR efflux [27].

Experimental studies of cross resistance amongst the vinca alkaloids have mainly been conducted in vivo with the VCR-resistant line of P388 leukaemia [2, 17, 22]. Schabel et al. [22] have reported that this tumour is partially resistant to VLB and resistant to VDS, and this has been confirmed by Maral et al.,

who also demonstrate cross-resistance to another semi-synthetic vinca, navelbine [17]. Recent studies with this same cell line have also shown that cross resistance is expressed in vitro to VLB and VDS, allowing the conclusion that this cross resistance is a function of the cellular properties of the drug-resistant cells and independent of any host factors [31]. In our studies reported in this paper, where resistance was developed in vitro to either VCR or VDS, we have shown that cross resistance between VCR and VDS is *not* invariable in these L5178Y lymphoblasts, but appears to be dose-dependent. The differences from these two studies are not easy to interpret, but the degree of resistance, the cell type used, or its inherent sensitivity to the vincas may be significant. In this last respect it should be noted that with the L5178Y cells the drug concentrations used are at least 1000-fold less than those employed with P388 cells. We are currently investigating VCR resistance further in other mammalian cell types. Alternatively, as suggested by Maral et al. [17], the resistant cell lines induced in vitro may not represent a valid in vivo model. However, this experimental study does provide some laboratory evidence that resistance to VCR and VDS is dose-dependent. This may be relevant in terms of the few conflicting suggestions that pretreatment with VCR can reduce the subsequent response to VDS [31, 32] amongst the more extensive literature reporting an absence of cross resistance between the two drugs [3, 16, 18, 24, 25].

In terms of patterns of cross resistance with other antitumour agents, as expected (i) either partial or complete resistance was observed to the other vincas, the anthracyclines, and ACT D, confirming other reports with VCR-resistant lines of the P388 leukaemia in vivo or in vitro [5, 13, 22, 29] or of the Ehrlich ascites tumour [7]; whilst (ii) full sensitivity was retained to MTX and 5-FU, as reported previously [7; R. K. Johnson, personal communication]. However, results with other drugs provided conflicting data. For example, these drug-resistant L5178Y cells proved sensitive to both the podophyllotoxin derivatives, but in vivo the VCR-resistant P388 leukaemia proved sensitive to only VP-16-213 [30] and partially resistant to VM26 (R. K. Johnson, personal communication), whilst in vitro studies with this tumour type demonstrated cross resistance to VP-16-213 [30].

Generally both the VCR-resistant and VDS-resistant lines exhibited comparable patterns of sensitivity or resistance to other antitumour drugs. This might be expected, since in vitro both drugs exert similar lethal and cell cycle kinetic effects [9]. However, certain differences have been noted: (i) VDS has been shown to be quantitatively superior to VCR in arresting various cell types in mitosis, including L5178Y cells [9], L1210 cells [12], and CHO cells [26]; (ii) their pharmacokinetic properties differ [20]; and (iii) they appear to have a different spectrum of clinical antitumour activity [3, 16, 21]. In addition, this study provides provides further evidence of a quantitative nature since the two resistant lines differed in their responses to treatment with ACT D and mAMSA. The VDS-resistant line exhibited cross resistance to both drugs, but the VCR-resistant line showed only slight resistance to ACT D and full sensitivity to mAMSA. This suggests that mAMSA may prove valuable in specifically overcoming resistance to VCR. This lack of cross resistance between VCR and the mAMSA was also noted in vivo with P388 vincristine-resistant cells (R. K. Johnson, personal communication). Our studies also suggest that resistance to VDS in these L5178Y cells appears less easily overcome than resistance to VCR, which might argue in favour of using VDS as second-line therapy in tumours equally responsive initially to the two vincas.

To our knowledge this is the first presentation concerning experimentally induced VDS resistance. We are now expanding these studies to consider other cell types, which will be used to screen for antitumour drugs to which these resistant cells exhibit collateral sensitivity.

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