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New insights into the P-glycoprotein-mediated effluxes of rhodamines

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Multidrug resistance (MDR) in tumour cells is often caused by the overexpression of the plasma drug transporter P-glycoprotein (P-gp). This protein is an active efflux pump for chemotherapeutic drugs, natural products and hydrophobic peptides. Despite the advances of recent years, we still have an unclear view of the molecular mechanism by which P-gp transports such a wide diversity of compounds across the membrane. Measurement of the kinetic characteristics of substrate transport is a powerful approach to enhancing our understanding of their function and mechanism. The aim of the present study was to further characterize the transport of several rhodamine analogues, either positively charged or zwitterionic. We took advantage of the intrinsic fluorescence of rhodamines and performed a flow-cytometric analysis of dye accumulation in the wild-type drug sensitive K562 that do not express P-gp and its MDR subline that display high levels of MDR. The measurements were made in real time using intact cells. The kinetic parameter, $k_a = V_M/k_m$, which is a measure of the efficiency of the P-gp-mediated efflux of a substrate was similar for almost all the rhodamine analogues tested. In addition these values were compared with those determined previously for the P-gp-mediated efflux of anthracycline. Our conclusion is that the compounds of these two classes of molecules, anthracyclines and rhodamines, are substrates of P-gp and that their pumping rates at limiting low substrate concentration are similar. The findings presented here are the first to show quantitative information about the kinetic parameters for P-gp-mediated efflux of rhodamine analogues in intact cells.

Keywords: P-glycoprotein; multidrug resistance; membrane transport; rhodamine; efflux.

Since 1940, a broad variety of antibiotics active against many infectious organisms were discovered or developed. The widespread, and sometimes uncontrolled, use of these drugs has led to the emergence of defence mechanisms that, at present, are the major drawback of the drug-based treatment of infectious diseases and cancers. Such resistance is not restricted to the drugs (or analogues) used in the treatment but also involves several structurally and functionally unrelated compounds. This phenomenon, which has been termed multidrug resistance (MDR), can be caused by various mechanisms. However, over expression of the P-glycoprotein multidrug transporter (P-gp) in the plasma membrane is believed to be a major cause of resistance to multiple chemotherapeutic drugs [1–4].

P-gp is an unusual ABC protein in that it appears to be highly promiscuous: hundreds of compounds have been identified as substrates. The spectrum of MDR compounds includes a large number of anticancer drugs (e.g. anthracyclines, vinca alkaloids, taxanes) as well as steroids, fluorescent dyes, rhodamines, and the γ-emitting radio pharmaceutical ^{99m}Tc-MIBI. P-gp can transport neutral and positively charged molecules but not negatively charged ones. Despite the advances of recent years, we still have an unclear view of the molecular mechanism by which P-gp transports such a wide diversity of compounds across the membrane [5–9].

Recently, we have performed several studies using K562 intact cells to describe the kinetics of anthracycline transport in MDR cells in order to predict how modifications in the anthracycline molecule affect its transport characteristics [10–13]. In the present paper we have used the same cell line to characterize the transport of several rhodamines.

Most of the rhodamines are well known as P-gp substrates. Eytan *et al.* [7] have examined seven rhodamine dyes for their P-gp-mediated exclusion from MDR cells, their localization in wild-type drug-sensitive cells, their capacity to stimulate the ATPase activity of P-gp reconstituted in proteoliposomes, and their transmembrane movement rate in artificial liposomes. All these rhodamine dyes were accumulated in wild-type drug-sensitive cells and were localized mainly in the mitochondria. All the dyes tested

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Abbreviations: P-gp, P-glycoprotein; MDR, multidrug resistance; Rh 123, rhodamine 123; Rh 6G, rhodamine 6G; Rh B, rhodamine B base; TMR, tetramethylrosamine; Rh I, tetramethylrhodamine ethyl ester or rhodamine I; Rh II, tetramethylrhodamine methyl ester or rhodamine II; Rh 123_{hyd}, hydrolysis product of Rh 123; RhI,II_{hyd}, hydrolysis product of RhI and RhII; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)-phenylhydrazone.

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were substrates of reconstituted P-gp and cellular P-gp. Sharom *et al.* have studied the transport of tetramethylrosamine by P-gp in vesicles [14].

Although it is well known that rhodamines are P-gp substrates [7,14], quantitative data are lacking. In the present study, we have used a continuous fluorescence assay with different rhodamine analogues to quantify their transport by P-gp in intact cells. The rate of dye transport was measured in real time using two fluorescence-based methods: traditional fluorescence and cytofluorometry. Our aim was to get quantitative data on the P-gp-mediated efflux of rhodamines in order to compare the rhodamine analogues between each other and to other substrates of P-gp. Therefore we have determined in both K562/ADR cells and the parental cell line K562, in the absence of membrane potential, the gradient of rhodamine concentration generated by the presence of the pump, the free intracellular rhodamine concentration, the rate of their passive diffusion through the plasma membrane and then the kinetic parameters characteristic of their P-gp-mediated efflux. Our data show that the efficiencies of the P-gp-mediated efflux of Rh I, Rh II, TMR, Rh 6G, Rh B, RhI,II hydrolysed are similar to each other and to the efficiency of the P-gp-mediated efflux of anthracyclines. This work represents the first report, using intact cells, of real-time measurements of the rate of rhodamine transport.

Experimental procedures

Cell lines and cultures

K562 is a human leukemia cell line, established from a patient with a chronic myelogeneous leukemia in blast transformation [15]. K562/ADR cells resistant to doxorubicin were obtained by continuous exposure to increasing doxorubicin concentrations. This subline expresses a unique membrane glycoprotein with a molecular mass of 180 000 Da [16]. Total RNA was prepared from frozen cells according to a CsCl-guanidinium isothiocyanate method proposed by Maniatis *et al.* [17] and adapted by Ferrandis *et al.* [18]. Transcript levels of the MDR1 gene were measured comparatively to that of the KB-8-5 cell line that shows an expression of 30 (arbitrary units) [19]. Our K562/ADR cells exhibited an MDR1 gene transcript level of 800. K562 cells and the P-gp expressing K562/ADR cells were cultured in RPMI 1640 (Sigma Chemical Co.) medium supplemented with 10% fetal bovine serum (Bio Media Co) at 37 °C in a humidified incubator with 5% CO_2. The resistant K562/ADR cells were cultured with 400 nM DOX until 1–4 weeks before experiments. Cell cultures used for experiments were split 1 : 2 in RPMI 1640 medium 1 day before use in order to ensure logarithmic growth.

Cells (10^6 mL^{-1}; 2 mL per cuvette) were energy-depleted via preincubation for 30 min in Hepes buffer with sodium azide but without glucose.

Drugs and chemicals

Rhodamine 123 (Rh123), rhodamine 6G (Rh 6G), and rhodamine B base (Rh B) were purchased from Sigma. Tetramethylrosamine (TMR), tetramethylrhodamine ethyl ester or rhodamine I (Rh I), and tetramethylrhodamine methyl ester or rhodamine II (Rh II) were purchased from Molecular Probes. Rh 123_{hyd}, the hydrolysis product of Rh123, was obtained by basic hydrolysis. The basic hydrolysis of RhI and RhII yielded the same compound that will be hereafter named RhI,II_{hyd}. Stock solutions of rhodamines at 10^{-3} M, were prepared in ethanol. Triton X-100, valinomycin, carbonyl cyanide *p*-(trifluoromethoxy)-phenylhydrazone (FCCP), verapamil were from Sigma. Valinomycin and FCCP were dissolved in ethanol. Synthesis of the radio labelled compound [hexakis(methoxyisobutylisonitrile) technetium (I)] (^{99m}Tc-MIBI) was performed with a one-step kit formulation as described previously [9]. 2-[4-(Diphenylmethyl)-1-piperazinyl]ethyl-5-(*trans*-4,6-dimethyl-1,3,2-dioxaphosphorinan-2-yl)-2,6-dimethyl-4-(3-nitrophenyl)-3-pyridinecarboxylate P oxide (PAK-104P) was a gift from Drs N. Shudo, T. Iwasaki and S.I. Akiyama, Nissan, Chemical Industries, Ltd. All the reagents were of the highest quality available and deionized double-distilled water was used throughout the experiments. Some experiments were performed in Hepes/Na^+ buffer solutions containing 20 mM Hepes plus 132 mM NaCl, 3.5 mM KCl, 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$, 5 mM glucose at pH 7.3. However, in order to dissipate membrane potential, as the plasma membrane potential of most eukaryotic cells is thought to be primarily of potassium diffusion potential [20], high extra cellular potassium (130 mM) and low chloride were used to depolarize the plasma membrane. The addition of the ionophore valinomycin (10 nM) and of the protonophore FCCP (1 μM) in such buffer prevented the accumulation of lipophilic cations [21]. Therefore most of the experiments were performed in Hepes/K^+ buffer solutions containing 20 mM Hepes plus 133 mM K-methanesulfonate, 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$, 5 mM glucose, 10 nM valinomycin and 1 μM FCCP at pH 7.3. This buffer will hereafter named K^+-buffer. At these concentrations neither valinomycin nor FCCP inhibits the P-gp-mediated efflux of drug [22]. It has previously been observed that FCCP and valinomycin in combination can precipitate in the presence of potassium [23,24]. However, under our experimental conditions, the FCCP concentration was 100-fold higher than that of valinomycin. Therefore, even if a valinomycin-K^+-FCCP complex precipitates, the modification of the FCCP concentration would not exceed 2%. K-methanesulfonate was made by titration of methanesulfonic acid with KOH prior to addition to buffer [25].

Real-time fluorescence measurement of drug transport in living cells

Fluorescence measurements were carried out using a Perkin Elmer LS50B spectrofluorometer equipped with a temperature-controlled sample compartment. The appropriate concentration of rhodamine was preincubated in 2 mL of buffer and allowed to equilibrate for about 200 s to stabilize the fluorescence intensity. A volume of 200 μL of buffer containing 2×10^6 cells was quickly added to the cuvette with magnetic stirring. The fluorescence intensity of Rh B, Rh123, TMR, RhI, RhII and Rh6G was measured continuously until steady-state was reached (excitation 553, 493, 502, 543, 553, 554 and 527 nm and emission at 580, 521, 527, 575, 580 and 553 nm, respectively). During

the time course of these experiments, aliquots were taken at various interval of time and used as such for flow cytometry measurements. A Becton Dickinson FACScan flow cytometer equipped with a spectra Physics argon-ion laser was used. The fluorescence signal was gated on the forward angle light scatter signal to exclude dead cells debris from analysis. The argon-ion laser was tuned to 488 nm and used at a power of 15 mW. For rhodamine 123, emission was detected through an emission filter that collects radiations from 515 to 545 nm. For the other rhodamines, an emission filter that collects radiations from 563 to 607 was used. In order to minimize the re-equilibration of the fluorescent probe in the various intracellular compartments of the cells that occur when probes from the extracellular medium are removed, cells were not washed. We have estimated that one cell remains about 0.01 s in the sheath fluid [26] and therefore that the decrease of the intracellular rhodamine should not exceed 1%. These experiments were performed in K^+-buffer.

Mathematical calculations

The mathematical symbols used are the following: $N{\cdot}10^9$ is the number of cells per litre; V_{cell} is the volume of one cell ($\approx 10^{-12}$ L per cell); C_e is the extracellular drug concentration; C is the concentration of internal rhodamine bound to its receptors; C_i is the concentration of free internal rhodamine; C_T, the total concentration of rhodamine added to the cells, and is equal to the concentration of rhodamine in the extracellular medium plus the concentration of rhodamine, bound and free, inside the cells.

$$C_T = C_e + N{\cdot}10^9{\cdot}(\mathscr{C} + \mathscr{C}_i){\cdot}V_{cell} \quad (1)$$

K is the mean binding constant for rhodamine to all its receptors, whatever they are; [receptors] is the concentration of all internal receptors for rhodamine, whatever they are; $K = \mathscr{C}/\mathscr{C}_i{\cdot}$[receptors] or $K = \beta{\cdot}$[receptors] with $\beta = \mathscr{C}/\mathscr{C}_i$; F is the molar fluorescence of rhodamine free in the cytosol; ρ is the fluorescence quantum yield for rhodamine bound to its receptors. $A = F(1 + \beta{\cdot}\rho)$ is the proportionality constant between the fluorescence intensity recorded via flow cytometry and $\mathscr{C}_i$; V_+, the rate of passive uptake for rhodamine, is equal to the number of moles that enter by passive diffusion into one cell per second; V_-, the rate of passive efflux for rhodamine, is equal to the number of moles that leave one cell by passive diffusion per second; k is the passive permeability rate constant (which takes into account the permeability constant of the molecule, the membrane exchange area per cell); $V_+ = k{\cdot}C_e$ and $V_- = k{\cdot}\mathscr{C}_i$; V_a, the rate for outward pumping is equal to the number of moles that are pumped out by P-gp per cell and per second; k_a is the rate constant for outward pumping at limiting low substrate concentration; $V_a = k_a{\cdot}\mathscr{C}_i$.

We intend to derive from the data k_a, the rate constant for outward pumping at limiting low substrate concentration; for this purpose we need to determine: (a) the concentration of free internal rhodamine; (b) the binding constant for rhodamine to its receptors whatever they are; (c) the fluorescence quantum yield; (d) the passive permeability rate constant; and (e) the rate constant for outward pumping at limiting low substrate concentration.

The determination of the kinetic parameters, e.g. the maximum rate (V_M) and the Michaelis–Menten constant (K_m), characteristic of the transporter-mediated efflux of drugs required the measurement of V_a and $\mathscr{C}_i$. When V_a can be determined for various intracellular free drug concentrations $\mathscr{C}_i$, V_M and the apparent K_m can be computed by nonlinear regression analysis of transport velocity (V_a) vs. ($\mathscr{C}_i$) assuming that the transport follows the Michaelis equation.

$$V_a = V_M{\cdot}\mathscr{C}_i/(K_m + \mathscr{C}_i) \quad (2)$$

In many cases, the complete curve $V_a = f(\mathscr{C}_i)$ cannot be obtained and therefore it is not possible to obtain these two parameters characteristic of a transporter. However, if $\mathscr{C}_i$ is much lower than K_m, Eqn (2) becomes:

$$V_a \approx (V_M/(K_m){\cdot}\mathscr{C}_i \text{ or } V_a = k_a{\cdot}\mathscr{C}_i \quad (3)$$

In this work, the rate of P-gp-associated efflux of rhodamine was calculated at the steady-state, taking into account the following points: (a) the diffusion (influx and efflux) of rhodamine through the membrane is passive, so it obeys Fick's law; (b) whatever the type of cells i.e. either drug-sensitive or drug-resistant, at the steady-state (s) the rate of rhodamine influx $(V_+)_s$ is equal to that of rhodamine efflux; (c) for drug-resistant cells, the efflux is composed of two terms: a passive efflux of the molecule $(V_-)_s$, and a P-gp-mediated efflux of the molecule $(V_a)_s$. It follows that:

$$\begin{aligned} (V_+)_s &= (V_-)_s + (V_a)_s \text{ or} \\ (V_a)_s &= (V_+)_s - (V_-)_s \\ (V_a)_s &= k{\cdot}(C_e - \mathscr{C}_i)_s \end{aligned} \quad (4)$$

Taking into account Eqns (2) and (4), it becomes

$$k_a = k{\cdot}[(C_e/\mathscr{C}_i) - 1] \quad (5)$$

Therefore, the determination of k_a requires those of k, C_e and $\mathscr{C}_i$.

As will be demonstrated below, the determination of k requires the knowledge of F, the molar fluorescence of rhodamine free in the cytosol, of ρ, the fluorescence quantum yield for rhodamine bound to its receptors, and β, that is $\mathscr{C}/\mathscr{C}_i$. For this purpose, sensitive cells were incubated with rhodamine in K^+-buffer. Under these experimental conditions, where $\Delta\psi = 0$, the positively charged rhodamines cannot accumulate inside mitochondria. However, they can interact with different receptors within the cell. The intracellular concentration of rhodamine bound to these receptors ($\mathscr{C}$) is in thermodynamic equilibrium with the rhodamine free in the cytosol ($\mathscr{C}_i$). A mean binding constant can be defined as $K = \mathscr{C}/\mathscr{C}_i$[receptors]. As we were working under experimental conditions where the receptors were in large excess compared to the intracellular rhodamine concentration, the concentration of free receptors could be considered as constant. It follows that the binding of the different rhodamine to the receptors can be characterized by $\beta = \mathscr{C}/\mathscr{C}_i$. The concentration of rhodamine inside the cells is therefore:

$$\mathscr{C} + \mathscr{C}_i = \mathscr{C}_i(1 + \beta) \quad (6)$$

As the sizes of the cells used are almost homogeneous, we can therefore consider that the number of moles per cell does not vary much between cells. The fluorescence of one cell measured using flow cytometry (F_{cyto}) is therefore proportional to the concentration of rhodamine in the cell. F_{cyto} is composed of two terms: the fluorescence of the free internal rhodamine ($F{\cdot}\mathscr{C}_i$) and that of the rhodamine bound to its receptors ($\rho{\cdot}F{\cdot}\mathscr{C}$).

Therefore:

$$F_{cyto} = F{\cdot}\mathscr{C}_i{\cdot}(1 + \rho{\cdot}\beta) \quad (7)$$

Let us consider sensitive cells, $N{\cdot}10^9\ L^{-1}$, in K^+-buffer, incubated with rhodamine at concentration C_T. At steady state, $C_e = \mathscr{C}_i$ and taking into account Eqns (1) and (6), it becomes

$$\mathscr{C}_i = C_T/[1 + 10^{-3}{\cdot}N{\cdot}(1 + \beta)] \quad (8)$$

and

$$F_{cyto} = F{\cdot}[C_T{\cdot}(1 + \beta{\cdot}\rho)]/[1 + 10^{-3}{\cdot}N{\cdot}(1 + \beta)] \quad (9)$$

F, ρ and β can then be calculated by a nonlinear analysis of F_{cyto}, measured at fluorescence steady-state, vs. N.

The parameter k was determined from the continuous monitoring of the fluorescence signal, F_{cyto} (flow cytometry), when sensitive cells in K^+-buffer were incubated with rhodamine. In fact, when cells are incubated with rhodamine, before reaching the steady state, rhodamine continuously enters into the cells. According to Eqn (6), during dt, the increase in the intracellular rhodamin concentration is $(1 + \beta){\cdot}dC_i$ and the increase of the number of moles per cell and per second is

$$V_{cell}{\cdot}(1 + \beta){\cdot}(d\mathscr{C}_i)/dt = k{\cdot}(C_e - \mathscr{C}_i) \text{ or} \quad (10)$$

$$(d\mathscr{C}_i)/dt = k{\cdot}(C_e - \mathscr{C}_i)/(1 + \beta){\cdot}V_{cell} \quad (11)$$

The integration of this equation yields

$$\mathscr{C}_i = C_e{\cdot}(1 - \exp[-kt/(1 + \beta){\cdot}V_{cell}]) \quad (12)$$

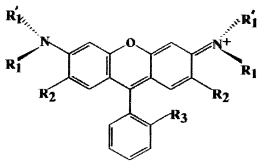
On the other hand, according to Eqn (7), it becomes

$$F_{cyto} = (1 + \beta{\cdot}\rho){\cdot}F{\cdot}C_e\,(1 - \exp[-kt/(1 + \beta){\cdot}V_{cell}]) \quad (13)$$

In this expression, C_e can be taken equal to C_T, and the other parameters are constant. It follows that the term $k/(1 + \beta){\cdot}V_{cell}$ and therefore k, can be computed by a nonlinear analysis of F_{cyto} vs. t data. It should be emphasize that these calculations are valid if the rate of interaction of the dye with its receptors is much higher than the rate of its passive diffusion through the membrane.

Results

The rhodamines used in the present study (Fig. 1) can be classified into two categories: those which have a permanent positive charge: Rh 123, TMR, Rh I, Rh II, and Rh 6G (class I) and those which in addition have an acidic function and are therefore zwitterionic: Rh 123_{hyd}, RhI,II_{hyd} and Rh B.



Class		R_1	R'_1	R_2	R_3
I	Tetramethylrosamine	CH_3	CH_3	H	H
	Tetramethylrodamine ethyl ester (Rhodamine I)	CH_3	CH_3	H	$COOCH_2CH_3$
	Tetramethylrodamine methyl ester (Rhodamine II)	CH_3	CH_3	H	$COOCH_3$
	Rhodamine 6 G	CH_2CH_3	H	CH_3	$COOCH_2CH_3$
	Rhodamine 123	H	H	H	$COOCH_3$
II	Rhodamine B	CH_2CH_3	CH_2CH_3	H	COOH
	Rhodamine I, II hydrolysed	CH_3	CH_3	H	COOH
	Rhodamine 123 hydrolysed	H	H	H	COOH

Fig. 1. Structure of the rhodamines used in this study.

The aim of the present work was to determine the efficiency of the P-gp-mediated efflux of these rhodamines which can be characterized, as we have shown in the experimental section, by the coefficient of active efflux $k_a = k{\cdot}[(C_e/\mathscr{C}_i) - 1]$. The determination of k_a requires the measurement of (a) the gradient of concentration generated by the pump, e.g. the extracellular C_e and the cytosolic $\mathscr{C}_i$ free drug concentrations at steady-state and (b) the passive permeability rate constant k. The following experiments were designed to determine these three parameters.

If we consider resistant cells in Na^+-buffer, the gradient of concentration through the plasma membrane depends on two parameters: (a) the plasma membrane potential which create a 'positive' gradient which tends to make the cytosolic concentration of positively charged rhodamines higher than the extracellular concentration ($\mathscr{C}_i > C_e$), and (b) the P-gp-mediated efflux of rhodamine which tends to create a 'negative' gradient ($\mathscr{C}_i < C_e$). Under these conditions it is impossible to determine the gradient of concentration generated by the pump only. However, experiments performed in the absence of membrane potential, e.g. in K^+-buffer, can solve the problem since the gradient of concentration through the plasma membrane is then only due to the pump.

Determination of C_e, the extracellular rhodamine concentration

A continuous spectrofluorometric monitoring (Perkin Elmer LS50B spectrofluorometer) of the fluorescence signal of the rhodamine during incubation with cells in a 1-cm quartz cuvette containing K^+-buffer was performed. Sensitive or resistant cells, $10^6\ mL^{-1}$, were incubated with 0.2 μM rhodamine. No modification of the fluorescence signal was observed. At steady state, cells were centrifuged and the fluorescence of the supernatant measured. The intensity of the signal was very similar to that observed in the presence of cells. Our conclusion was that it is

reasonable, under these conditions, to consider that C_e is equal to C_T.

Determination of $\mathscr{C}_i$, the cytosolic free rhodamine concentration

In a first set of experiments, sensitive cells, 10^6 mL^{-1}, were incubated in K^+-buffer, in the presence of different concentrations of rhodamine ranging from 0.02 to 0.2 μM. At steady state, the flow cytometry signal (F_{cyto}) was recorded. Figure 2 shows for different rhodamines the plot of F_{cyto} as a function of C_T which is equal, as we have shown above, to C_e. In addition, and this is the very important point, at steady state when $\Delta\psi = 0$, there is a transmembrane equilibrium and the free rhodamine concentration should be the same on both side of the plasma membrane, e.g. $C_e = \mathscr{C}_i$. As shown, there is a linear dependency of F_{cyto} as a function of $C_T = \mathscr{C}_i$ and therefore, $F_{cyto} = A{\cdot}\mathscr{C}_i$. The fluorescence signal recorded from the cells is due not only to the rhodamine free (C_i) in the cytosol but also to the rhodamine bound (C) to intracellular sites with a mean binding constant $K = \beta/[\text{receptors}]$ ($\beta = \mathscr{C}/\mathscr{C}_i$). As we have shown in the experimental section, $F_{cyto} = F{\cdot}(1 + \beta{\cdot}\rho){\cdot}\mathscr{C}_i$ and therefore $A = F{\cdot}(1 + \beta{\cdot}\rho)$.

In a second set of experiments, resistant cells, 10^6 mL^{-1}, were incubated in K^+-buffer, in the presence of different concentrations of rhodamine ranging from 0.02 to 0.2 μM. At steady state, the flow cytometry signal (F_{cyto}) was recorded and the plot of F_{cyto} as a function of C_T is shown in Fig. 2. As can be seen, for the same extracellular drug concentration, the fluorescence signal is higher in sensitive cells than in resistant cells. Similar experiments were performed in energy-depleted cells [10] and the values of F_{cyto} obtained were similar to that determined for sensitive cells. This allowed us to say that the parameter A value was the same for both sensitive and resistant cells. Therefore, from the F_{cyto} value measured in resistant cells we can easily calculate the $\mathscr{C}_i$ value and then the gradient of concentration $\mathscr{C}_i/C_e$ generated by the pump. This calculation was performed for the various concentrations of rhodamines used (0.02–0.2 μM) and we did not observed significant variation of the gradient value indicating that we were working under conditions were the P-gp was far from being saturated. Mean values are reported in Table 1. The gradients generated by the pump for the positive charged rhodamine, RhI, RhII and TMR, are very similar but about fourfold higher than for Rh6G. In the case of zwitterionic rhodamine, Rh B and hydrolysed rhodamine, this gradient is smaller, about fourfold lower than for TMR.

Determination of $\beta = \mathscr{C}/\mathscr{C}_i$

In this set of experiments, cells were incubated with always the same rhodamine concentration $C_T = 0.2$ μM but the number of cells used during the incubation in K^+-buffer was varied from 0.1×10^9 to 50×10^9 cells per L (i.e. N was varied from 0.1 to 50). The flow cytometry signal was measured at steady state. Figure 3 shows typical records of F_{cyto} as a function of the cell number for TMR, Rh 6G, Rh B. As can be seen, the intensity of the signal decreased when the number of cells increased. Data points of F_{cyto} vs. cells number were fitted to Eqn (9) of the experimental section and the values of F, ρ and β were estimated. To check if the β constants in resistant cells were similar to those observed in sensitive cells, experiments were performed with energy-depleted resistant cells. The values of the three parameters were the same, respectively, than those determined for sensitive cells. They are reported in Table 2. The β-values for most of the rhodamines were within the range 16–40, independent of the charge of the molecule; however, the value wass fivefold higher for TMR and very low for $Rh123_{hyd}$.

Determination of the passive permeability rate constant *k*

A continuous cytofluorometric monitoring (FACScan) of the fluorescence signal of sensitive cells incubated in

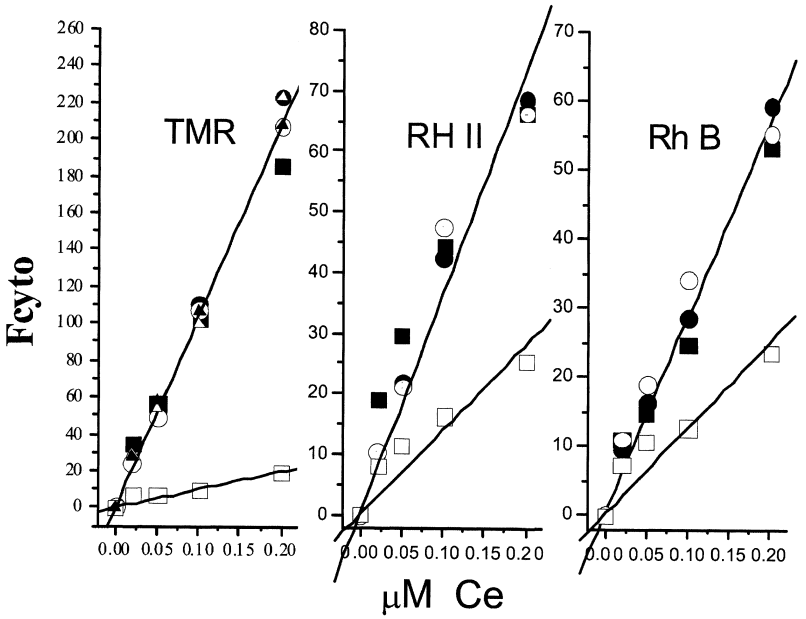


Fig. 2. Intensity of the flow cytometry signal recorded at steady-state fluorescence from sensitive and resistant K562 cells incubated with TMR, Rh II or Rh B. The intensity of the signal (F_{cyto}) is plotted as a function of the extracellular concentration, C_e, of rhodamine. Cells, 10^6 mL^{-1}, were incubated with various concentrations of rhodamine in K^+-buffer in ATP-rich and ATP-depleted cells. ATP-rich sensitive cells (■), resistant cells (□) and resistant cells in presence of 50 μM PAK-104P (△); in ATP-depleted sensitive (●) and resistant (○) cells. The data points are from a representative experiment.

Table 1. Kinetic parameters for rhodamine and anthracycline derivatives. k_a, the rate constant for outward pumping at limiting low substrate concentration; k, the passive permeability rate constant; $\mathscr{C}_i/C_e$, the gradient of concentration generated by P-gp through the plasma membrane; K_m the Michaelis constant. The data are the means ± SEM of at least five determinations.

Rhodamine	$\mathscr{C}_i/C_e$	$k \times 10^{-13}$ (L per cell per s)	$k_a \times 10^{-12}$ (L per cell per s)	Reference	K_m (μM) [ref]
Rh I	0.15 ± 0.02	1.7 ± 0.2	1.0 ± 0.2	This work	ND
Rh II	0.15 ± 0.02	1.8 ± 0.2	1.0 ± 0.2	This work	ND
TMR	0.15 ± 0.02	18 ± 2	10 ± 2	This work	0.3 [14]
Rh 6G	0.035 ± 0.005	0.8 ± 0.1	2.3 ± 0.4	This work	ND
Rh 123 hydro	0.63 ± 0.07	0.12 ± 0.02	0.007 ± 0.001	This work	ND
Rh B	0.75 ± 0.06	> 40	> 1	This work	ND
Rh I,II hydro.	0.71 ± 0.06	> 30	> 1	This work	ND
Daunorubicin	0.13 ± 0.02	2 ± 0.2	1.5 ± 0.2	[28]	2.1 [34]
Idarubicin	0.60 ± 0.06	40 ± 3	1.9 ± 0.2	[28]	1.0 [34]
Pirarubicin	0.30 ± 0.03	35 ± 3	6.2 ± 1.1	[28]	0.4 [10]

K^+-buffer with various rhodamine concentrations (0.02–0.2 μM) was performed. Figure 4 shows such a record for Rh6G. Data points of F_{cyto} vs. time (or the experimental records) were fitted to Eqn (13) of the experimental section and the value of $k/V_{cell.}$ (1 + β) and then k were estimated. The k-values are reported in Table 1. The k-values for the positively charged RhI, RhII and Rh6G were similar but that of TMR was about 10-fold higher and that for $Rh123_{hyd}$ about 100-fold lower. The rates of uptake of the Rh B and RhI,II_{hyd} were too high to be measured by this technique but we could estimated that the values were higher than 3×10^{-12} L per cell per s.

Determination of the active efflux coefficient k_a

Once the parameters k, C_e and $\mathscr{C}_i$ measured, it was easy to calculate k_a according to Eqn (5). The values are reported in Table 1. As it was impossible to measure k for Rh B and RhI,II_{hyd} it was also impossible to calculate their k_a value. However it is possible to estimate that, in both cases, k_a should be higher than 1×10^{-12} L per cell per s.

Control experiments

After having established the principle of the experiments as explained above, a set of control experiments was performed in order to further validate the use of the experimental model to analyze the transport kinetics of rhodamines.

First, we have checked that in K^+-buffer plasma and mitochondrial potentials were dissipated. We have performed a continuous spectrofluorometric of the fluorescence signal of a cationic rhodamine (TMR 0.2 μM) during incubation with sensitive cells in a 1-cm quartz cuvette containing Na^+-buffer on the one hand and K^+ buffer on the other hand. In Na^+-buffer a strong decrease of the fluorescence signal was observed due to the accumulation of the lipophilic cation mainly in the mitochondrial compartments, leading to a quenching of the fluorescence signal. However, when the same experiment was performed in K^+-buffer, no quenching of the fluorescence was observed from which we inferred that there was no accumulation of TMR inside the cells and therefore that the potentials were eliminated.

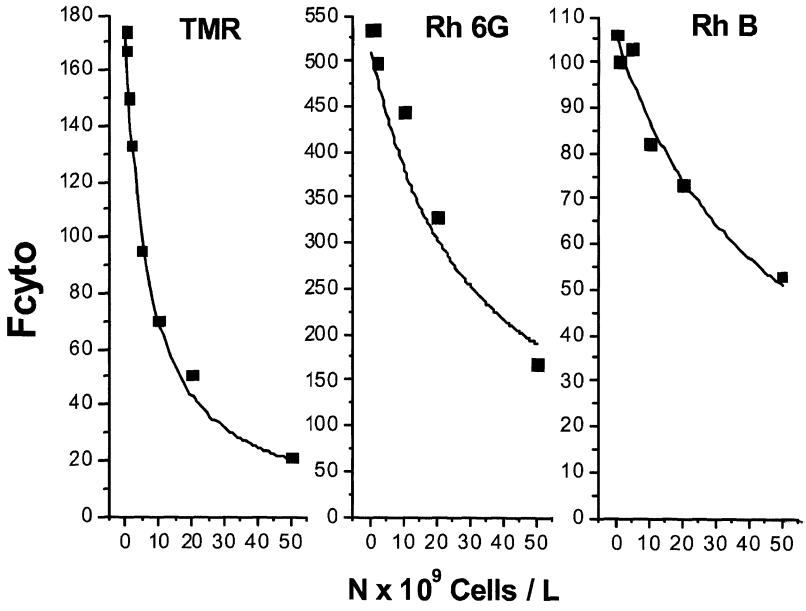


Fig. 3. Intensity of the flow cytometry signal recorded at steady-state fluorescence from sensitive cells incubated with 0.2 μM TMR, Rh 6G or Rh B in K^+-buffer. The intensity of the signal (F_{cyto}) recorded plotted as a function of the number of cells per L. The data points are from a representative experiment. They were fitted to Eqn (9) of the experimental section $F_{cyto} = F{\cdot}[C_T{\cdot}(1 + \beta{\cdot}\rho)]/[1 + 10^3{\cdot}N{\cdot}(1 + \beta)]$, as shown by the solid line, and the values of F, β and ρ were estimated.

Table 2. Parameters characteristic of the interaction of rhodamines with cells. The data are the means ± SEM of three determinations. $\mathscr{C}$ is the concentration of internal rhodamine bound to its receptors; $\mathscr{C}_i$ is the concentration of free internal rhodamine; *F*: molar fluorescence (arbitrary units) of rhodamine free in the cytosol; ρ, fluorescence quantum yield of rhodamine bound to its intracellular binding.sites. ND, not determined.

Rhodamine	$\beta = \mathscr{C}/\mathscr{C}_i$	$F \times 10^{-6}$	ρ
Rh I	22 ± 3	150 ± 20	0.1
Rh II	25 ± 4	130 ± 15	0.08
TMR	150 ± 20	190 ± 30	0.03
Rh 6G	33 ± 4	590 ± 70	0.10
Rh 123 hydro	1.0 ± 0.2	ND	ND
Rh B	20 ± 3	186 ± 30	0.09
Rh I,II hydro.	15 ± 2	160 ± 20	0.006

To be sure that in K^+-buffer plasma and mitochondrial potentials were dissipated, we used a totally different method to check it. We measured the Tc-MIBI accumulation in sensitive cells as described in [9]. Due to its lipophilic cationic nature, Tc-MIBI may distribute across biological membranes in response to the transmembrane potential in a manner similar to cationic rhodamines. We have determined Tc-MIBI accumulated inside the cells after 1 h of incubation with 1 nM Tc-MIBI. Taking into account the volume occupied by the cells (the volume of one cells having been estimated to 10^{-12} L) we have calculated the intracellular concentration of Tc-MIBI inside the cells. When the incubation was performed in Na^+-buffer, the intracellular Tc-MIBI concentration was about 15-fold higher than the extra cellular one. However, when the incubation was performed in K^+-buffer the intra- and extracellular Tc-MIBI concentrations were very similar. This implied a lack of potential-dependent accumulation of Tc-MIBI by cells under K^+-buffer conditions.

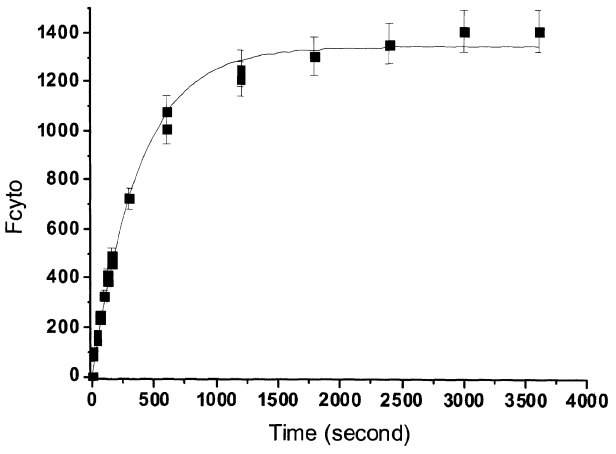


Fig. 4. Uptake of Rh 6G by sensitive cells. Cells, 10^6 mL^{-1}, were incubated with 0.2 μM Rh 6G in K^+-buffer. The cytofluorometry signal (F_{cyto}) was recorded as a function of time. The values represent mean ± SD of two independent experiments performed on 2 different days. They were fitted to Eqn $F_{cyto} = (1 + \beta\cdot\rho)\cdot F\cdot C_c\cdot[1 - \exp(-k\cdot t/V_{cell}\cdot(1 + \beta)]$ and the values of *k* was calculated.

Second, we have checked that the P-gp-mediated efflux of molecules did not depend on the membrane potential: to verify that point, we have compared the accumulation of daunorubicin in K562/ADR cells in Na^+-and K^+-buffer, respectively. The accumulation of anthracycline in sensitive cells did not depend on the membrane potential and this molecule did not accumulate in mitochondria. We have observed using a previously described method [10] that the accumulation of DNR in resistant cells did not depend on $\Delta\psi$.

A third control was carried out to check the ATP intracellular level under the different experimental conditions. The ATP concentration was determined using the luciferin-luciferase test [25]. In both cell lines, the presence of azide under glucose-free conditions yielded 90% ATP depletion.

A fourth control was performed to check that P-gp inhibitors inhibit rhodamine transport. For this purpose, two well-known P-gp inhibitors, verapamil and PAK-104P were used with TMR [27]. Cells were incubated in K^+-buffer with TMR and either 100 μM verapamil or 50 μM PAK-104P (see Fig. 2). In both cases the flow cytometry signal was similar to that observed with sensitive cells, indicating that these classical P-gp inhibitors were able to block the P-gp-mediated efflux of rhodamines.

Discussion

Most of rhodamine dyes are P-gp substrates and among them Rh123 is a marker widely used in cellular dye-exclusion assays aimed at monitoring MDR. Rh123 is also widely used as a structural marker for mitochondria as an indicator of mitochondrial activity [28–30].

Measurement of the kinetic characteristics of substrate transport, is a powerful approach to enhancing our understanding of their function and mechanism. In this paper, we present data that further characterize the transport of several rhodamine analogues. We took advantage of the intrinsic fluorescence of rhodamines and performed a flow-cytometric analysis of dye accumulation in the wild-type drug sensitive K562 that do not express P-gp and its MDR subline which display high level of MDR (the resistance factor for daunorubicin was equal to 20 [31]). The measurements were made in real time using intact cells. The kinetics parameters are compared with previous data obtained with others P-gp substrates. The findings presented here are the first to show quantitative information about the kinetics parameters for P-gp-mediated efflux of rhodamine analogues in intact cells.

The ability of ABC transporters to actively transport compounds against a concentration gradient across the cell membrane has allowed the development of a number of functional assays to measure the level and function of transporter present [32]. The efflux of fluorescent compounds from cells expressing ABC proteins can be quickly and easily measured by flow cytometry and many fluorescent compounds have been used to characterize it. However such measurements must be made with high cautions and we took great care to specify what we were exactly measuring.

To characterize the P-gp-mediated efflux of compounds, the parameter k_a was calculated. As shown in the Materials

and methods and in [10,31], at low substrate concentration, k_a is proportional to the ratio V_M/K_m and is very convenient to evaluate the efficiency of a transporter. This parameter is very useful because its value can be estimated from few measurements while the determination of the kinetics parameters V_M and K_m requires a very large number of measurements and the use of high substrate concentrations needed to saturate the transporter and reach the maximal rate. It is not always possible to use such conditions, especially with living cells. Thus, in the present case we did not observe saturation of the rhodamine efflux.

The determination of k_a requires the measurement of the gradient of concentration, i.e. C_e vs. $\mathscr{C}_i$, which is generated by the presence of the pump. A problem inherent to almost all studies of P-gp is the lack of control of the experimenter over the intracellular free drug concentration, $\mathscr{C}_i$, which can often be roughly estimated. $\mathscr{C}_i$, however, is one of the most important parameters determining the transport rate. This problem is even more crucial for positively charged rhodamine analogues because the gradient of concentration across the plasma membrane can be generated by both the P-gp and the potential membrane. For this reason we have used cells without membrane potential after having checked that the P-gp-mediated efflux of drug was not dependent on potential. Here we have developed new concepts to determine $\mathscr{C}_i$ using flow cytometry and macrospectrofluorometry. One important piece of data from our study is the demonstration that thanks to the use of two independent fluorometric techniques, macrofluorescence and flow-cytometry, it is possible to directly determine the free rhodamine concentration in the cytosol and in the extracellular medium. Actually, our data clearly show that the cytofluorometric signal, in cells without membrane potential, is proportional to the amount of rhodamine free in the cytosol. This observation allows the further determination of the concentration of drug free in the cytosol of resistant cells.

The determination of k_a requires also the measurement of the rate of passive diffusion of the dye through the plasma membrane. This cannot be done by the simple measurement of the increase of the fluorescence signal (F_{cyto}) of the cells when they are incubated with the dye. Actually, the dye can interact with various components inside the cells yielding modifications of the fluorescence. For this reason, we have determined the ratio of the drug bound to the drug free in the cytosol, which subsequently allows the determination of the real number of molecules that penetrate per second into one cell and therefore the true rate of passive diffusion of the dye.

As can be seen in Table 1, the gradient of concentration is about fivefold higher in the case of positively charged rhodamine compared to the zwitterionic one. However, this does not mean that the positively charged rhodamine analogues are better substrates than the zwitterionic ones because one must take into account the rate of passive diffusion which is very high for RhI,II_{hyd} and for Rh B. This rate is so high that it cannot be measured with the conventional technique used here. However, we have estimated that k was higher than 30×10^{-13} L per cell per s and therefore that k_a was higher than 1×10^{-12} L per cell per s, i.e. similar to the k_a of the positively charged rhodamines. We were unable to get such parameters for Rh123 because it was impossible to reach a steady state incorporation. We can propose the following explanation for this observation: Rh I, RhII, Rh6G and Rh123 are esters that can be hydrolysed by intracellular esterases but this hydrolysis is rather slow. In the case of RhI, RhII and Rh6G that penetrate rapidly inside the cells, the steady state is reached within a few minutes and the amount of hydrolysed compound is very low. However in case of Rh123, whose rate of uptake is very low the rate of hydrolysis is not negligible when compared to the rate of uptake, it follows that the dye continues to accumulate and that no steady state is reached.

Let us compare our data with those from the literature. To our knowledge, only Eytan *et al.* [7] have examined several rhodamine dyes for their P-gp-mediated exclusion from MDR cells. In an effort to define the dye characteristics that allow P-gp to efficiently extrude rhodamine dyes and MDR-type drugs from MDR cells, these authors have compared the levels of dye accumulation in MDR cells using the following parameters: the affinity toward P-gp evident as the apparent K_m of ATPase-activity modulation of reconstituted P-gp; the level of maximal stimulation of P-gp ATPase activity; the level of dye binding to artificial membrane; the transmembrane movement rate; and the hydrophobicity. The best and only clear correlation observed was with the transmembrane movement rate. Thus, they observed that Rh B, the poorest cellular substrate, exhibited high affinity towards reconstituted P-gp, but was the fastest membrane-traversing dye. In contrast, TMR, the best cellular substrate, although exhibiting an affinity toward reconstituted P-gp similar to Rh B, was the slowest to traverse membranes among the rhodamine dyes. We agree with their observation that TMR is the best cellular MDR probe as we have found that k_a for TMR is fivefold to tenfold higher than that for Rh6G, RhI and RhII. However, we disagree with their conclusion that Rh B was the poorest P-gp substrate: we have shown that k_a for Rh B is equal to or higher than that observed for RhI and RhII. In any case, it is also difficult to compare the data obtained by these authors with ours because (a) the experiments were performed with cells having membrane potential and (b) the cells were washed before the cytofluorometry measurement and under those conditions there is a rapid redistribution of the dye between intracellular compartments and extracellular medium. In addition, these authors didn't provide quantitative data allowing a true comparison with other P-gp substrates.

One of our aims was to compare the P-gp-mediated efflux of these rhodamine analogues to that of anthracyclines. To help this comparison, the values of $\mathscr{C}_i/C_e$, k and k_a for three anthracycline derivatives are reported in Table 1. We have chosen daunorubicin for which $\mathscr{C}_i/C_e$ is high and k rather low and idarubicin for which $\mathscr{C}_i/C_e$ is rather low and k very high. However, for these two molecules the k_a values are similar and very close to those determined for rhodamine analogues. Our conclusion is that the compounds of these two classes of molecules, anthracyclines and rhodamines, are substrates of P-gp and that their pumping rates at limiting low substrate concentration are very similar. This is corroborated by the observation of Lu *et al.* [14] who monitored the transport of TMR in proteoliposomes containing reconstituted P-gp and determined a K_m of

0.3 μM for TMR; as can be seen in Table 1, this value is similar to that we observed for anthracycline derivatives.

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