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Reduced influx is a factor in accounting for reduced vincristine accumulation in certain verapamil-hypersensitive multidrug-resistant CHO cell lines

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The rates of accumulation, influx and efflux of vincristine have been examined in a series of multidrug-resistant Chinese hamster ovary cell lines which show exceptionally high levels of hypersensitivity (collateral sensitivity) to several resistance modifiers. The more highly resistant members of the series show significantly reduced levels of vincristine influx compared to the control cell line from which they were derived. It is possible that resistance modifier hypersensitivity and reduced vincristine influx may be due to a common change in membrane composition which has arisen during prolonged selection for vincristine resistance in these cell lines.

Multidrug resistance (CHO cells); Vincristine; Drug influx

1. INTRODUCTION

Multidrug resistance involves the simultaneous development of resistance to a wide range of structurally and functionally unrelated cytotoxic drugs to which cells have not previously been exposed [1,2]. Multidrug resistant cells characteristically overexpress a group of plasma membrane glycoproteins with an M_r of 170,000–180,000, termed P-glycoproteins [3,4] encoded by a family of three closely related genes in rodents and two genes in humans. Multidrug resistance can usually be reversed by a range of compounds which are known collectively as resistance modifiers, the most extensively studied of which is verapamil [5]. P-glycoproteins are thought to act as energy-dependent efflux pumps and the reduced drug accumulation which is commonly observed in multidrug resistant cell is normally attributed primarily to increased drug efflux consequent upon the elevated levels of P-glycoprotein in the cell membranes of these cells. However, some studies have indicated that the mechanism of reduced accumulation may be more complex in some cases, possibly involving reduced influx or redistribution of drugs within the cell. For example, Sirotonak et al. [6] observed a 24-fold decrease in influx of vincristine but only a 2-fold increase in vincristine efflux in highly vincristine resistant (2,750-fold) Chinese hamster lung cells. Trans-inhibition studies showed that efflux of preloaded, unlabelled drug inhibited the influx of labelled drug, suggesting that influx and efflux pathways interact and are not completely independent. Bradley et al. [1], in reviewing the mechanisms underlying multidrug resistance, concluded that the available experimental data on drug transport suggested that the two proposed mechanisms of drug efflux and drug influx permeability barrier are not mutually exclusive, but may represent different aspects of a pleiotropic alteration in membrane function that results in reduced drug accumulation. Jardillier et al. [7] have recently reported a reduction in vinblastine influx in vinblastine resistant human leukaemic lymphoblast CEM cells.

In the present study, we have analysed the rates of vincristine influx and efflux in multidrug resistant Chinese hamster ovary cell lines which show much lower levels of vincristine resistance than those studied by Sirotonak et al. [6]. These cell lines comprise the VRT series, VRT5, VRT15, and VRT25, which were selected by 5, 15 and 25 sequential exposures to increasing concentrations of vincristine respectively, and the VRA series, VRA5 and VRA15, which were independently selected by 5 and 15 rounds of vincristine selection, respectively. We have previously shown that the cell lines obtained after 15 and 25 rounds of selection exhibit an unusually high degree of hypersensitivity (collateral sensitivity) to verapamil and to other resistance modifiers such as nicardipine, diltiazem and quinidine sulphate [8,9]. The cell lines VRA15 and VRT15 have been shown to have reduced verapamil accumulation [10].

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Abbreviations: CHO, Chinese hamster ovary; MDR, multidrug resistant.

They are cross-resistant to cyclosporin A, which can act as a multidrug resistance modifier [11].

In the present work, measurement has been made of the vincristine accumulation, influx and efflux on these cell lines in order to broaden our understanding of the membrane physiology of these multidrug resistant cell lines which show an exceptional level of hypersensitivity to resistance modifiers, and also to complement Sirotnak's studies [6] on very much more highly vincristine resistant hamster cell lines.

2. EXPERIMENTAL

The origin of the cell lines VRA5, VRA15, VRT5, VRT15 and VRT25 has been described previously [8,9]. VRA15 and VRT15 were referred to as VCR/A and VCR/T respectively in [8]. Cell lines were cultured in Glasgow MEM with 10% foetal calf serum in a 5% CO_2 humidified incubator.

For accumulation, influx and efflux measurements, cells were grown in monolayer culture and harvested by 5 min exposure to 0.25% trypsin. After addition of an equal volume of medium, the cell suspension was pelleted by centrifugation, washed in 2 ml medium containing 20 mM HEPES (pH 7.2) and resuspended in 3 ml of medium containing HEPES.

For accumulation and influx measurements, the cells were incubated with shaking for 1 h to recover from the trypsin treatment. To commence the experiment, cold and [^{3}H]vincristine (Amersham) were added to a total concentration of 10 μM and 0.025–0.04 MBq/ml. Duplicate samples were removed at time intervals and rapidly added to 10-fold volume of ice-cold 0.14 M NaCl, 0.01 M KPO_4 pH 7.2 buffered isotonic saline. The cells were washed twice by centrifugation and resuspension in ice-cold buffered saline. Finally the cell pellet was resuspended in 1% (w/v) SDS to lyse the cells before scintillation counting. For accumulation measurements, the initial drug bound to the cells during the first 15 s of the experiments was excluded from the calculation. For comparisons of intracellular drug concentrations, drug accumulation per cell was converted to drug concentrations for each cell line using cell volume measurements made with a Coulter ZM counter.

For efflux experiments, cells prepared as above were preloaded by incubation with gentle shaking for 1 h with 10 μM vincristine, containing between 0.025–0.06 Mq/ml [^{3}H]vincristine. After this period, cells were pelleted and washed briefly in ice-cold PBS solution. Finally, the pellet was resuspended in 2.5 ml of prewarmed (37°C) medium containing HEPES and transferred to a 37°C shaking waterbath. At various time intervals, duplicate aliquots were removed and spun in a microcentrifuge for 30 s and the pellets washed briefly before being resuspended in 1% (w/v) SDS prior to scintillation counting. Significance testing of differences between influx or efflux rates of different cell lines was performed by covariance analysis using a program written by Dr. J.G. Hastewell.

3. RESULTS

3.1. *Vincristine accumulation*

The time course of vincristine accumulation by the sensitive cell line E29 and the VRT series of multidrug-resistant cell lines is shown in Fig. 1. It can be seen that that accumulation is rapid for the first 10 min, after which time the rate of accumulation is considerably reduced. The independently selected cell lines, VRA5 and VRA15 showed very closely similar time course for vincristine accumulation as VRT5 and VRT15 respectively. It has been shown previously by HPLC analysis that [^{3}H]vincristine is not metabolised in CHO cells [12], so the accumulated material is assumed to be in the form of the original drug. Values for total drug accumulation for all cell lines after 30 min, expressed as a ratio of intracellular to extracellular drug concentrations, are presented in Table I. It can be seen from Fig. 1 and Table I that there is an inverse correlation between drug accumulation and vincristine resistance levels. The least resistant members, VRA5 and VRT5, show only slight reduction in drug accumulation. The moderately resistant cell lines VRA15, VRT15 and VRT25 do have a highly significant reduction in accumulation, as would be expected. However, although VRT25 is a more highly resistant derivative of VRT15, we were not able

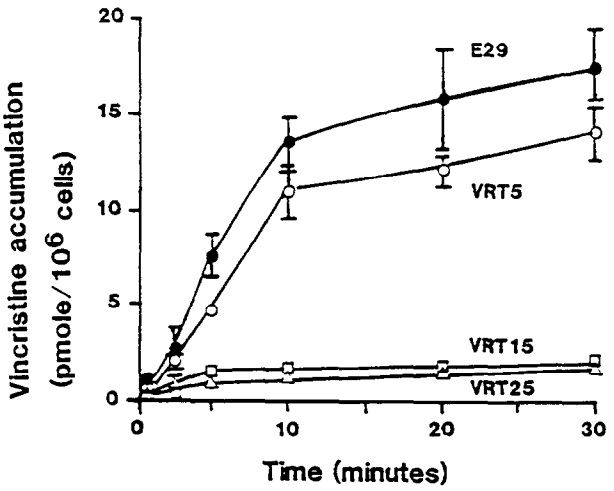

Fig. 1. Time course of vincristine accumulation by the sensitive cell line, E29 and by the multidrug-resistant cell lines, VRT5, VRT15 and VRT25, incubated in 10 μM vincristine at 37°C. Each point represents the mean of 6 measurements. Vertical lines represent the S.E.M.

Table I

Vincristine accumulation, efflux and influx of the VRA and VRT series of multidrug resistant CHO cells

Cell line	Vincristine resistance	Vincristine accumulation	Vincristine efflux	Vincristine influx
E29	(1)	(1)	(1)	(1)
VRA5	3.2	0.88	1.10	1.15
VRA15	61	0.13***	0.89	0.21**
VRT5	9.7	0.57**	0.81	0.81*
VRT15	58	0.07***	0.63	0.21***
VRT25	87	0.08***	0.86	0.14***

All values are expressed relative to the sensitive cell line, E29. Significance levels are given for each value relative to the corresponding value for E29, with * for 5%, ** for 1% and *** for 0.1% significance levels, respectively. Tests of significance are by Student's *t*-test for accumulation and by covariance analysis for efflux and for influx. Values for relative vincristine resistance levels have been published previously [9], and are given for comparison with the accumulation, efflux and influx data.

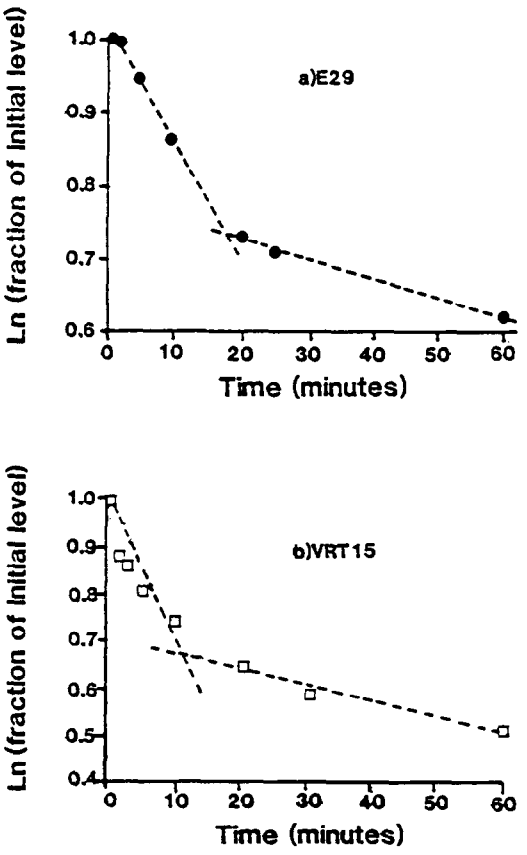

Fig. 2. Loss of vincristine from sensitive cells, E29, and multidrug-resistant cells, VRT15, following 30 min preloading with 10 μM vincristine. Each point is the mean of 6 measurements.

to detect a significant difference in the levels of vincristine accumulation between these two cell lines.

3.2. *Vincristine efflux*

Preliminary experiments showed that there was no detectable drug efflux from sensitive or resistant cell lines at 0°C (data not shown), although very rapid efflux occurred at 37°C from both sensitive and resistant cell lines. This preliminary experiment established that efflux did not continue during the washing on stop buffer, and was necessary before performing experiments to determine the contribution of increased efflux to the reduced accumulation of vincristine at 37°C. Efflux rates at 37°C were shown to be biphasic in sensitive and resistant cells (Fig. 2). This phenomenon has been reported previously by Sirotonak et al. [6] and it is thought that the initial rapid component of 10–15 min represents efflux of exchangeable, unbound drug, whereas the subsequent second, slower component represents the rate of dissociation (off rate) of intracellularly bound drug. The rates of efflux of free drug from the cell lines were therefore measured during the first 10 min of efflux and are given in Table I. Slight differences in efflux rates exist in the resistant cell lines in comparison with the sensitive cell line E29, but none of the differences were found to be significant at the 5% probability level by covariance analysis.

3.3. *Vincristine influx*

Influx measurements were performed in order to assess the role of changes in influx on the decreased accumulation observed in these multidrug resistant cell lines. During the period of 5 min in which the measurements were made, entry of the drug was linear with time at a concentration of 10 μM vincristine in parental and drug resistant cell lines (Fig. 3). There was a linear relationship between the external concentration of vincristine and the rate of vincristine influx over a wide range of concentrations (Fig. 4).

Rates of vincristine influx of the sensitive cell line, E29 and the resistant cell lines of the VRA and VRT series are shown in Table I. The low level resistant cell lines VRA5 and VRT5 have similar rates of influx to those of E29 cells. However, the rate of influx is reduced fivefold in VRA15 and in VRT15 and sevenfold in VRT25. Overall, there is a very strong negative correlation (correlation coefficient, −0.95) between increased levels of resistance and influx rates.

4. DISCUSSION

The multidrug resistant CHO cell lines studied here show a reduction in vincristine accumulation which is approximately proportional to resistance levels, as has been widely observed for many multidrug resistant cell lines [1]. There is not a simple linear relationship between the two, however, because we did not detect a difference in the levels of vincristine accumulation between VRT15 and VRT25, although they have a significantly different level of resistance.

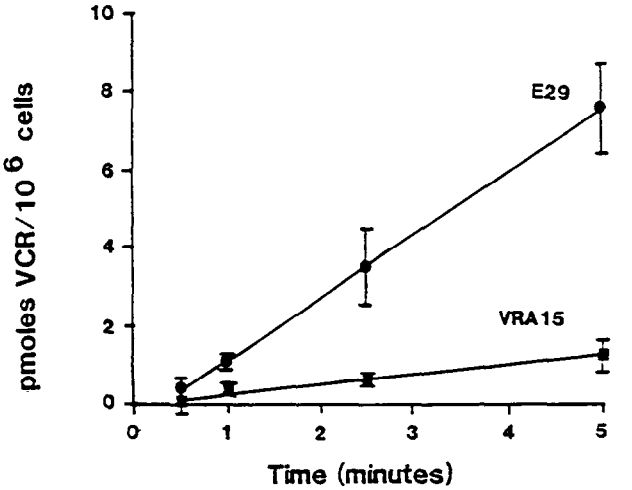

Fig. 3. Time course for the influx of vincristine at 37°C in the sensitive cell line, E29, and the multidrug-resistant cell line, VRA15, during incubation in 10 μM vincristine. Each point represents the mean of 6 measurements. Vertical lines represent the S.E.M.

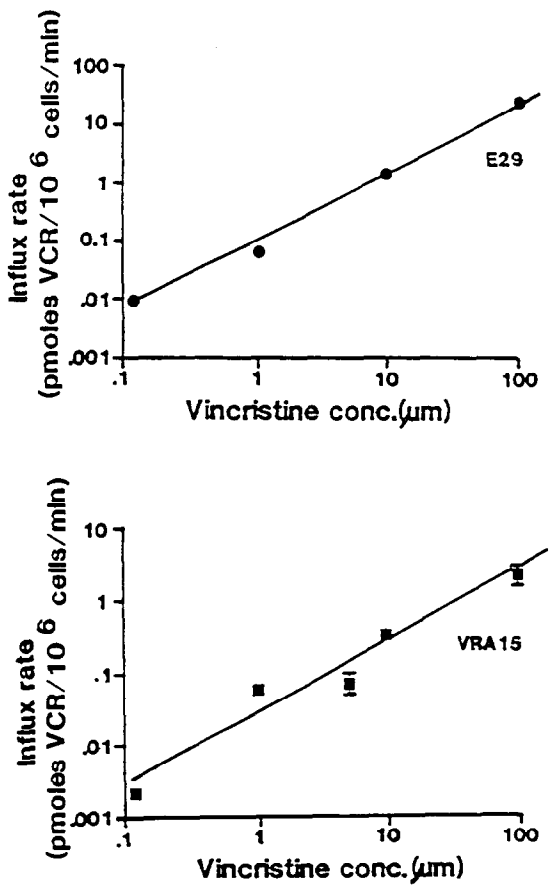

Fig. 4. Concentration dependence of vincristine influx in the sensitive cell line, E29, and the multidrug-resistant cell line, VRA15 at 37°C. Each point represents the mean of 6 measurements. Vertical lines represent the S.E.M.

The unusual feature of the reduction in drug accumulation observed in these cells is that it does not appear to be simply attributable to increased drug efflux and there is an appreciable reduction in influx in all except the least resistant cell lines. These results complement earlier observations by Sirotnak et al. [6] who were working with very much more highly vincristine resistant cells. These authors observed that in Chinese hamster lung cells, which were approximately thirty fold more resistant than the most highly resistant cell lines studied here (2,750-fold compared with 87-fold), decreased vincristine accumulation involved a 24-fold reduction in influx, and only a twofold increase in vincristine efflux. Our results suggest that this general conclusion also holds for some hamster cell lines with a very much lower level of vincristine resistance.

It is of interest that the multidrug resistant cells studied by Sirotnak were selected on the basis of vincristine resistance, as in the case of the cells studied in this work, and also that the daunomycin cross-resistance of the cells studied by Sirotnak did not involve any change in daunomycin influx. It may therefore be possible that the involvement of reduced drug influx is a feature of vincristine resistance in a subset of multidrug resistant cells which have been selected for resistance by prolonged culture in vincristine, not occurring in multidrug resistance to other drugs or selected by exposure to other drugs. It is possible to speculate on how this may arise. Vinca alkaloids are known to interact directly with membranes to induce changes in phospholipid composition [13] and in physical properties [14,15]. These drug induced changes are likely to have detrimental effects on the normal functioning of the cell membranes and hence on cell growth and survival. During prolonged exposure to high vincristine concentrations, selection pressure will be exerted in favour of those variants which have compensatory changes in membrane composition and structure which go some way to reverse the drug induced membrane abnormalities. Thus, multidrug resistant cells selected by prolonged exposure to vincristine may have characteristic membrane changes which in turn may influence some aspects of membrane functioning, possibly including those involved in vincristine influx. Sirotnak et al. [6] have presented evidence suggesting that a carrier is involved in influx of vincristine. The speculated membrane changes outlined above may have led to reduced accessibility of such a carrier and hence reduced influx of vincristine.

Although there have been several reports of multidrug resistant cell lines showing hypersensitivity (collateral sensitivity) to resistance modifiers [16–18], cell lines of this series have higher levels than is generally observed in other multidrug resistant cell lines. Hypersensitivity arises in these cells to a wide range of modifiers, which include the calcium channel blockers verapamil, diltiazem and nicardipine and the membrane active agent, quinidine sulphate. It has been proposed that this hypersensitivity arises in cells of this series due to the presence of abnormal composition and structure of membranes which render them unusually sensitive to membrane destabilising effects of resistance modifiers [8–10]. Such membrane abnormalities may be the same, or share some common features with, those membrane abnormalities which are proposed here to contribute to reduced vincristine influx. In order to explore this possibility further, it would be of interest to examine whether the highly vincristine resistant cell lines with reduced influx studied by Sirotnak [6] exhibit hypersensitivity to resistance modifiers.

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REFERENCES

[1] Bradley, G., Juranka, P.F. and Ling. V. (1988) Biochim. Biophys. Acta 948, 87–128.

[2] Beck, W.T. and Danks, M. (1991) in: Molecular and Cellular Biology of Multidrug Resistance in Tumor Cells (I.B. Roninson, ed.) pp. 3–55, Plenum, New York.
[3] Juranka, P.F., Zastawny, R.L. and Ling, V. (1989) FASEB J. 3, 2583–2592.
[4] Roninson, I.B. (1991) in: Molecular and Cellular Biology of Multidrug Resistance in Tumor Cells (I.B. Roninson, ed.) pp. 189–214, Plenum, New York.
[5] Tsuruo, T. (1991) in: Molecular and Cellular Biology of Multidrug Resistance (I.B. Roninson, ed.) pp. 349–372, Plenum, New York.
[6] Sirotnak, F.M., Yang, C., Mines, L.S., Orib, E. and Biedler, J.L. (1986) J. Cell. Physiol. 126, 266–274.
[7] Jardillier, J.C., Warren, L., Colin, M. and Madoulet, C. (1992) Proc. Am. Assoc. Cancer Res. 33, 474.
[8] Warr, J.R., Brewer, F., Anderson, M. and Fergusson, J. (1986) Cell. Biol. Int. Rep. 10, 389–399.
[9] Stow, M.W. and Warr, J.R. (1991) Biochim. Biophys. Acta 1092, 7–14.
[10] Warr, J.R., Anderson, M. and Fergusson, J. (1988) Cancer Res. 48, 4477–4483.
[11] Twentyman, P.R., Fox, N.E. and Bleehen, N.M. (1986) Int. J. Radiat. Oncol. Biol. Phys. 12, 1355–1358.
[12] Meyers, M.B., Kreis, W.M., Degnan, T.J. and Biedler, J.L. (1983) Proc. Am. Assoc. Cancer Res. 24, 42.
[13] Schroeder, F., Fontaine, R.N., Feller, D.J. and Weston, K.G. (1981) Biochim. Biophys. Acta 643, 76–88.
[14] Ter-Minassian-Saraga, L., Madelmont, G., Hort-Legrand, C. and Metral, S. (1981) Biochem. Pharmacol. 30, 411–415.
[15] Ter-Minassian-Saraga, L. and Madelmont, G. (1983) Biochim. Biophys. Acta 728, 394–402.
[16] Cano-Gauci, D.F. and Riordan, J.R. (1987) Biochem. Pharmacol. 36, 2115–2123.
[17] Cano-Gauci, D.F. and Riordan, J.R. (1991) in: Molecular and Cellular Biology of Multidrug Resistance in Tumor Cells (I.B. Roninson, ed.) pp. 337–347, Plenum, New York.
[18] Twentyman, P.R., Fox, N.E. and White, D.J.G. (1987) Br. J. Cancer 56, 55–57.