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Research Article

Saturable Process Involved in Active Efflux of Vincristine as a Mechanism of Multidrug Resistance in P388 Leukemia Cells

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Kinetic analysis of vincristine (VCR) efflux in multidrug-resistant and parental P388 leukemia cells was performed to investigate the difference in activity between the two cell lines. Efflux velocities of VCR were directly determined from the slope of the initial release of drug induced by resuspending the preloaded cells in VCR-free medium, representing unidirectional efflux from intracellular free or loosely bound drug pools. Further, the equilibrium binding of VCR to whole-cell homogenates was analyzed by ultrafiltration to estimate intracellular unbound drug concentrations. A two-site binding model was found to fit the data best for both cell lines, and depletion of ATP by the addition of apyrase decreased binding. The binding parameters were similar between the two cell lines. A Hofstee plot of efflux demonstrated the existence of both linear and saturable transport of VCR in both cell lines. The greater maximum velocity observed with VCR efflux in the resistant cells suggests that an increased number of transporters causes greater activity of this process in the resistant cells.

KEY WORDS: multidrug resistance; active efflux; vincristine; kinetic analysis; carrier-mediated transport.

INTRODUCTION

Drug resistance, which is a major problem in cancer chemotherapy, has been studied using drug-resistant cell lines as an experimental model of this phenomenon (1,2). Resistant cell lines often exhibit cross-resistance to a broad range of agents such as anthracyclines, vinca alkaloids, colchicine, and etoposide, which is called multidrug resistance (3–5). Energy-dependent reduced uptake of such drugs has been reported as a mechanism in most multidrug-resistant cell lines (6–9).

Several hypothetical mechanisms of the reduced drug uptake have been proposed, for example, the active efflux-pump model (6,8–10), reduced drug-binding model (11), and active permeability barrier model (7). Of these, the active efflux-pump model seems the most likely mechanism for multidrug resistance by biochemical studies on a membrane glycoprotein, called P-glycoprotein or P-170, which is overexpressed in most multidrug-resistant cell lines (12). Cell-transferred genes of this molecule demonstrated the multidrug-resistant phenotype (13,14). In addition, a photoaffinity analogue of vinca alkaloid was found to bind to P-glycoprotein (15,16). Based on the strong homology of the amino acid sequence between P-glycoprotein and bacterial active transport proteins, its function was suggested to be active transport (17–19).

The kinetic basis of this transport still remains unclear. In a previous report (20), we determined the kinetics of active efflux of VCR,[4] chosen as a model drug, in multidrug-resistant and parental P388 leukemia cells by indirect assessment of both efflux and intracellular binding. Saturable efflux in both cell lines and a greater maximum velocity and lower Michaelis constant in the resistant cells were observed in that study. However, those analyses assumed that both influx and binding of VCR are not influenced by cellular ATP suppression. It was thus desirable to analyze both efflux and binding based on their direct assessment to elucidate the kinetic basis of the active efflux. In this report, we attempted to analyze directly the outward transport phenomenon of VCR according to the following methods. First, the velocity of VCR efflux is determined by the initial release rate of VCR from the drug-loaded cells at varied intracellular concentrations suspended in VCR-free medium. Second, analysis of the equilibrium binding of VCR to the whole-cell homogenate determined by ultrafiltration is investigated to estimate the intracellular free concentration of VCR. Finally, we analyzed the relationship between the velocity of efflux and the intracellular free concentration of VCR.

MATERIALS AND METHODS

Chemicals [^{3}H]VCR sulfate (4.4–6.1 Ci/mmol) and

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[4] Abbreviations used: VCR, vincristine; P388, P388 leukemia cells; P388/ADR, adriamycin-resistant P388 leukemia cells; HBSS, Hanks' balanced salt solution; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; DNP, 2,4-dinitrophenol.

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^3H_2O (5 mCi/ml) were purchased from Amersham International plc (Buckinghamshire, England). [^{14}C]Inulin (3.0 mCi/g) was purchased from New England Nuclear Corporation (Boston, Mass.) and used after reexamination of its radiochemical purity. Unlabeled VCR sulfate (Oncovin) was purchased from Shionogi & Co., Ltd., Osaka, Japan. All other chemicals were obtained commercially and were analytical grade.

Cells. P388 and P388/ADR cell lines were supplied by the Mammalian Genetics and Animal Production Section, Division of Cancer Treatment, National Cancer Institute (Bethesda, Md.). P388/ADR having multidrug-resistant phenotypes were cross-resistant to VCR, e.g., concentrations giving 50% inhibition of cell growth *in vitro* obtained by a 1-hr exposure to VCR in P388 and P388/ADR were 0.01 and 0.64 μ*M*, respectively, and lower accumulation of VCR was observed with P388/ADR as compared with P388, as reported previously (5,10). P388 and P388/ADR were maintained in DBA/2 and BALB/c × BDA/2 ($CD2F_1$) mice (Charles River Japan, Atsugi), respectively, by weekly i.p. transplantation of 10^6 cells/mouse.

Efflux Studies. Ascites fluid was collected from the peritoneal cavity of tumor cell-bearing mice 7 days after inoculation of 10^6 cells. The harvested cells were washed two or three times with cold HBSS without glucose and were initially suspended in glucose-free HBSS containing 15 m*M* HEPES (glucose-free incubation buffer, pH 7.3) to a density of 8×10^6 cells/ml. Cells were pre-loaded with VCR in glucose-free incubation buffer containing 1 m*M* DNP to obtain a high level of intracellular VCR, because P388/ADR accumulate only low levels of intracellular VCR due to the active efflux when cells are incubated in glucose-containing medium without DNP (normal condition). An equal volume of glucose-free incubation buffer containing 2 m*M* DNP was added to the cell suspension. After temperature equilibration at 37°C for 5 min, a small volume of both [^{3}H]VCR and cold VCR solution was added to produce various extracellular concentrations of VCR (0.06–20 μ*M*). Following incubation at 37°C for 25 min, preloading with VCR was terminated by rapid chilling at 4°C. After centrifugation at 1000*g* for 1 min, the supernatant was decanted and cells were washed with ice-cold glucose-free incubation buffer. Efflux was initiated by resuspending cells in prewarmed either normal incubation buffer containing glucose or glucose-free incubation buffer containing 1 m*M* DNP. Cells were separated from the medium at each sampling time according to our previous report (20). Briefly, 1-ml aliquots of cell suspension were transferred onto an oil-layered 1.5-ml microcentrifuge tube, followed by rapid centrifugation at 14,000 rpm for 30 sec in a Kubota microcentrifuge (Model KM-152000). One-half milliliter of 0.5 *N* KOH was added to dissolve cells and then left overnight. The radioactivity was then determined using a Beckmann Model LS-355 liquid scintillation counter in 10 ml of Aquasol-2 (New England Nuclear, Boston, Mass.). The velocity of the VCR efflux under normal conditions, when cells were incubated in glucose-containing medium without DNP, was determined from the slope of the initial linear phase (10–30 sec) in the release of VCR from drug-loaded cells.

Binding Studies Cells were suspended in 15 m*M* HEPES buffer (pH 7.3) with 2 m*M* $CaCl_2$ at a density of 2.67 × 10^8 cells/ml and incubated at 37°C for 5 min. Swollen cells were homogenized by 50 strokes in a Dounce homogenizer and the whole-cell homogenate was diluted with fourfold concentrated incubation buffer so as to achieve a density of 2×10^8 cells/ml. Specific binding of VCR to the whole-cell homogenate was determined by filtration through DE-81 disks (21) (Watman Ltd., Maidstone, England). The whole-cell homogenate (final density of 10^8 cells/ml) in incubation buffer with 0.1 m*M* GTP (binding buffer) in the presence or absence of 2 m*M* ATP was preincubated at 37°C for 10 min for the temperature equilibration and then [^{3}H]VCR was added. Two hundred-microliter aliquots of the homogenate were removed at 10, 20, 30, and 40 min and were filtered through DE-81 disks, followed by washing with 2 ml cold binding buffer. These filtration procedures were completed within 15 sec. In some experiments, apyrase (Sigma Chemical Co., Ltd., Dorset, U.K.) was added at a final concentration of 10 U/ml 20 min later to deplete external and endogenous ATP. Nonspecific binding was determined by blank experiments in the presence of excess cold VCR (100 μ*M*) and was subtracted from the total binding at each sampling time point.

Equilibrium binding of VCR to the whole homogenate was determined by an ultrafiltration method (22). To examine ATP dependence of VCR binding to the homogenate, neutralized ATP solution or apyrase was added at a final concentration ranging from 0.1 to 10 m*M* or 10 U/ml. These samples were incubated at 37°C for 30 min to reach equilibrium, as described for the filtration method. Five hundred-microliter aliquots were transferred to the Amicon ultracentrifuge filter device (MSP-3) and were centrifuged at 2000*g* for 30 min at 37°C. To decrease interaction between VCR and filters, the filters were precoated with bovine serum albumin solution. Blank experiments were performed in the absence of the homogenate. The free and total concentrations of VCR were obtained by measuring radioactivities of the ultrafiltrates and filters, respectively, and bound VCR was determined and corrected by the use of these obtained concentrations and blank value. Scatchard analysis was investigated by the use of data about equilibrium binding of VCR to the whole-cell homogenate in the presence of 2 m*M* ATP at various concentrations of VCR, ranging from 0.01 to 100 μ*M*.

Determination of ATP Levels. ATP was determined by the method of Stanley and Williams (23) with minor modifications using the ATP analytical kit (Analytical Luminescence Laboratories, Inc., San Diego). Cells suspended in either glucose-free incubation buffer containing 1 m*M* DNP or normal incubation buffer were incubated at 37°C for 30 min. The cells were transferred into cuvettes and Extralight in Firelight buffer, pH 7.75 (Analytical Luminescence Laboratories, Inc.) was added. The cuvette was kept for 2 min at room temperature to release cellular ATP. Luciferine-luciferase (Firelight) was added and the emitted light at 560 nm was determined in a Monolight 500. A standard solution of ATP was used for the calibration curve. To calculate the intracellular concentration of total ATP, intracellular volume was determined by the method of Beck *et al.* (11) using ^3H_2O and [^{14}C]inulin as an extracellular marker.

RESULTS

Efflux of VCR from drug-loaded cells was suppressed

more significantly in both P388 and P388/ADR cells resuspended in glucose-free medium containing 1 m*M* DNP than in control medium, and a greater rate of efflux was observed in P388/ADR cells compared with P388 cells in glucose-containing medium without DNP (Figs. 1 and 2). The cellular ATP level was suppressed in both P388 and P388/ADR cells (Table I), when cells were incubated in glucose-free medium containing 1 m*M* DNP. The velocity of VCR efflux was determined from the initial release rate up to 30 sec (Fig. 2). Various degrees of efflux were plotted against varied intracellular drug levels in P388 and P388/ADR cells (Fig. 3), demonstrating enhanced efflux activity in P388/ADR as compared with P388 cells.

VCR binding to the whole-cell homogenate was also investigated. The binding, determined by the filtration method, was increased in both P388 and P388/ADR cells in the presence of 2 m*M* ATP, similar to intracellular concentrations of ATP (Table I). Bound fractions of VCR were similar between P388 and P388/ADR cells in the presence or absence of ATP (Fig. 4). The addition of apyrase at a final concentration of 10 U/ml to deplete external and endogenous ATP clearly decreased the binding, and the binding determined by the filtration method would reflect only tight binding. Similar results were also obtained with equilibrium binding of VCR as determined by the ultrafiltration method, which gives both tight and loose bindings (Fig. 5). Furthermore, no difference was observed in the dissociation rate of VCR bound to the whole-cell homogenate between the two cell lines (data not shown).

Scatchard analysis of equilibrium binding of VCR determined by the ultrafiltration method was made with different densities of the homogenates, 10^8 and 2×10^8 cells/ml, to ascertain that binding is not influenced by dilution of the homogenate. No difference was observed in either cell line between the two densities of the cell homogenates (Fig. 6), if the bound amounts of VCR were normalized by the cell densities. The two-site binding model exhibited the best fitting among several binding models based on statistical consideration using the AIC value calculated by the nonlinear least-squares regression method (24). Accordingly, VCR bound to the whole-cell homogenate could be expressed as follows:

$$A_{bound} = \frac{n_1 \times C_{free}}{K_{d1} + C_{free}} + \frac{n_2 \times C_{free}}{K_{d2} + C_{free}} \quad (1)$$

where K_{d_1} and n_1 are the dissociation constant and the binding capacity for the high-affinity site, respectively, K_{d_2} and n_2 are those for the low-affinity site, and A_{bound} and C_{free} are the amount of bound VCR and the concentration of free VCR, respectively. Estimation of the binding parameters to the whole-cell homogenate was made by the use of a nonlinear least-squares regression method (Table II). No difference was observed with the parameters for the high-affinity site. On the other hand, the binding parameters (K_{d_2}, n_2) for the low-affinity site in P388 cells were about twofold greater than those in P388/ADR cells.

This analysis made it possible to estimate the intracellular free concentration of VCR ($C_{cell,free}$). Based on analysis

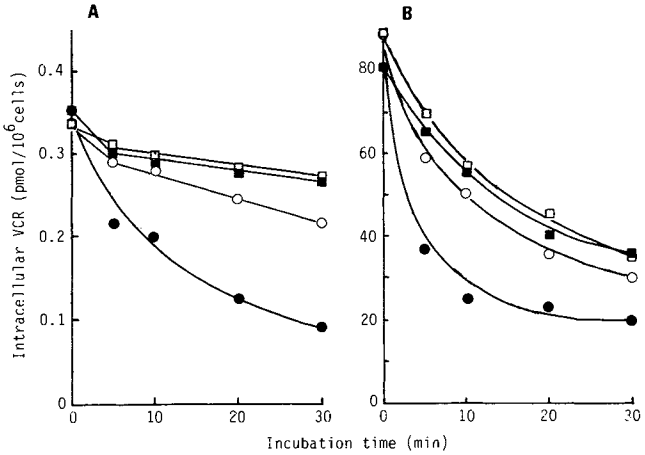

Fig. 1. Release of VCR from P388 and P388/ADR. Cells of P388 (○, □) and P388/ADR (●, ■) were loaded with [^{3}H]VCR at 0.04 μ*M* (A) or 60 μ*M* (B) in glucose-free incubation buffer with 1 m*M* DNP for 25 min. Release of VCR was measured as described under Materials and Methods. Cells were incubated in VCR-free buffer with glucose and without DNP (○, ●) or without glucose and with 1 m*M* DNP (□, ■). Each point represents the mean of triplicate determinations.

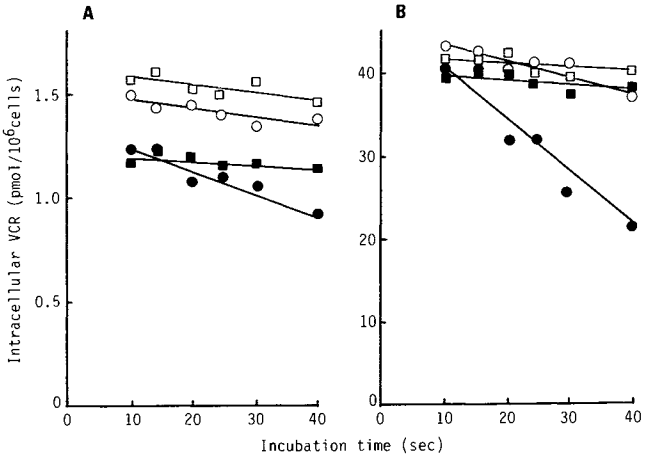

Fig. 2. Initial time course of VCR release from P388 and P388/ADR. Cells of P388 (○, □) and P388/ADR (●, ■) were loaded with [^{3}H]VCR at 0.1 μ*M* (A) or 30 μ*M* (B) as shown in Fig. 1. Cells were incubated in VCR-free buffer with glucose and without DNP (○, ●) or without glucose and with 1 m*M* DNP (□, ■). Intracellular VCR was determined in triplicate. See Materials and Methods for further details.

Table I. Cellular ATP Levels Under Normal or ATP-Suppressed Conditions

Medium condition	Cell line	Cellular ATP (pmol/10^6 cells)	Intracellular ATP (m*M*)[a]
Normal	P388	603 ± 31	1.39 ± 0.07
	P388/ADR	457 ± 15	1.03 ± 0.03
ATP suppressed[b]	P388	35.3 ± 1.8	0.082 ± 0.004
	P388/ADR	24.4 ± 1.9	0.055 ± 0.004

[a] Calculated using intracellular space.
[b] Glucose-free HBSS containing 1 m*M* DNP.

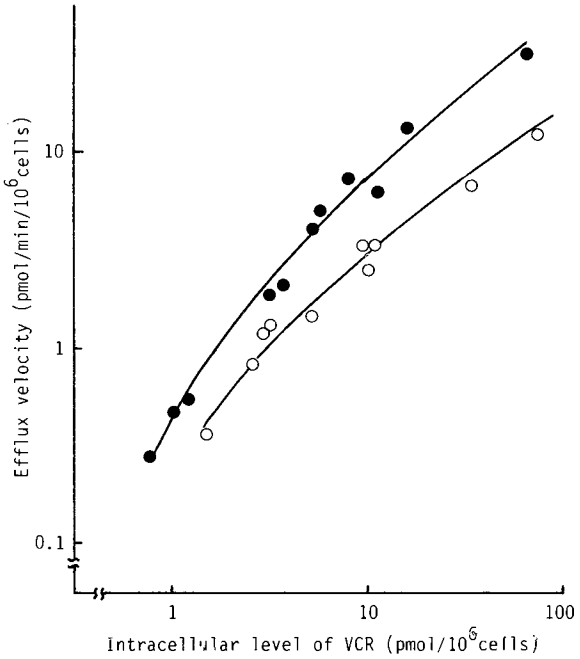

Fig. 3. Relationship between initial intracellular VCR levels and velocity of VCR efflux. Velocity of VCR efflux was determined from the slope of the initial release of VCR up to 30 sec, which was induced by resuspending the cells preloaded with [^{3}H]VCR at various intracellular levels in VCR-free incubation buffer. P388, ○; P388/ADR, ●.

of equilibrium binding to the whole-cell homogenate, intracellular VCR (A_{cell}) is expressed as follows:

$$A_{cell} = \frac{n_1 \times C_{cell,free}}{K_{d1} + C_{cell,free}} + \frac{n_2 \times C_{cell,free}}{K_{d2} + C_{cell,free}} + V \times C_{cell,free} \quad (2)$$

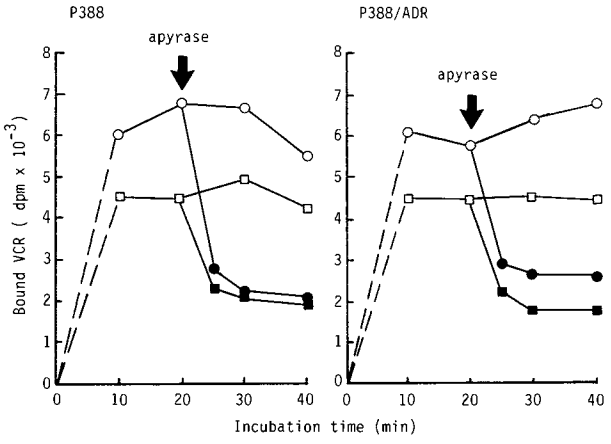

Fig. 4. Binding of VCR to the whole-cell homogenate determined by the filtration method. The whole-cell homogenate prepared from P388 (left) or P388/ADR (right). Binding of VCR to the cell homogenate at 37°C with (○) or without (□) 2 m*M* ATP was determined in triplicate by the filtration method. To deplete external and endogenous ATP, apyrase was added at a final concentration of 10 U/ml 20 min after the beginning of incubation (●, ■). The standard deviation of each point was less than 15% of the mean value.

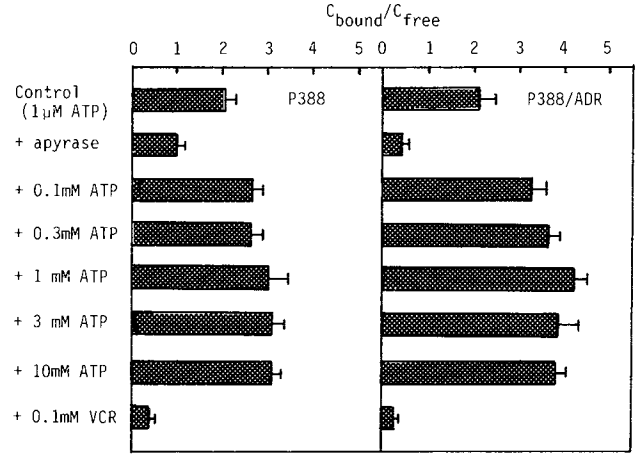

Fig. 5. Binding of VCR to the whole-cell homogenate determined by the ultrafiltration method. The whole-cell homogenate, prepared from P388 (left) or P388/ADR (right), was incubated with [^{3}H]VCR in the presence or absence of varied concentrations of ATP. In some experiments, apyrase was added at a final concentration of 10 U/ml to deplete endogenous ATP. The ratio of bound to free VCR concentration was determined by the ultrafiltration method. The bar represents the standard deviation of the mean value of triplicate determinations.

where V is the intracellular space. Equation (2) was converted as follows:

$$(C_{cell,free})^3 + [V(K_{d1} + K_{d2}) + n_1 + n_2 - A_{cell}]/V \times (C_{cell,free})^2 + [VK_{d1}K_{d2} + n_1K_{d2} + n_2K_{d1} - A_{cell}(K_{d1} + K_{d2})]/V \times C_{cell,free} - A_{cell}K_{d1}K_{d2}/V = 0 \quad (2a)$$

Intracellular free concentrations were estimated by the use of obtained parameters shown in Table II and the A_{cell} values

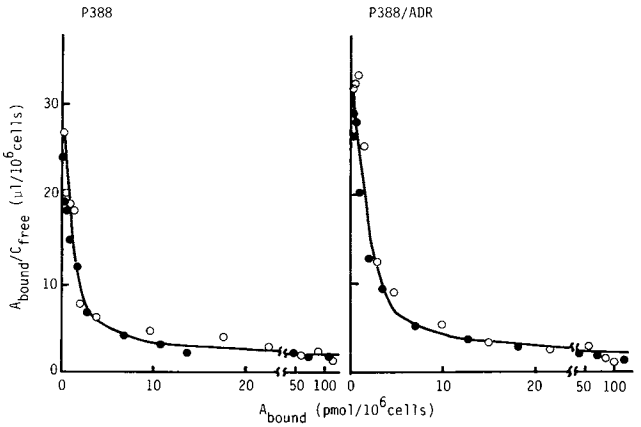

Fig. 6. Scatchard plot of equilibrium binding of VCR to the whole-cell homogenate determined by the ultrafiltration method. The whole-cell homogenate prepared from P388 (left) or P388/ADR (right) was incubated at 37°C with [^{3}H]VCR at various concentrations in a binding buffer with 2 m*M* ATP for 30 min to reach equilibrium. Then VCR binding was determined by the ultrafiltration method. Curves were calculated by the use of the two-site binding model [Eq. (1)] with an iterative nonlinear least-squares regression method. The calculated parameters are shown in Table II. See the text for details. (○) 10^8 cells/ml; (●) 2×10^8 cells/ml.

Table II. Parameters of Vincristine Binding to Cellular Homogenate

Cell line	High-affinity site		Low-affinity site	
	Dissociation constant (K_{d1}) (μM)	Binding capacity (n_1) (pmol/10^6 cells)	Dissociation constant (K_{d2}) (μM)	Binding capacity (n_2) (pmol/10^6 cells)
P388	0.111 ± 0.025	2.43 ± 0.39	129 ± 32	338 ± 84
P388/ADR	0.111 ± 0.020	3.13 ± 0.47	69.0 ± 16.4	208 ± 33

determined at 10 sec in the efflux experiments, based on Eq. (2a) using the Hitchcock–Bairstow method, a general method to solve equations of high degrees numerically.

Thus obtained pairs of velocity of efflux and intracellular free concentration in P388 and P388/ADR are presented in Fig. 7 as a Hofstee plot. Curve fitting of these plots with a computer indicated that VCR efflux has two transport systems (i.e., Michaelis–Menten-type saturable transport and linear type transport) not only in P388/ADR cells but also in P388 cells. Accordingly, VCR efflux in P388 and P388/ADR cells can be expressed by the following equation:

$$V_{eff} = \frac{V_{max,eff} \times C_{cell,free}}{K_{m,eff} + C_{cell,free}} + P_{eff} \times C_{cell,free} \qquad (3)$$

where V_{eff} is the velocity of VCR efflux, $V_{max,eff}$ and $K_{m,eff}$ are the maximum velocity and the Michaelis constant for the saturable process, respectively, and P_{eff} is the diffusion coefficient. The Michaelis constant, maximum velocity, and diffusion coefficient were calculated based on this equation (Table III). The V_{max} value obtained was greater in P388/ADR than in P388 cells, suggesting that an increased number of the transporters of this process causes greater activity in P388/ADR cells, whereas the Michaelis constant is almost the same between the two cell lines, and the diffusion coefficient in P388 is smaller than in the P388/ADR cells.

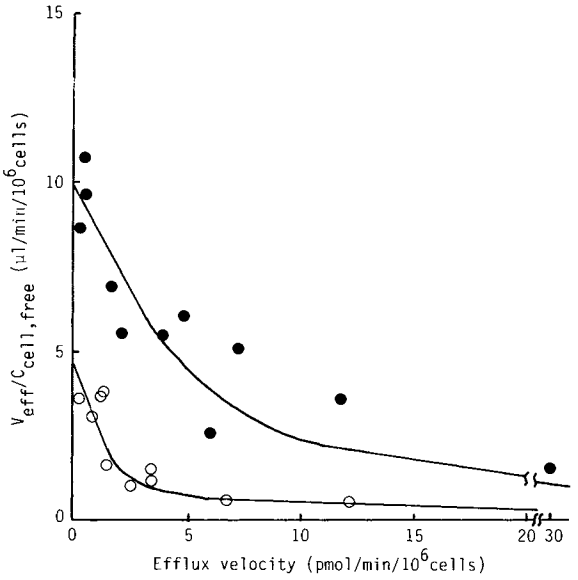

Fig. 7. Hofstee plot of VCR efflux in P388 and P388/ADR cells. The velocity of VCR efflux in P388 (○) and P388/ADR (●) was determined by the initial drug release rate, as shown in Fig. 2. The intracellular free concentration of VCR was estimated from the initial intracellular VCR at 10 sec based on the analysis of equilibrium binding to the whole-cell homogenate. Curves were calculated by the use of Eq. (3) with an iterative nonlinear least-squares regression method. The calculated parameters are shown in Table III. See the text for details.

DISCUSSION

It is difficult to distinguish between outward transport and release from the cell surface in the direct assessment of drug efflux from drug-loaded cells in drug-free medium. We thus defined the initial release rate up to 30 sec as an efflux velocity after washing the drug-loaded cells with cold medium to minimize any VCR associated with the cell surface membrane. Further, the VCR amount at the cell surface membrane, as obtained by extrapolation to time 0 in the initial time course of VCR uptake, was less than 20% of the total uptake at 30 min in various extracellular VCR concentrations under the same conditions as the experiments in Figs. 1 and 2 (data not shown). A saturable efflux, which had been demonstrated in previous analysis (20), was also observed by a Hofstee plot (Fig. 7) in the present study. These findings strongly suggest the existence of carrier-mediated active efflux of VCR in both cell lines.

Inaba *et al.* (28,29) reported that active efflux of both VCR and anthracyclines was inhibited not only by the anthracycline analogues but also by nonantitumor vinca alkaloids. These facts might support mediated efflux. As for kinetic parameters of efflux, a significantly greater maximum velocity of efflux was observed in P388/ADR cells, presenting a kinetic basis for their enhanced efflux activity. The difference in diffusion coefficient is insufficient to account for the different efflux activities between the two cell lines at intracellular VCR concentrations less than K_m, that is, 0.6–0.7 μM. However, the contribution of a passive component to the different VCR efflux cannot be neglected at higher drug concentrations where the mediated process is saturated. The obtained K_m value, 0.6 μM, was comparable with the dissociation constant for VCR binding to membrane vesicles prepared from multidrug-resistant K562 myeloma, reported as 0.2 μM by Naito *et al.* (30), in which ATP-dependent VCR binding was shown, and this resistant subline of K562 cells overexpressed P-glycoprotein (31), that is, vinca alkaloid binding protein (15,16). Furthermore, Northern analysis using *mdr1*, a multidrug-resistance gene coding for P-glycoprotein, as a probe clearly showed that messenger RNA of P-glycoprotein was detected at high levels in P388/ADR as compared to P388 (32). These findings support P-glycoprotein as transporter of the mediated efflux of VCR, as presented by biochemical approaches (12).

Table III. Kinetic Parameters of Vincristine Efflux

Cell line	Maximum velocity ($V_{max,eff}$) (pmol/min/10^6 cells)	Michaelis constant ($K_{m,eff}$) (μM)	Diffusion coefficient (P_{eff}) (μl/min/10^6 cells)
P388	2.36 ± 0.59	0.577 ± 0.221	0.370 ± 0.08
P388/ADR	5.76 ± 1.85	0.693 ± 0.265	1.25 ± 0.32

VCR binding to the whole-cell homogenate was studied for the following three purposes: (a) to examine the validity of the hypothesis of energy-dependent binding, presented by Beck *et al.* (11); (b) to confirm the assumption in the previous analysis of VCR binding (20); and (c) to estimate the intracellular free concentration necessary for the efflux studies by quantitative analysis of VCR binding.

ATP-dependent binding was observed in both cell lines, and the bound fractions were comparable between the two cell lines or slightly greater in P388/ADR (Figs. 4 and 5). These results argue against the hypothesis of energy-dependent reduction of VCR binding as a mechanism for cross-resistance to VCR in P388/ADR cells. The ATP-independent bound fraction, which was observed under ATP depletion induced by apyrase, was similar to that in the presence of excess cold drug, suggesting nonspecific binding (Fig. 5). The mechanism(s) for this ATP dependence of VCR binding is unknown. Bowman *et al.* reported that the stability of the [^{3}H]VCR–tubulin complex in kidney cytosol was increased in the presence of ATP and suggested that ATP may compete with GTP or serve as a phosphate donor through kinase (33).

To confirm the assumption in the previous analysis of VCR binding (20) that VCR binding is ATP independent, the intracellular concentration of total ATP was measured and found to be approximately 0.1 and 1 mM under ATP-suppressed and normal conditions, respectively (Table I). When VCR binding was compared between these two ATP concentrations, little difference was found (Fig. 5). Reduced VCR binding in the presence of apyrase (Fig. 4 and 5) suggests ATP dependence of this binding. However, the effective ATP concentration seems to be very low, probably less than 1 μM. In this respect, the previous assumption may be considered acceptable.

As for VCR binding to cytosol prepared from VCR-sensitive and -resistant human xenografts in nude mice, Houghton *et al.* (25) reported the dissociation constants for the high- and low-affinity sites to be 0.06–0.2 and 40–100 μM, respectively, in a two-site binding model. The association constant for both the high- and the low-affinity sites in our present study agreed well with these values, although our analysis did not use cytosol but whole-cell homogenate. Owellen *et al.* (26) reported tubulin as an intracellular binding site specific for vinca alkaloid. However, Safa *et al.* (27) identified specific binding sites of vinca alkaloids which were not immunoprecipitated with antitubulin antibodies in P388 cells. Therefore, it is unclear whether the binding site analyzed in this study can be attributed only to tubulin.

Some differences in efflux parameters were observed between previous (20) and the present studies, but they may be a consequence of different methodologies between these analyses. However, common results obtained from previous and the present studies strongly suggest (i) the existence of a saturable process involved in the active outward transport of vincristine and (ii) a greater maximum velocity in the resistant cells as the basis of its enhanced efflux activity.

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