

Ascorbic acid increases drug accumulation and reverses vincristine resistance of human non-small-cell lung-cancer cells

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A human lung-cancer PC-9 subline with acquired resistance to vincristine (VCR), a chemotherapeutic agent, was established with incremental increases of the drug. The resistant PC-9 subline (PC-9/VCR) shows a 12-fold increase in resistance to VCR and a unique cross-resistance pattern: high cross-resistance to the potent VCR analogue colchicine (6.9-fold) and vinblastine (2.5-fold); lower cross-resistance to actinomycin D (1.8-fold), cisplatin (1.2-fold) and adriamycin (1.3-fold) and a sensitivity to melphalan and VP-16 which is similar to that of the parental cell line. A reduced accumulation of VCR in the resistant cells was demonstrated. Interestingly, the VCR resistance of the PC-9/VCR cell line was partially reversed by ascorbic acid, and the drug uptake was enhanced. In contrast, ascorbic acid had no effect on drug tolerance and drug accumulation was not observed in either PC-9 parental cells or known multidrug-resistant (MDR)

cells, suggesting that VCR resistance in PC-9/VCR cells results essentially from reduced drug accumulation. It is worth noting that, whereas reduced drug accumulation in the PC-9/VCR cells was susceptible to modulation by ascorbic acid, the increased efflux rate characteristic of the resistant cells was not. Further, there was a higher efflux rate in resistant cells than in parental cells. DNA Southern- and RNA Northern-blot hybridization analyses indicate that PC-9/VCR cells do not contain amplified *mdr* genes or overexpress P-glycoprotein. In addition, the calcium-channel blocker verapamil, which acts as a competitive inhibitor of drug binding and efflux, did not affect the resistant phenotype of PC-9/VCR cells. These findings suggest an ascorbic acid-sensitive drug uptake mechanism which is important in mediating VCR resistance *per se* in human lung-cancer cells; this differs from the P-glycoprotein-mediated MDR mechanism.

INTRODUCTION

Vincristine (VCR), a *Vinca* (periwinkle) alkaloid, is an effective chemotherapeutic agent. It is widely used in combination with other agents in clinical cancer treatment, including lung cancer. However, resistant cells occasionally arise, causing a great hindrance in the fight against cancer. Extensive studies in dealing with drug resistance have been accumulated with *in vitro* cell model systems, but they are not necessarily reproduced *in vivo*. VCR-resistant tumour-cell lines have previously been established. Among these, cell lines selected by microtubule blockers, such as VCR and colchicine, are often associated with multidrug resistance (MDR), a phenomenon resulting from overexpression of a membrane-anchored P-glycoprotein, which functions like an efflux pump for the drug [1,2]. Nevertheless, the extent of MDR in these cells is not always consistent with the level of P-glycoprotein, suggesting that additional mechanisms may also participate in the MDR phenotype. For example, an increased level of GSH was detected in an MDR small-cell lung-cancer cell line that does not overexpress P-glycoprotein [3]. Non-P-glycoprotein-mediated MDR has also been documented, some of which is believed to precede P-glycoprotein expression during the development of MDR [4–12]. It has been shown that human cells with slight resistance (less than 10-fold) to colchicine or VCR do not overexpress the P-glycoprotein genes; however, cells which are treated by stepwise exposure to the selecting agents and which acquire greater resistance are associated with overexpression of P-glycoprotein (see, e.g. [13]). Thus obtaining more information about non-P-glycoprotein pathway(s) is important for the understanding of the development of MDR.

In the present study we established a VCR-resistant subline from human lung-cancer PC-9 cells which displays a reduced

drug accumulation and does not overexpress P-glycoprotein. More interestingly, we found that L-ascorbic acid could inhibit cellular resistance to VCR, the inhibition being associated with restoration of drug accumulation. L-Ascorbic acid is effective in modulating the growth of human leukaemia cells [14–16], and potentiating the inhibitory effect of certain agents on growth of cultured neuroblastoma cells [17]. A potential value of ascorbic acid in cancer treatment comes from differential responses of cancer cells and their normal counterpart to the agent (see, e.g., [18]). It has been shown at the molecular level that ascorbic acid causes DNA strand breaks and cross-links in human neuroblastoma cells and prevents their growth without affecting normal neuronal cells [19]. We demonstrate here that ascorbic acid could also reverse cellular resistance to VCR by modulating drug accumulation.

EXPERIMENTAL

Chemicals and medium

RPMEI-1640 medium was purchased from Hyclone (Logan, UT, U.S.A.). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum and penicillin/streptomycin were obtained from Gibco, Gaithersburg, MD, U.S.A. VCR, colchicine, adriamycin, cisplatin, and melphalan (L-PAM) were purchased from Sigma, St. Louis, MO, U.S.A. Other chemicals and supplies were also obtained from Sigma, unless otherwise indicated.

Cell cultures and determination of resistance

Human lung-cancer PC-9 cell line (obtained from Dr. Y. Hayata, Tokyo Medical College, Tokyo, Japan), and human colon-cancer SW620 parental and MDR cells [20] were maintained in

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; L-PAM, melphalan; MDR, multidrug resistance; VCR, vincristine.

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RPMI-1640 and DMEM respectively. The resistant cell line PC-9/VCR was established by intermittent exposure of PC-9 cells to VCR. The drug concentration was increased from $5 \times 10^4 \mu\text{g/ml}$ initially to $0.02 \mu\text{g/ml}$ in 2-fold increments. Exposure to each concentration of the drug for 4–6 days was followed by 2–4 weeks growth in the absence of drug to allow for recovery between each treatment. The selected resistant cell line, named PC-9/VCR, was cultured continuously in medium containing $0.02 \mu\text{g/ml}$ VCR to maintain the resistant phenotype. The acquired resistance of the cells to the drug is defined as the ratio of IC_{50} , the drug concentration inhibiting cell survival by 50% of that of the parental cells. Cytotoxicity was assayed with the MTT dye 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (Sigma) as previously described [21]. In some cases, the colony-forming assay [22] was also used to determine clonogenicity of cells.

Drug-accumulation analysis

Drug-uptake and -efflux determinations were carried out by a modification of the method of Lemontt et al. [28]. For drug-uptake analysis, at specific intervals after mixing 10^6 cells with 0.5 ml of serum-free medium containing 5 nM of [^3H]VCR (7.2 Ci/mmol; Amersham International), labelled cells were layered on to 0.2 ml of a silicone oil/mineral oil mixture (5:1, v/v) and centrifuged for 10 s at 12000 g. The cell pellets were washed four times with the medium. ^3H radioactivity was counted in 2 ml of scintillation cocktail fluid (Beckman) in a liquid-scintillation counter. For drug-efflux analysis, all the cells were depleted of ATP with 20 ng/ml rotenone (Sigma) before being loaded with 5 nM [^3H]VCR/ 10^6 cells. The cells were centrifuged, and efflux was initiated by resuspending the cell pellets in the medium and incubating at 25 °C. At specific times, 0.8 ml of the cell suspension (1×10^6 cells) were extracted as described above. The aqueous medium (0.8 ml) remaining over the oil was removed for counting of ^3H radioactivity. All the drug-accumulation studies were carried out at 25 °C. For the effects of ascorbic acid on drug uptake, medium containing $25 \mu\text{g/ml}$ ascorbic acid was preincubated with cells for 20 min before the labelled drug was added. For drug-efflux determinations, cells were preincubated with the labelled drug for 3 h before ascorbic acid was added. Cells were then incubated for 1, 2 or 3 h before harvesting for radioactivity counting.

DNA and RNA blot hybridizations, and probes

Hybridization analyses of DNA and RNA blots were performed by standard methods [24]. For Southern DNA blots, 10 μg of genomic DNA was digested with the restriction enzymes *Hind*III or *Eco*RI (New England Biolabs) and separated in a 1% agarose gel. For Northern RNA blots, 10 μg of total RNA from the cell lines was fractionated by electrophoresis on 1% agarose containing 6.7% formaldehyde. DNA or RNA was transferred to a Hybond-N filter (Amersham) and cross-linked with a u.v. cross-linker (Stratalinker; Stratagene) and hybridized at 42 °C for 16 h in hybridization buffer ($6 \times \text{SSC}/50\%$ deionized formamide/ $10 \times \text{Denhardt's}$ solution/ $10 \text{ mM EDTA}/0.1\%$ SDS; $1 \times \text{SSC}$ is 0.15 M NaCl/0.015 M sodium citrate) containing 5×10^5 c.p.m. of probe/ml. The filter was then washed at 65 °C in $2 \times \text{SSC}/0.1\%$ SDS, followed by exposure on X-ray film with an intensifying screen at -80 °C. The X-ray film was scanned in a densitometer to estimate the density of the bands.

The human *mdr1* gene probe was a 368 bp *Sac*I-*Eco*RV fragment ($-137/+231$; numbered from the first ATG codon [25] of the full-length human *mdr1* cDNA clone cut from pGEM3Zf(-)-*mdr1* (obtained from Dr. P. Borst, Netherlands

Cancer Institute, Amsterdam, The Netherlands) as previously described. Probes were purified by electrophoresis on an NA45 membrane (Schleicher and Schuell) and radiolabelled by the random-priming method with [α - ^{32}P]dCTP to a specific radioactivity of 10^8 c.p.m./ μg of DNA as described in [26]. The labelled probe was purified by passage through a resin (Sephacrose G-50; Pharmacia)-loaded spin column.

RESULTS

Characteristics of the sensitive and resistant cells

A VCR-resistant human lung-cancer cell line (PC-9/VCR) was established. Cytotoxicity, determined by the MTT-dye method (Figure 1a) or colony-forming assay (Figure 1b), exhibited a 12-fold and 10-fold increase in resistance respectively to the selecting drug. The patterns of the survival curves by both methods are similar, showing a 'shoulder' response at low concentrations of VCR (within $10^{-2} \mu\text{g/ml}$) in the resistant cells. In contrast, the parental cells lacked a similar tolerance response. At drug concentrations higher than $10^{-2} \mu\text{g/ml}$, both cell lines displayed similar exponential responses. The IC_{50} was 0.008 and $0.1 \mu\text{g/ml}$ for parental and resistant cells respectively (also see below). There is a 12.5-fold increase in resistance in PC-9/VCR cells. As estimated by clonogenic ability, VCR cells acquired 10-fold resistance, results comparable with the MTT-dye data. The acquired resistance phenotype was stable, as evidenced by the persistence of drug resistance after removal of VCR from the testing medium for at least 1 month (results not shown). Some other characteristics of the established PC-9/VCR cells are shown in Table 1. Approx. 40% of the resistant cells attached to the culture vessel, and the other 60% grew stably as the parental cells in suspension. The significance of adherent growth of the resistant cells has yet to be studied.

Cross-resistance of PC-9/VCR cells to anticancer agents

Resistance of PC-9/VCR cells to other anticancer agents was determined by the MTT-dye assay. Calculated IC_{50} values are listed in Table 2. PC-9/VCR cells also showed high resistance to

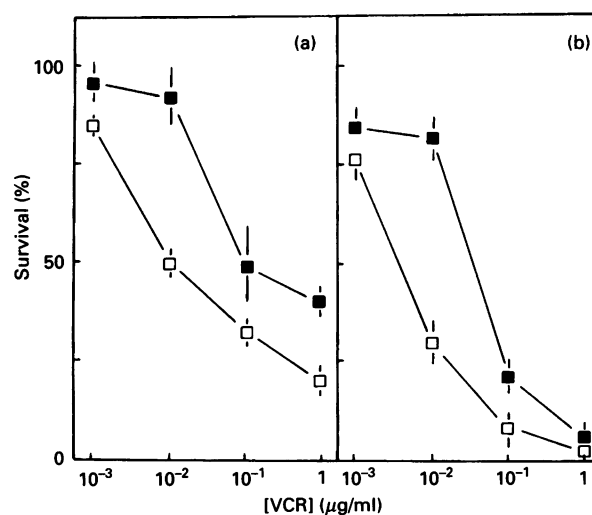


Figure 1 Relative sensitivity of PC-9 and resistant PC-9/VCR cells to VCR

(a) MTT-dye assay; (b) colony-forming assay. □, PC-9 cells; ■, PC-9/VCR cells. Results are means \pm S.D. (bars) of three determinations.

Table 1 Characteristics of PC-9 and VCR-resistant PC-9/VCR cells

Cell line	Culture pattern	Plating efficiency*(%)	Cell size [diam. (μm)]†	Doubling time* (h)
PC-9	Suspension	85.3 \pm 4.1	19.3 \pm 0.3	31.1 \pm 1.1
PC-9/VCR	Suspension (60%)‡	76.1 \pm 2.1	17.9 \pm 0.2	35.2 \pm 1.7

* Means \pm S.D. for four experiments.
† Means \pm S.D. for three determinations with 10^4 cells each.
‡ There is approx. 40 and 60% of adherence and suspension respectively for PC-9/VCR cells. With PC-9, nearly 100% of cells grow in suspension.

colchicine (6.9-fold) and vinblastine (2.5-fold); modest resistance to actinomycin D (1.8-fold), cisplatin (1.4-fold) and adriamycin (1.2-fold); and a sensitivity as parental cells to L-PAM and VP-16. The modest (1.2–1.8 fold) resistance of PC-9/VCR to actinomycin, cisplatin and adriamycin are not convincing. First, the differences are marginal in view of the S.D. measurements. Secondly, differences of this magnitude are often observable between unselected subclones of the same line. The MDR of PC-9/VCR cells is probably not the same as that of the P-glycoprotein-mediated MDR phenotype (see below) but, rather, that the cells are simply cross-resistant to several other microtubule inhibitors.

Inhibition of drug resistance by ascorbic acid in resistant cells

The effect of ascorbic acid on cellular response to VCR was measured by the MTT-dye assay. To eliminate complications from the modulating agent itself, a non-toxic or low cytotoxic concentration of ascorbic acid was used. Figure 2(a) shows cellular response to ascorbic acid. Concentrations below 10 $\mu\text{g}/\text{ml}$ had no profound toxic effect on either parental (\square) or resistant (\blacksquare) cells. At 25 $\mu\text{g}/\text{ml}$, ascorbic acid slightly inhibited cell growth in both to a similar extent. The modulating effect of ascorbic acid on the VCR response of cells was studied with this background. For parental cells (Figure 2b), no significant sensitization by 25 $\mu\text{g}/\text{ml}$ ascorbic acid (\blacktriangle) was detected. Neither of the lower concentrations (1 and 10 $\mu\text{g}/\text{ml}$) of ascorbic acid addressed the modulating effect on the VCR cytotoxicity (results not shown). In contrast, 25 $\mu\text{g}/\text{ml}$ ascorbic acid significantly lowered the resistant-cell proliferation (Figure 2c, \bullet). Lower concentrations of ascorbic acid were also effective in sensitizing VCR toxicity. These results indicate that a non-toxic (10 $\mu\text{g}/\text{ml}$) or toxic (25 $\mu\text{g}/\text{ml}$) concentration of ascorbic acid is effective in sensitizing the killing effect of VCR in resistant cells, but not in parental cells.

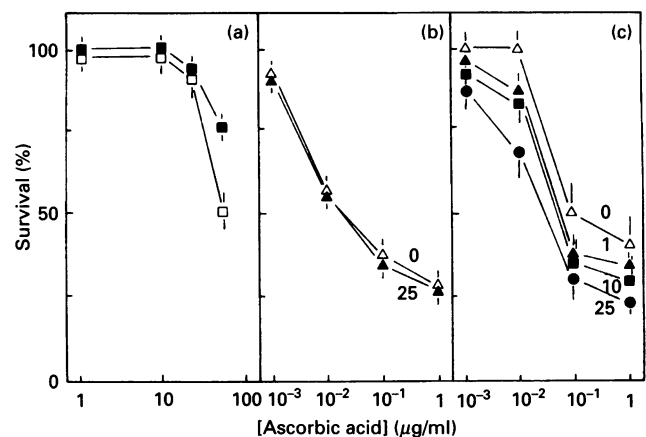
To investigate whether the same cytotoxic modification exists in the MDR cells, effects of ascorbic acid on VCR toxicity were compared in SW620 and resistant SW620/MDR cells (Table 3). IC_{50} of adriamycin toxicity with or without ascorbic acid during treatment is shown in the Table. The extent of reduction in toxicity (right column of the Table) was calculated from the IC_{50} values without ascorbic acid divided by the IC_{50} with ascorbic acid. For SW620 and SW620/MDR cells it was 1.04 and 0.97 respectively. The data indicate that ascorbic acid effectively reversed acquired resistance of PC-9/VCR cells (see Figure 2); by contrast, ascorbic acid caused very little, if any, effect on VCR resistance of SW620/MDR cells (Table 3). These results are consistent with the conclusion that the mechanism of drug resistance in PC-9/VCR cells is different from the P-glycoprotein-mediated resistance in SW620/MDR cells.

Table 2 IC_{50} values to anticancer agents of PC-9 and PC-9/VCR cells

Agent	IC_{50} ($\mu\text{g}/\text{ml}$)*		Fold resistance†
	PC-9	PC-9/VCR	
VCR	0.008 \pm 0.0002	0.1 \pm 0.025	12.5
Colchicine	0.096 \pm 0.003	0.661 \pm 0.002	6.9
Vinblastine	0.0035 \pm 0.0003	0.0086 \pm 0.0006	2.5
Actinomycin D	0.0012 \pm 0.0009	0.0022 \pm 0.0008	1.8
Cisplatin	2.2 \pm 0.6	3.0 \pm 0.8	1.4
Adriamycin	0.036 \pm 0.003	0.043 \pm 0.007	1.2
L-PAM	42.3 \pm 1.8	52.8 \pm 2.0	1.2
VP-16	9.2 \pm 0.9	10.69 \pm 1.1	1.2

* IC_{50} is the drug concentration effective in inhibiting 50% of the cell growth measured by the MTT-dye assay after 4 days of continuous exposure to the drug; results are means \pm S.D. for three determinations.

† Fold resistance was determined by the ratio of IC_{50} of PC-9/VCR to the IC_{50} of PC-9.

**Figure 2** Modulation of VCR responses by ascorbic acid

(a) Cellular responses to ascorbic acid ($\mu\text{g}/\text{ml}$) alone. \square , PC-9 cells; \blacksquare , PC-9/VCR cells. (b) Sensitivity of PC-9 cells to VCR ($\mu\text{g}/\text{ml}$) in the absence (Δ) or presence (\blacktriangle) of 25 $\mu\text{g}/\text{ml}$ ascorbic acid. (c) Sensitivity of PC-9/VCR cells to VCR ($\mu\text{g}/\text{ml}$) in the absence (Δ) or presence of 1 (\blacktriangle), 10 (\blacksquare) or 25 (\bullet) $\mu\text{g}/\text{ml}$ ascorbic acid. Results are means \pm S.D. (bars) of four determinations.

Table 3 Effects of ascorbic acid on IC_{50} of adriamycin in MDR cells

Cell line	IC_{50} ($\mu\text{g}/\text{ml}$)*		Fold reduction†
	– Ascorbic acid	+ Ascorbic acid†	
SW620	0.025 \pm 0.004	0.024 \pm 0.005	1.04
SW620/MDR	1.05 \pm 0.15	1.08 \pm 0.1	0.97

* IC_{50} is the drug concentration effective in inhibiting 50% of the cell growth measured by the MTT-dye assay after 4 days of continuous exposure to the drug; results are means for three determinations.

† Ascorbic acid concentration was 25 $\mu\text{g}/\text{ml}$.

‡ The ratio of IC_{50} without ascorbic acid divided by IC_{50} with ascorbic acid.

Reduced drug accumulation in resistant cells

To analyse the role of the cellular membrane barrier on VCR accumulation, [^3H]VCR was used to study drug uptake and

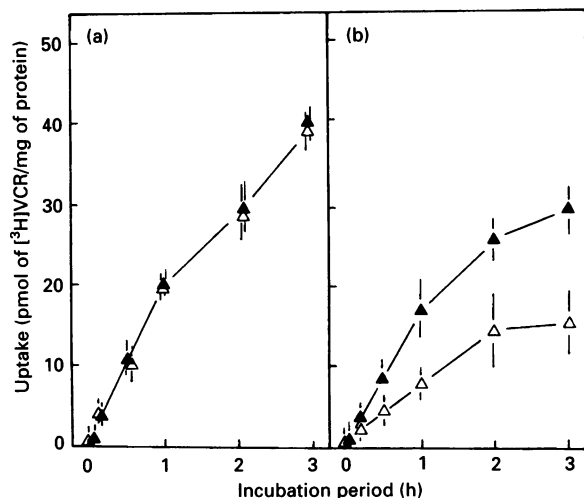


Figure 3 Modulation of drug uptake by ascorbic acid

(a) ³H-labelled VCR (5 nM) uptake in PC-9 cells for 5 min, 10 min, 30 min, 1 h, 2 h or 3 h in the absence (Δ) or presence (\blacktriangle) of 25 μ g/ml ascorbic acid. (b) VCR uptake in PC-9/VCR cells. Symbols are as in (a). Results are means \pm S.D. (bars) of five or six determinations.

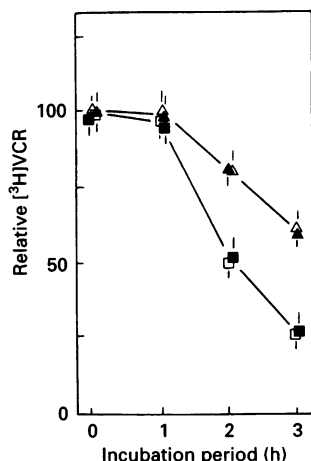


Figure 4 Modulation of drug efflux by ascorbic acid

VCR efflux in PC-9 cells (Δ , \blacktriangle) or PC-9/VCR cells (\square , \blacksquare) for 1 h, 2 h or 3 h in the absence (open symbols) or presence (closed symbols) of 25 μ g/ml ascorbic acid. Cells were preincubated with 5 nM [³H]VCR for 3 h before drug-efflux assay. The points for 5, 10 and 30 min are omitted from the connecting curves. Results are means \pm S.D. (bars) of five or six determinations.

efflux in parental and resistant PC-9 cells. For drug uptake (Figure 3), parental cells showed a nearly linear pattern of drug accumulation after 5 min, 15 min, 30 min, 1 h, 2 h or 3 h incubation in the presence or absence of ascorbic acid. For both conditions the drug uptake rate is about 15 pmol/h per mg of protein. It is apparent that drug uptake in parental cells was not affected by ascorbic acid. In contrast, profound restoration of drug uptake was detected in resistant cells. There is a reduced uptake of VCR (8 pmol/h per mg of protein) in the absence of ascorbic acid (Δ). The drug uptake reached a plateau after 2 h incubation. However, when ascorbic acid was included in the reaction mixture, accumulation of VCR within 2 h incubation increased from 8 to 15 pmol/h per mg of protein, an uptake rate

comparable with that estimated in the parental cells. There is a 2-fold increase in drug-uptake rate. After 3 h incubation in this study, resistant cells take up 30 and 15 pmol per mg of protein in the presence or absence of ascorbic acid respectively. Prolonged incubation (e.g. 5 h) did not increase the level of drug uptake (results not shown). In contrast, parental cells take up 40 pmol/mg of protein VCR after the same period of incubation and continued to take up the drug. The results indicate that the drug-uptake rate in PC-9/VCR cells is at least 2-fold lower than in parental cells. Furthermore, the resistant cells accumulated severalfold less VCR than parental cells after prolonged incubation.

Enhancement of drug-uptake in resistant cells by ascorbic acid

To test whether ascorbic acid could also modulate drug efflux, cells were incubated with labelled VCR for 3 h, and retention of the drug by cells was analysed at 5 min, 15 min, 30 min, 1 h, 2 h or 3 h after drug removal (Figure 4). In parental cells, no reduction in the amount of drug was detected within the first hour, followed by a linear pattern of drug release (20%/h). This response was consistently observed in the presence or absence of ascorbic acid. In contrast, resistant cells effectively lost the drug from the cells after drug removal (35%/h). Ascorbic acid did not affect the pattern of drug efflux compared with the reaction without ascorbic acid. The efflux rate in resistant cells is 1.8-fold higher than the efflux rate in parental cells. In any case, the function of drug efflux is independent of ascorbic acid in both cell lines. Therefore this modulating agent only acts on uptake, not efflux, of the drug.

Lack of overexpression of the *mdr1* gene in resistant cells

We have previously shown that a P-glycoprotein-mediated MDR human colon-cancer cell line has acquired the ability to decrease drug accumulation inside the cell by pumping the drug out [27]. This characteristic holds true for a variety of hydrophobic drugs, which is typical of an MDR phenotype. It is reasonable to speculate then that the PC9/VCR cells may overexpress P-glycoprotein because they developed an enhanced ability to pump the drug out (see Figure 4). However, the DNA Southern- and RNA Northern-blot hybridizations did not indicate a detectable increase in amplification or overexpression of the P-glycoprotein (or *mdr1*) gene in the PC9/VCR cells (Figure 5). For DNA analysis, *Hind*III- or *Eco*RI-digested genomic DNA showed no detectable *mdr* in PC-9 and PC-9/VCR. As controls, samples from the above-described MDR colon-cancer cell line and its parental line were run in parallel. Significant amounts of *mdr*-gene fragments are amplified in MDR cells. For RNA analysis, the steady-state level of *mdr* mRNA was not increased in PC-9/VCR compared with the parental cells. In contrast, the MDR control cells overexpressed at least 30-fold greater amounts of *mdr* message than their parental cells. After a longer exposure to X-ray film of both DNA and RNA blots, *mdr1*-corresponding bands with similar intensity were visible in SW620, PC-9 and PC-9/VCR cells (results not shown). In addition, immunostaining of cells with a commercial antibody (C219, Centocor, Malvern, PA, U.S.A.) against P-glycoprotein did not show increased expression of this protein in PC-9/VCR cells (results not shown).

The literature documents that amplification of *mdr* genes in human cells is usually observed only at higher levels of resistance. Moreover, the SW620/MDR line used here as a positive control is an order of magnitude more resistant than the PC-9/VCR isolate. To verify further that the acquired VCR resistance of PC-9/VCR cells is independent of P-glycoprotein, effects of a

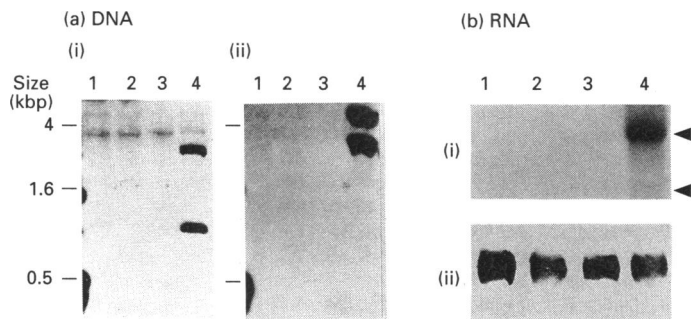


Figure 5 DNA Southern- and RNA Northern-blot hybridization of *mdr1* gene in PC-9 and resistant PC-9/VCR cells

(a) DNA hybridization. A 10 μ g portion of genomic DNA were digested with *Hind*III (i) or *Eco*RI (ii). Lanes: 1, PC-9 cells; 2, PC-9/VCR cells; 3, SW620; 4, SW620/MDR. Size markers are indicated on the left. (b) RNA hybridization. 10 μ g of total cellular RNA from PC-9 (lane 1), PC-9/VCR (lane 2), SW620 (lane 3) or SW620/MDR (lane 4) cells were loaded. (i) *mdr1* hybridization pattern; positions for 28 S and 18 S rRNA are indicated with arrowheads. (ii) The same RNA blot re-probed with rat β -actin cDNA.

Table 4 Effects of verapamil on the IC_{50} of VCR

Cell line	IC_{50} (μ g/ml)*		Fold reduction†
	–Verapamil	+Verapamil†	
PC-9	0.01 \pm 0.002	0.0093 \pm 0.001	1.07
PC-9/VCR	0.12 \pm 0.007	0.11 \pm 0.01	1.09
SW620	0.031 \pm 0.004	0.03 \pm 0.005	1.03
SW620/MDR	3.2 \pm 0.25	0.1 \pm 0.3	32

* IC_{50} is the drug concentration effective in inhibiting 50% of the cell growth measured by the MTT-dye assay after 4 days of continuous exposure to the drug. Results are means \pm S.D. of three determinations.

† Verapamil concentration was 1 μ g/ml, which did not cause cytotoxic effect.

‡ The ratio of IC_{50} without verapamil divided by IC_{50} with verapamil.

sublethal concentration of verapamil (1 μ g/ml) on VCR toxicity was compared in parental and resistant cells (Table 4). SW620 and SW620/MDR cells were used as controls. The IC_{50} of the two pairs of cell lines is indicated in the presence or absence of verapamil. Resistance to VCR is reduced by verapamil only 1.07- and 1.09-fold respectively in PC-9 and PC-9/VCR cells. By contrast, the resistance of SW620 and SW620/MDR cells to VCR is reduced by verapamil 1.03- and 32-fold respectively. The results indicate that verapamil affected VCR toxicity only in P-glycoprotein-overexpressing SW620/MDR cells, but not in PC-9/VCR cells, supporting the notion that the drug resistance in PC-9/VCR cells is probably not due to P-glycoprotein.

DISCUSSION

In the present study we have established and characterized a VCR-resistant lung-cancer cell subline. The resistant PC-9/VCR cells are highly cross-resistant to the VCR analogues colchicine and vinblastine, but show marginal or lack of resistance to other anticancer agents, including actinomycin D, cisplatin, adriamycin, L-PAM and VP-16. The results indicate that PC-9/VCR cells are especially resistant to microtubule inhibitors. It has previously been demonstrated that VCR, after entering cells, immediately interacts with microtubules [28–31], subsequently

inhibiting the formation of mitotic spindles, thereby preventing cells from dividing [29,31,32]. In addition, VCR may modulate the shape of cells through interaction with the cytoskeleton and may affect functions of the cell membrane [16]. Therefore, control by the cell membrane of VCR accumulation may play a role in determining the drug's effect on cells. It is conceivable that loss of cell response to VCR may result from an altered membrane function. This is supported by studies on drug accumulation (see Figures 3 and 4) showing that PC-9/VCR cells displayed a reduced drug uptake and an increased drug efflux. It is worth noting that altered drug accumulation in PC-9/VCR cells is insensitive to verapamil, an effective inhibitor of the P-glycoprotein pump. In addition, PC-9/VCR cells do not overexpress P-glycoprotein. Taken together, these results strongly suggest that the altered membrane function of PC-9/VCR cells is unlikely to be dependent on P-glycoprotein. Recently, Cole et al. [33] have isolated a drug transporter gene from human small-cell lung cancer cells that is distinct from P-glycoprotein. The mRNA of the gene encodes a member of the ATP-binding cassette transmembrane transporter superfamily. The overexpression of this gene in MDR HeLa cells and inherently resistant non-small-cell lung-cancer cell lines suggests that this protein may play a role in resistance in the PC-9/VCR cells.

Interestingly, ascorbic acid effectively inhibited the resistance of PC-9/VCR cells to VCR. It has been shown that the oxidized form of ascorbic acid alone inhibits the growth of Ehrlich ascites tumour, L1210 leukaemia, P388 leukaemia, and prolongs animal lifespan [34,35]. Furthermore, it has recently been reported that ascorbic acid can serve as an essential antioxidant in the presence of severe GSH deficiency, and that it protects animals against cataracts [36]. Although the exact mechanism whereby ascorbic acid sensitizes PC-9/VCR cells to VCR is not clear, it is possible that the drug-accumulation-associated membrane activity detected in this study is modulated by ascorbic acid through an oxidation mechanism. Drug accumulation analyses indicate that the reduced drug uptake in PC-9/VCR cells is restored by ascorbic acid and is associated with the loss of VCR resistance. The results suggest that the altered membrane drug-transport activity in resistant cells is important in determining VCR resistance. Since ascorbic acid also has an inhibitory effect on the resistance and the uptake of colchicine in PC-9/VCR cells (E.-J. Song, C. D. Chiang, V. C. Yang and C. C.-K. Chao, unpublished work), the altered membrane activity is likely also to be responsible for 'gating' colchicine from the cells. Thus the resistant PC-9/VCR subline is especially useful in studying responses of cells to microtubule inhibitors. Perhaps alteration of this ascorbic acid-sensitive membrane activity is an early step towards the establishment of MDR phenotype. However, the membrane activity is unlikely to be due to P-glycoprotein, since the drug-sensitivity of the MDR colon-cancer cells (described in [32]) is not affected by ascorbic acid. This novel mechanism of drug resistance may be an additional resistance pathway encountered in clinical cancer therapy.

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