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A kinetic study of Rhodamine123 pumping by P-glycoprotein

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Abstract

The MDR1 P-glycoprotein (P-gp) actively extrudes a wide variety of structurally diverse cytotoxic compounds out of the cell, is widely expressed in the epithelial cells of kidney, liver and intestine, and in the endothelial cells of brain and placenta, and plays an important role in drug resistance. We measured the accumulation of Rhodamine 123 (Rho123), a substrate of P-gp, into a drug sensitive and a drug resistant strain of the human leukemia cell line K562, as function of Rho123 concentration. With the aid of a mathematical transformation, we used the accumulation of Rho123 into the sensitive cells as a surrogate measure for the internal concentration of the probe in the resistant cells, and were thus able to measure the kinetic parameters of drug efflux pumping by P-gp. Drug pumping was half-saturated at an external Rho123 concentration of 7.2E–06±1.1E–06 M, and displayed a co-operative behaviour with a Hill number of 1.94±0.32. Verapamil could be shown to inhibit Rho123 efflux uncompetitively.
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Keywords: Pglycoprotein; Rhodamine 123; Kinetics; Resistance; Fluorescence; Quenching

1. Introduction

The MDR1 P-glycoprotein (P-gp) actively extrudes a wide variety of structurally diverse cytotoxic compounds out of the cell. P-gp is widely expressed in the epithelial cells of kidney, liver and intestine, and in the endothelial cells of brain and placenta [1]. P-gp transports a wide variety of drugs out of the cell in an energy-dependent manner, and has also been implicated in multidrug resistance found during cancer and AIDS chemotherapy [2]. The broad substrate specificity and wide expression of P-gp makes it play a very important role in the regulation of ADME (drug absorption, distribution, metabolism and excretion) [1], and has made it a target of intense investigation. The arrangement of the domains in P-gp in the primary sequence is: TMD1–NBD1–TMD2–NBD2, but no atomic resolution structural information has been available for P-gp until now [3] due to its low abundance, difficult purification, membrane location, and the lack of a high-expression system [4]. Thus data are very limited on the interaction mechanism between P-gp and its substrates, information which is of crucial importance both in the development of MDR reversers and in decision-making with regard to drug candidates. The number and the primary structure of the binding sites of substrates and inhibitors in P-gp have been reported. However, the data were mostly acquired from P-gp binding and P-gp ATPase activity, such as photolabeling, cysteine-scanning mutagenesis, and P-gp ATPase determination [5,6], rather than from P-gp transport. The kinetic study of P-gp transport thus provides a useful additional approach to understand the interaction mechanism between P-gp and compounds, when atomic resolution structural information for P-gp is not available. In the present paper, we introduce a kinetic approach to study P-gp transport, based on the influx of Rhodamine 123 (Rho123), a much-used probe for P-gp. We confirm that P-gp is an effective efflux transporter of Rho123 and show that there is co-operative interaction between Rho123 molecules (probably in pairs) as they bind to P-gp during efflux pumping. We estimate the apparent

Abbreviations: P-gp, P-glycoprotein; ABC, ATP-binding cassette; TM, transmembrane; NBD, nucleotide-binding domain; Rho123, Rhodanmine123

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affinity of Rho123 for the pump's binding sites. Verapamil addition diminishes the rate of efflux pumping and does so in an uncompetitive fashion.

In our experiments, we have used K562 cells, a human leukemia cell line, established from a patient with chronic myelogeneous leukemia. K562/ADR cells resistant to doxorubicin were obtained by continuous exposure to increasing doxorubicin concentrations [7]. The K562/ADM subline expresses P-gp at the membrane surface at a high level, whereas the parent line does not. Indeed, a recent single cell immunoassay study using capillary electrophoresis with laser induced fluorescence detection suggests that the resistant subline we use possesses some four times as much PgP as does the parent, sensitive line [8]. The parent line (K562/S), which is sensitive to doxorubicin, has been used as a negative control against the K562 drug resistant subline in a P-gp related study [7].

In order to avoid possible complications in data analysis that can arise due to the self-quenching of Rho123 within the loaded cells, we have developed a method in which the drug sensitive cells are used to provide a calibration curve for the amount of

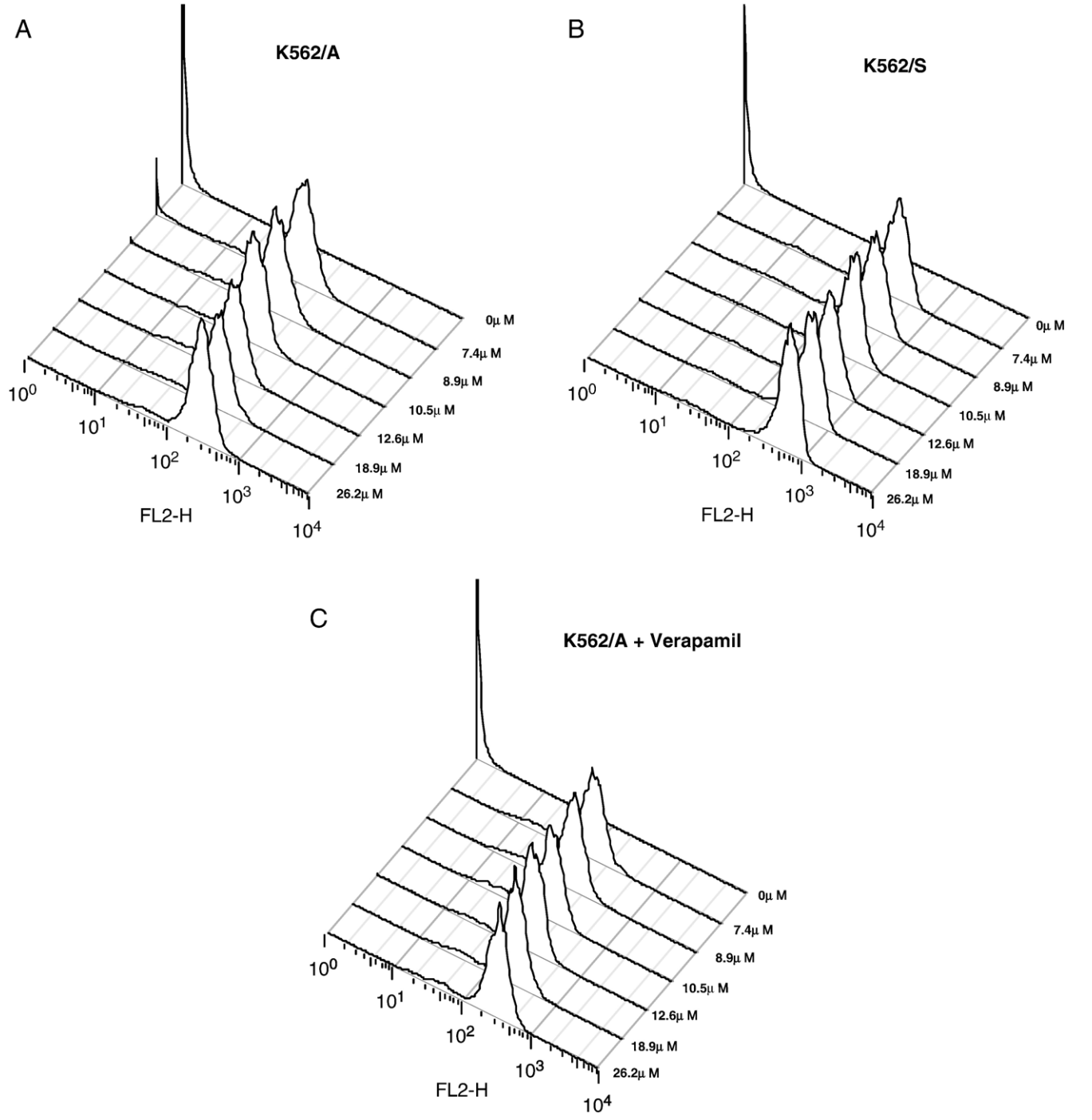

Fig. 1.

Rho123 accumulated within the cells at any level of external probe. This enables us to calculate, for the resistant cells, the relative amount of probe that has been pumped out of the cells. We use this to obtain a measure of the fraction of the probe that is pumped out of the cell, at any level of external Rho123. It is from this fraction and its dependence on the level of external probe that we derive the kinetic data for Rho123 pumping.

2. Materials and methods

2.1. Materials

R-(+)-Verapamil and Rhodamine123 were purchased from Sigma. All the other chemicals were reagent grade and were obtained commercially.

2.2. Cell culture

Both the drug-sensitive K562 (K562/S) cell line and the doxorubicin-resistant cell subline (K562/ADR) were purchased from the Tianjin Institute of Hematopathy. K562/A and K562/S cells were cultured in PRMI1640(Gibco) medium, supplemented with 10% fetal bovine serums at 37 °C in a humidified incubator with 5% CO2.

2.3. Rho123 influx

An initial influx study was performed in pH 7.4 standard phosphate buffered saline (PBS) supplemented with 0.63 mM $CaCl_2$, 0.74 mM $MgSO_4$, 5.3 mM glucose and 0.1 mM ascorbic acid (PBSA) [9]. Cells (K562/A and K562/S) were distributed into 6 tubes at a density of 4×10^5 cells/ml (0.5 ml per tube). Then they were loaded with Rho123 at a concentration of 8.92 μM. Influx was stopped by centrifugation, at 0.5 min, 5 min, 10 min, 15 min, 20 min, and 25 min and the cells were then washed twice with ice-cold PBS containing Verapamil 100 μM. Controls without Rho123 were used to define fluorescence thresholds. A total of 5000 events were acquired per sample. Flow cytometric analysis of Rho123 fluorescence at 585 nm was carried out with the Fluorescence Activated Cell Analyser (FACS Vantange, Becton Dickinson, San Jose, CA) with Cell Quest software (Becton Dickinson Immunocytometry Systems, San Jose, CA).

For the determination of the kinetic parameters of Rho123 transport by P-gp and the interaction mechanism of verapamil with P-gp, both K562/A and K562/S cells were incubated at 37 °C at a density of 4×10^5 cells/ml (0.5 ml per tube) and loaded with Rho123 (the concentration of Rho123 ranged from 8.92 μM to 41.99 μM) . To some samples of the K562/ADR cells, 0.10 μMol/L verapamil was added at the same time. The cells were incubated at 37 °C for 5 min, centrifuged and washed twice with ice-cold PBS containing Verapamil 100 μM. Controls without Rho123 were used to define fluorescence thresholds. Rho123 were determined by flow cytometry as indicated above. In all cases, the fluorescence data are reported here as the median fluorescence of the data output plotted as a histogram.

2.4. Data analysis

The data on the uptake of Rho123, measured as the median fluorescence as described above, plotted as a function of the external concentration of probe for the drug sensitive cells, were fitted by the simple hyperbolic equation:

$$F = \mathrm{Fmax}^{*}S/(S+K_{1/2}) \quad (1)$$

where S is the external concentration of Rho 123, F is the median fluorescence, measured as described above, Fmax is the extrapolated maximum fluorescence and $K_{½}$ is the concentration of Rho123 at which one-half of the maximum fluorescence is reached. This provides a calibration curve for subsequent studies using the drug-resistant cells. Eq. (1) transforms simply to yield S at any F as:

$$S = \mathrm{Fmax}^{*}K_{1/2}/(F-\mathrm{Fmax}) \quad (2)$$

Thus using the values of Fmax and $K_{½}$ found from the calibration curve using the drug-sensitive cells, with measured values of F found for the drug-resistant cells, incubated at concentration S, we can estimate the external probe concentration, Sres, that, for the sensitive cells, would give the same flow cytometry signal. We do not

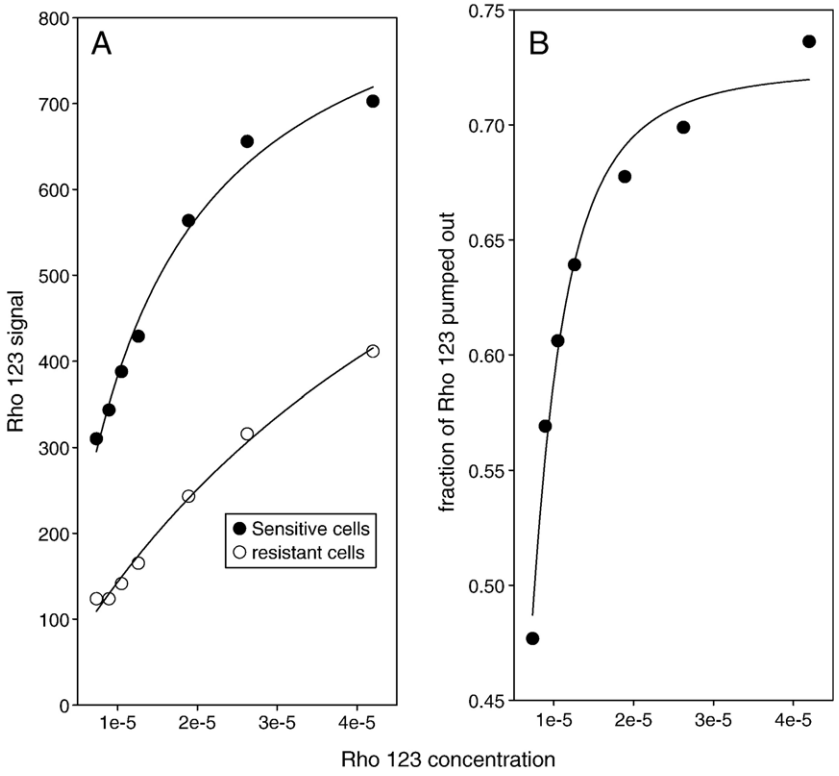

Fig. 2.

know the absolute probe concentration inside the cells in any condition, but it will be some factor, α, times the external probe concentration. The factor α will depend on the binding constant for Rho123 to its internal binding sites and on the amount of such sites present per cell. At any external concentration S, the amount of probe inside the cell will be given by α S. But Sres is the external probe concentration in the sensitive cells that gives the same fluorescence signal (and hence the same internal probe concentration) as that found when resistant cells are incubated with probe at concentration S. Thus the internal concentration of probe in the resistant cells is αSres. Subtracting αSres from αS yields the relative decrease in internal probe concentration due to pumping. Finally, (α S-α Sres)/ α S is the fraction of probe that is pumped out of the cells in the drug-resistant cells, when these are incubated with probe at external concentration S. Since α is, by assumption, everywhere the same, this reduces to (S−Sres)/S, which are all now known or measurable quantities. We can then plot this fraction against S to analyze the concentration dependence of the effectiveness of the drug pump.

3. Results

Uptake of Rho123 by the drug-sensitive cells was linear with time for up to 10 min (data not shown). For the kinetic studies the incubation times with probe were thus set at 5 min to ensure that initial rates were being measured. Fig. 1 S, A, and A+V depict the fluorescence scans at this time for the three conditions, sensitive cells, resistant cells, and resistant cells in the presence of verapamil, as a function of the concentration of Rho123.

Fig. 2A depicts a plot of the median value of the fluorescence signal, after 5 min of incubation, as measured in Materials and methods, against the external probe concentration for the drug-sensitive cells (filled circles) and drug-resistant cells (empty circles). At every probe concentration, the fluorescence signal for the resistant cells is lower than that for the sensitive cells, consistent with the ability of P-gp to bring about efflux of Rho123 from the resistant cells. The data for the sensitive cells appear to lie on a saturation curve. This would be consistent with the cells having a limited capacity for uptake of Rho 123, but this is contradicted by the fact that in the preliminary time course study, probe uptakes at 25 min were more than three fold those at 5 min, so a limited binding capacity is not the explanation of the apparent saturation found for the sensitive cells in Fig. 1A. Self-quenching of fluorescent probes is a well-studied phenomenon and would give results similar to those of Fig. 2A. Assuming self-quenching to be present, we used the data for the sensitive cells as a calibration curve for estimating the effect of drug pumping in the resistant cells. Curve fitting a simple ligand-binding equation (Eq. (1) of Materials and methods) to the data yielded the operative kinetic parameters of

$$\mathrm{Fmax} = 1015 + /-58$$

$$K_{1/2} = 1.65E{-}005 + /-2.02E{-}006,$$

which were used to derive the equivalent probe concentration, from the data plot for the resistant cells (open symbols in Fig. 2A), using Eq. (2), as described in Materials and methods. From this, the fraction pumped out at each probe concentration was calculated (see Materials and methods) and these results plotted against the respective probe concentration, in Fig. 2B. An attempt to fit these data to a simple binding-ligand failed. A good fit was obtained, however, using the co-operative kinetics for the Hill plot, yielding the solid line in Fig. 2B, with parameters Rsq=0.981, maximum fraction pumped of 0.725±0.014, K_m for binding of 5.47E–06± 3.59E–07 and a Hill number of 2.43±0.43. Three similar experiments yielded mean values for the maximum pumped fraction of 0.76±0.02, for the K_m for binding of 7.2E–06±1.1E–06, and for the Hill number of 1.94±0.32, with each mean value being followed by its SE (n=3).

Fig. 3 depicts two additional experiments where verapamil at 0.1 μ M is added to the drug resistant cells in the incubation medium during probe uptake (middle curve, the triangles, in each

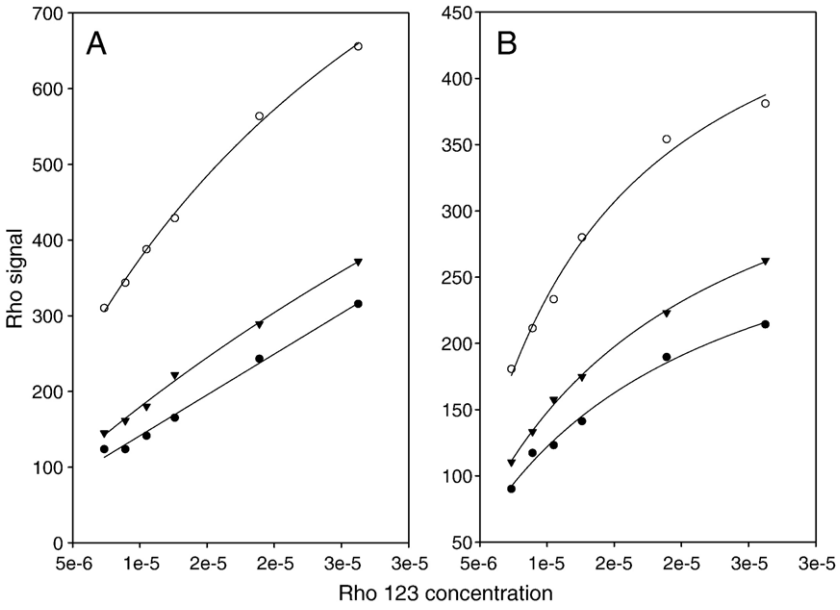

Fig. 3.

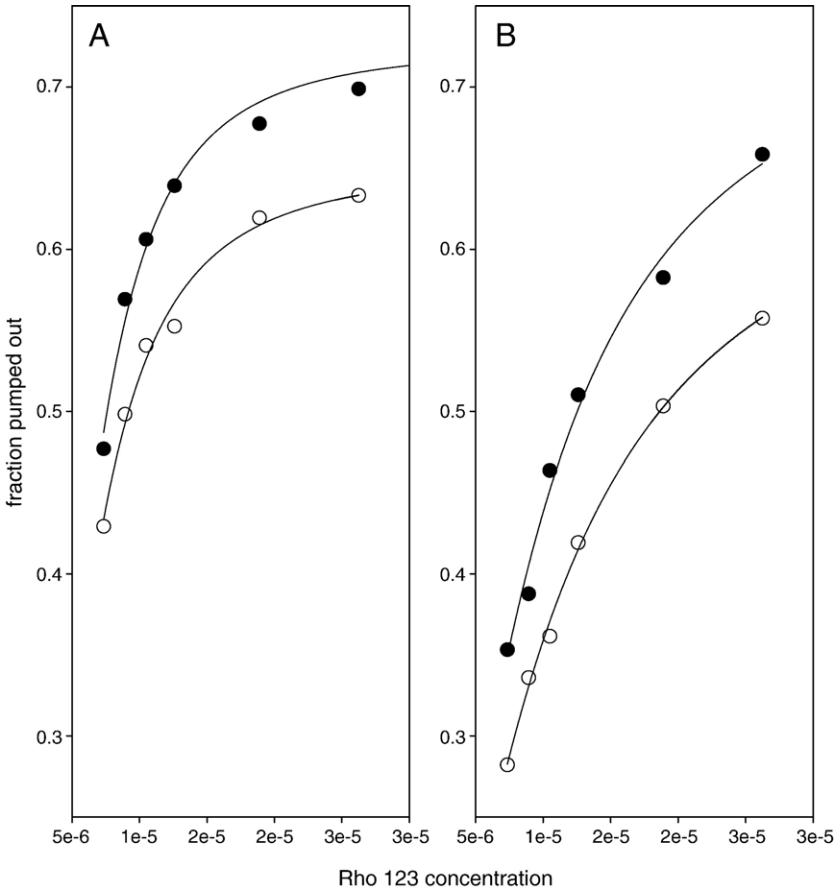

Fig. 4.

figure). Verapamil has the effect of raising the Fluorescence signal at each probe concentration, consistent with its reported ability to block pumping of P-gp substrates (such as Rho123). Analyzing the data as for Fig. 2 yielded the fraction of probe pumped at each probe concentration. These are plotted in Fig. 4, for the points obtained in the absence of verapamil (filled circles) or when verapamil is present (empty circles). The fraction of drug pumped is always lower when verapamil is added. Fitting the data in each case to the Hill equation yielded the pump parameters listed in the figure legend. In both experiments the Hill number and probe binding parameter is not affected by verapamil, but the maximum amount of probe pumped is reduced 11 ± 0.5% by the addition of 0.1 μM verapamil. This gives a Ki for verapamil of 1.1 μM.

4. Discussion

We have developed a simple algorithm that enables data on the uptake of Rho123 as a function of concentration in drug-sensitive and -resistant cells to be interpreted in terms of the kinetics of Rho123 pumping by P-gp. Our experimental data and its analysis confirm that P-gp can bring about the efflux of Rho123 from drug-resistant cells. Additionally, it would appear from our data that two molecules of Rho123 interact co-operatively during drug efflux and that verapamil is an effective inhibitor of the drug pumping process, affecting the maximum rate of transport but not the apparent affinity between Rho123 and P-gp. The maximum amount of Rho123 pumped out of the cells is about 75% of the total, in these cells and under our conditions. The apparent affinity of Rho123 for P-gp is some 7.2 μM, in terms of the external probe concentration. How do these points compare with previous studies of P-gp?

As a widely expressed transporter, P-gp transports an enormous variety of chemical compounds from interior to exterior of cells actively. Previous research discovered that P-gp plays an important role in the regulation of drug ADME properties [1] and is frequently responsible for multi-drug resistance during cancer chemotherapy [2]. But how P-gp interacts with so many kinds of chemicals still remains unknown. Key steps in understanding the transport process are binding of drug substrates within the transmembrane domains, initiation of ATPase activity, and subsequent drug efflux [10]. Photolabeling and cysteine-scanning mutagenesis in combination with a thiol-specific substrate have been widely employed in research concerning the binding sites of the substrate in P-gp, and play a crucial role in the identification of the number and location of its substrate binding sites [5,6,11]. It appears that there is a common substrate-binding pocket in P-gp, which is large enough to accommodate different substrates simultaneously. The substrates occupy different regions in the common drug-binding pocket. The binding of substrates with P-gp induces conformational changes in the protein, with a concomitant change of the ATPase activity of P-gp [12,13]. The two nucleotide-binding domains

would appear to form an integrated entity containing two bound ATP with just one of the two ATP being hydrolyzed per transport event. It is the ATP-binding and its subsequent hydrolysis that provides the primary driving force for transport. The two NBD cooperate with each other during drug transport [14].

Shapiro et al have determined the K_m for P-gp transport of Rho123 by plasma membrane vesicles. Their result for K_m, 15 μM, is not far from ours, 7.2 ± 1.2 μM [15]. They also show co-operation between ligands during binding to P-gp. Other investigators who have studied the interaction between P-gp and substrates by approaches such as P-gp ATPase activity determination, substrate binding analysis, and accumulation analysis [5,10,11] have often encountered co-operativity between ligands during the pump's action, as we do in this study for Rho123.

We also show that verapamil, as a typical inhibitor of P-gp, inhibits Rho123 transport uncompetitively, which indicates that Rho123 and verapamil bind with P-gp at different sites. This is consistent with the research of Loo, Clarke and their colleagues, which indicates that I340 (TM6), A841 (TM9), L975 (TM12), V981 (TM12), and V982 (TM12) contribute to the binding of Rhodamine dyes, while S222 (TM4), I306 (TM5), I868 (TM10), and G872 (TM10) contribute to the binding of verapamil [10,16,17].

Since P-gp-mediated drug efflux has been implicated as a major factor contributing to multi-drug resistance (MDR) in cancer chemotherapy, much effort has been put into the development of P-gp inhibitor drugs to reverse MDR and also to regulate the ADME of possible drugs. While photolabeling, cysteine-scanning mutagenesis and P-gp ATPase analysis provide useful approaches for the identification of substrate binding sites in P-gp, transport kinetic studies such as described in the present paper can make a useful addition to our knowledge and help us understand the complex interactions between P-gp and its transportable substrates.

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