ELSEVIER

Biochemical Pharmacology 63 (2002) 1471–1479

Biochemical Pharmacology

Preferential efflux by P-glycoprotein, but not MRP1, of compounds containing a free electron donor amine

Milena Salerno[a], Teresa Przewloka[b], Izabela Fokt[b], Waldemar Priebe[b], Arlette Garnier-Suillerot[a,*]

[a]*Laboratoire de Physicochimie Biomoléculaire et Cellulaire (UMR 7033), Université Paris Nord, 74 Rue Marcel Cachin, 93017 Bobigny, France*
[b]*M. D. Anderson Cancer Center, The University of Texas, 1515 Holcombe Boulevard, Houston, TX 77030, USA*

Received 24 October 2001; accepted 8 February 2002

Abstract

Multidrug resistance (MDR) in model systems is known to be conferred by two different integral proteins, the 170-kDa P-glycoprotein (P-gp) and the 190-kDa multidrug resistance-associated protein (MRP1), both of which pump drugs out of MDR cells. The presence of a nitrogen atom, charged at physiological pH, has frequently been considered to be a hallmark of P-gp substrates and inhibitors. The present study was aimed at investigating the role of nitrogen in the ability of the pump to recognise substrate. We measured the kinetics of active efflux of seven new anthracycline derivatives in P-gp-expressing K562/ADR cells and in MRP1-expressing GLC4/ADR cells. Six of these compounds represent analogues of daunorubicin in which the amino sugar nitrogen is bound to an amino- or a nitro-substituted benzyl moiety, the seventh is a doxorubicin derivative in which benzyl group is bound with 4′-oxygen. We found that the compounds with a nitro group on the benzyl ring were poor substrates for P-gp despite the presence of a secondary amine that can be protonated. In contrast, compounds that have a free amino group were very good substrates even though this amine is not protonated in the pH range studied ($pK \sim 3$). These results show that the nitrogen atom does not interact with P-gp in a charged form but rather as an electron donating group.

Keywords: P-glycoprotein; Multidrug resistance; Daunorubicin; MRP1

1. Introduction

Multidrug resistance (MDR) involves cellular resistance to naturally derived anti cancer agents, such as anthracyclines, vinca alkaloids, epipodophyllotoxins, taxanes, and others. This form of resistance results from multifactorial mechanisms, such as decreased cellular topoisomerase II, increased cellular glutathione, and increased ATP-dependent efflux of the cytotoxic agent over the cellular plasma membrane drug efflux pumps [1–4]. Biochemical research has uncovered three types of drug pumps that may play a role in MDR. The most intensively studied is the 170-kDa P-glycoprotein (P-gp), which in humans is encoded by the MDR1 gene. A variety of studies have shown that P-gp acts as a drug pump in the plasma membrane of tumour cells, recognising an astonishing range of cytotoxic molecules to be removed from the cell. Two more recently discovered pumps are the 190-kDa multidrug resistance-associated protein (MRP1) [5–9] and cMOAT/MRP2, the major organic anion transporter in the canalicular membrane of hepatocyte: cMOAT/MRP2 has been shown to be overexpressed in cancer cell lines resistant to cisplatin, doxorubicin, and vincristine [10–12]. These three proteins—P-gp, MRP1, and cMOAT/MRP2—belong to the superfamily of the ATP binding cassette transport proteins or traffic ATPases, which depend on ATP hydrolysis to translocate substrates across the cell membrane. Multidrug transporters are present in all living cells, and most of the currently used naturally derived anti cancer drugs are good substrates for P-gp, MRP1, or both. Overexpression of these transporters by tumour cells is thought to be a significant factor in both intrinsic and acquired resistance to anticancer drugs. Consequently a great deal of interest is focused on identifying chemical agents that can either antagonise drug transport by these proteins or inhibit

* Corresponding author. Tel.: +33-1-48-38-77-48; fax: +33-1-48-38-77-77.
E-mail address: garnier@lpbc.jussieu.fr (A. Garnier-Suillerot).
Abbreviations: P-gp: P-glycoprotein; MRP1: multidrug resistance associated protein; DNR: daunorubicin.

0006-2952/02/$ – see front matter
PII: S0006-2952(02)00895-X

the proliferation of tumours cells despite the expression of these transporters [13–15]. Knowledge of the chemical nature of the transported substrate is important, since it is difficult to understand how a transporting system works without knowing which molecule is transported. Despite a huge amount of research on the mechanisms of P-gp-mediated efflux of drugs, the molecular parameters required for a molecule to be recognised and pumped out by P-gp and MRP1 are still unknown.

Anthracyclines are among the most useful antitumour drugs ever developed; most patients undergoing systemic cancer chemotherapy are given an anthracycline at some time during treatment. The mechanism of action of anthracyclines has been studied extensively, and new analogs are being designed and synthesised [16]. Priebe and colleagues in approach to overcome MDR have focused on identification of chemical modifications that minimise drug efflux by reducing the affinity of the drug for P-gp. This approach led them to the hypothesis that the amino group in general and in the sugar portion of doxorubicin particular has an important role in substrate recognition by the transporter. They have shown that removal of the basic amine in doxorubicin and replacement with an hydroxyl group confers a partial lack of cross-resistance [16–18]. The ability of such modified compounds (e.g. Annamycin (LIT), hydroxyrubicin (LIT)) to overcome MDR1-mediated resistance was confirmed in *in vitro* experiments against several MDR cell lines [16,19]. Even though these molecules can be recognised by P-gp, their affinity for this transporter is significantly lower than that of anthracyclines having a basic amino group at C-3′ [20].

Continuing this approach, we examined the kinetics of active efflux of eight anthracyclines in P-gp-expressing K562/ADR cells and in MRP1-expressing GLC4/ADR cells. Six compounds represent analogues of DNR in which the amino-sugar nitrogen is bound to an amino- or nitro-substituted benzyl moiety; the seventh is a doxorubicin derivative in which the 3′-amine is unalterated whereas O-benzyl moiety is at C-4′ position. We found that compounds with benzyl ring substituted with amine are pumped out by P-gp about 10 times more efficiently than those which have benzyl ring substituted with nitro group. However, the presence of a free amino group had no affect on the ability of MRP1 to pump out the anthracyclines.

2. Materials and methods

2.1. Cell culture and cytotoxicity assay

K562 leukaemia cells and the P-gp expressing K562/ADR cells [21], as well as GLC4 and the MRP1-expressing GLC4/ADR cells [8] were cultured in RPMI 1640 medium (Sigma Chemical Co) medium supplemented with 10% foetal calf serum (Bio media) at 37° in a humidified incubator with 5% CO_2. The resistant K562/ADR and GLC4/ADR cells were cultured with 400 nM or 1.2 μM doxorubicin, respectively until 1–4 weeks before the experiments. Cell cultures were split 1:2, 1 day before use to ensure logarithmic growth during the experiments.

The cytotoxicity of the anthracyclines was determined by incubating 10^5 cells with six different concentrations of anthracylines for 72 hr in standard 6-well plates. Then the IC_{50}s (50% inhibitory drug concentrations) were determined by counting the cells with a Coulter counter. The resistance factor (RF) was defined as the IC_{50} for the resistant cells divided by the IC_{50} for the corresponding sensitive cells.

2.2. Drugs and chemicals

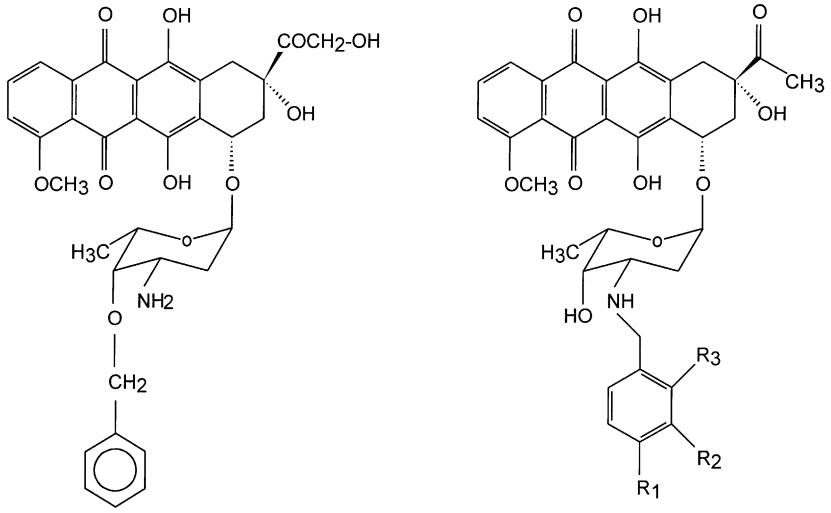
DNR was kindly provided by Roger Bellon laboratory. WP757, WP758, WP785, WP776, WP755, WP756 and WP744 (Fig. 1) were provided by Priebe and coworkers [16,17,22]. Stock solutions were prepared in water just before use. Concentrations were determined by diluting stock solutions in water to approximately 10 μM and using $\lambda_{480} = 11,500\ M^{-1}\ cm^{-1}$. Experiments were performed in buffer solutions containing 20 mM HEPES plus 132 mM NaCl, 3.5 mM KCl, 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$ at pH 7.3 in the presence of 10 mM sodium azide, with or without 5 mM glucose.

2.3. Cellular drug accumulation

The rationale and validation of our experimental set-up for measuring the kinetics of active transport of anthracyclines from tumour cells is described extensively and discussed before [13,23–25]. It is based on a continuous spectrofluorometric monitoring (Perkin Elmer LS50B spectrofluorometer) of the decrease of the fluorescence signal of the anthracycline at 590 nm ($\lambda_{ex} = 480$ nm) after incubation with cells in a 1-cm quartz cuvet. The fluorescence of the drug decreases during incubation through its being quenched as it is intercalated between the base-pairs of DNA.

2.4. Analysis of the P-gp- or MRP1-mediated efflux of anthracycline derivatives

Determination of the kinetic parameters the maximum rate (V_M) and the Michaelis–Menten constant (K_m), characteristic of the transporter-mediated efflux of drugs requires the measurement of V_a, the rate of drug efflux by the pump, and of $\mathscr{C}_i$, the intracellular free drug concentration. These measurements can be made easily with energy-depleted cells. For these experiments, cells (1×10^6 cells mL^{-1}; 2 mL per cuvet), are incubated for 30 min in HEPES buffer with sodium azide, but without glucose. Depletion of ATP in these cells is verified as being 90% with the luciferin-luciferase test and cell viability is confirmed with Trypan blue staining. The drug pumps in



WP744

Drug	R_1	R_2	R_3
WP757	NH_2	H	H
WP758	H	H	NH_2
WP785	H	NH_2	H
WP776	H	H	H
WP755	NO_2	H	H
WP756	H	H	NO_2

Fig. 1. Structures of the anthracyclines used.

energy-depleted cells are inactive; hence when the cells are incubated with anthracycline, at steady state, the free drug concentration ($\mathscr{C}_i$) in the cytosol susceptible to becoming a substrate for the pump is the same as that in the extracellular medium (C_e)—when the extracellular and intracellular pH levels are the same. At steady state, the addition of glucose leads to restoration of control ATP levels within 2 min and to an increase of the fluorescence signal due to the efflux of anthracycline from the cells. This ATP-dependent anthracycline efflux is determined from the slope of the tangent of the curve $F = f(t)$, where F is the fluorescence intensity at the time of addition of glucose. Under these conditions, the passive influx and efflux at the moment of glucose addition (when $\mathscr{C}_i = C_e$) are equal to each other, and the net initial efflux represents the MRP1- or the P-gp-mediated active efflux [13,21,25].

Thus, V_a can be determined for various intracellular free drug concentrations. In most cases, the maximum efflux rate (V_M), the apparent Michaelis–Menten constant (K_m), and the co-operativity constant (n_H) for the transport of anthracyclines can be computed by non-linear regression analysis of drug-transport velocity (V_a) vs. free cytosolic anthracycline concentration ($\mathscr{C}_i$) data by using the Mac curve fit program and by assuming that the transport follows the Hill equation [26]

$$V_a = \frac{V_M \mathscr{C}_i^{n_H}}{(K_m^{n_H} + \mathscr{C}_i^{n_H})} \quad (1)$$

In some cases, the complete curve $V_a = f(\mathscr{C}_i)$ cannot be obtained and therefore it is not possible to obtain these three characteristic transporter parameters by calculating the mean active efflux coefficient (k_a) according to the equation:

$$V_a = k_a \mathscr{C}_i \quad (2)$$

The relation between k_a and the parameters V_M, K_m and n_H [25] is

$$k_a = \left(\frac{V_M}{n_H K_m}\right)(n_H - 1)^{\left(1 - \frac{1}{n_H}\right)} \quad (3)$$

when $n_H = 2$, it follows that $k_a = V_M/2K_m$ is equal to the slope of the tangent to the curve $V_a = f(\mathscr{C}_i)$ when $\mathscr{C}_i = K_m$.

Thus, V_M, K_m and therefore k_a can be calculated using the Eq. (1). When the complete curve $V_a = f(\mathscr{C}_i)$ cannot be obtained (as it is the case of highly lipophilic drugs) a good estimation of the active coefficient k_a can be obtained from the ratio $V_a/\mathscr{C}_i$ at low $\mathscr{C}_i$ values.

3. Results

The structures of the molecules studied are collected in Fig. 1. Six compounds represent DNR with its amino sugar N-bound to benzyl moiety with either no substituent or amino or nitro substituent. One of the compound (WP744) studied is a doxorubicin derivative with its amino sugar (C-4′-O) bound to benzyl moiety. The amino and nitro groups bonded at different positions of a phenyl ring were also expected to modify the acidity of a daunosamine nitrogen. All N-substituted DNR monomeric derivatives were poorly soluble in water, this precluded the use of potentiometric method and calculations of the protonation constants were based only on the spectroscopic data made at 20 μM concentrations. The values for amino-derivatives are around 7.4 ± 0.3, while nitro-derivatives are characterised by more acidic amino-sugar function with pK very close to 5.8 ± 0.2. Compound WP776 having neither amino nor nitro group has an intermediary pK value equal to 6.6 ± 0.2. All pK values estimated for the anthracyclines studied here are distinctly lower than that found for parent DNR molecule (8.4). The pK values of the primary amino group of compounds WP757, WP758 and WP785 were found to be in the range between 2.5 and 3.5. The percentages of neutral form of the drugs at experimental pH values are indicated in Table 1.

3.1. Cell-growth inhibition

IC_{50} values obtained for the drugs for sensitive and resistant cells are shown in Table 1. The cytotoxicities of the three amino derivatives toward sensitive cells was 3–6-fold lower than that of DNR. The nitro derivatives were 50-fold less cytotoxic than DNR. However, their cytotoxicities toward resistant cells was almost the same yielding a RF close to 1. Also for WP776 very low RF was found. At the opposite the amino derivatives had lower cytotoxicities toward resistant cells than toward sensitive one yielding RF in the range 10–100 (Table 1).

3.2. Kinetics of the P-gp-mediated efflux of drugs

In all experiments, the intracellular and extracellular pH was the same and equal to 7.3. In each case, 10^6 cells mL^{-1} were incubated with varying amount of drug (1–15 μM). Fig. 2 shows the typical decrease of fluorescence at 590 nm as a function of time after addition, to energy-depleted K562/ADR cells, of WP757 and WP755, respectively (Fig. 2). For the seven derivatives, the time required to reach the steady state of anthracycline accumulation in energy-depleted cells was less than 30 min. At this point $\mathscr{C}_i = C_e$ was calculated from the non-quenched fluorescence: $C_e = C_T(F_0 - F'_n)/F_0$, where F_0 is the fluorescence of a C_T micromolar anthracycline solution and F'_n, the fluorescence at steady state. The addition of glucose yielded ATP synthesis and initiation of the active efflux component. The rate for the active efflux was calculated as $V_a = C_T/F_0(dF/dt)$, where dF/dt is the slope of the tangent to the curve. When similar experiments were performed using the parent cell line, no active efflux could be detected (data not shown). Figs. 3–5 shows the plot of V_a as a function of $\mathscr{C}_i$. For the four derivatives WP757, WP758, WP785 and WP744, bearing an amino group, V_a increased rapidly with $\mathscr{C}_i$ and then, in the case of WP758 and WP744, reached a plateau (Figs. 3 and 5). However, in the case of WP757 and WP785, the plot of V_a resulted in a bell-shaped dependence on the cytosolic free drug concentration (Fig. 3). For the three other derivatives which either had a nitro group instead of the amino one or had no substituent at all, the increase of V_a as a function of $\mathscr{C}_i$, was much less distinct (Fig. 4). Actually, for these

Table 1
Cross-resistance pattern of K562/ADR cells and kinetic parameters of anthracycline derivatives

Drug	% D°[a] at pH 7.3	IC_{50} (S)[b] nM	IC_{50} (R)[c] μM	RF[d]	k_a (P-gp)[e] $\times 10^{-12}$ L·cell^{-1}·s^{-1}
WP776	83	~75	~0.2	~3	0.6 ± 0.1
WP756 (o−NO_2)	97	~300	~0.5	~1.7	0.6 ± 0.1
WP755 (p−NO_2)	97	>500	0.5	~1	0.5 ± 0.1
WP758 (o−NH_2)	37	60	0.5	9	3.4 ± 0.3
WP785 (m−NH_2)	37	30	~2.5	83	5.4 ± 0.5
WP757 (p−NH_2)	37	30	>3	>100	5.6 ± 0.6
WP744	nd	10	0.3	30	4.3 ± 0.4
DNR	7	10	~0.2	~20	2.5 ± 0.3

[a] Percentage of drug in the neutral format pH 7.3.
[b,c] Drug concentration required to inhibit 50% of sensitive (S) or resistant (R) cell growth.
[d] Resistance factor calculated as resistant cell IC_{50}/sensitive cell IC_{50}.
[e] Active efflux coefficient. Data are mean ± SD from three to five independent experiments on different days.

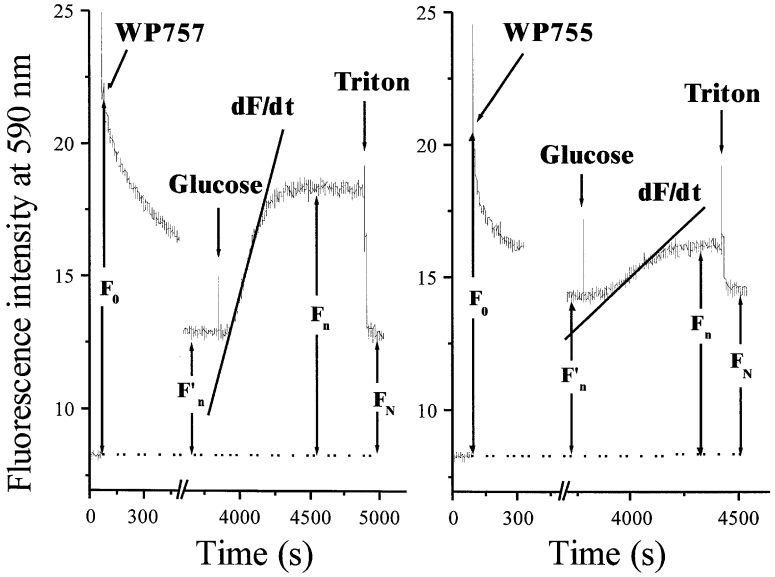


Fig. 2. Incorporation of WP757 (left) and WP755 (right) in energy-depleted K562/ADR cells and determination of the active efflux rate (V_a) from dF/dT after the addition of glucose.

compounds it was impossible to observe a saturation of the efflux even at intracellular concentration of drugs equal to 10 μM. For all these compounds the first part of the curve was linear and the slope yielded the mean active efflux coefficients k_a (Table 1). For a sake of comparison the value of k_a obtained for DNR under strictly analogous conditions is also reported. The k_a values for the compounds bearing an amino group (WP757, WP758, WP785 and WP744) were about 2-fold higher than that observed for DNR. However, for the compounds which either had no subsituent on the phenyl ring (WP776) or had a nitro group (WP755, WP756), the k_a values were about 10 times lower than those observed for the amino-bearing compounds.

3.3. Kinetics of the MRP1-mediated efflux of DNR, WP756 and WP758

We have determined the MRP1-mediated efflux of one compound of each class only: one bearing an amino group,

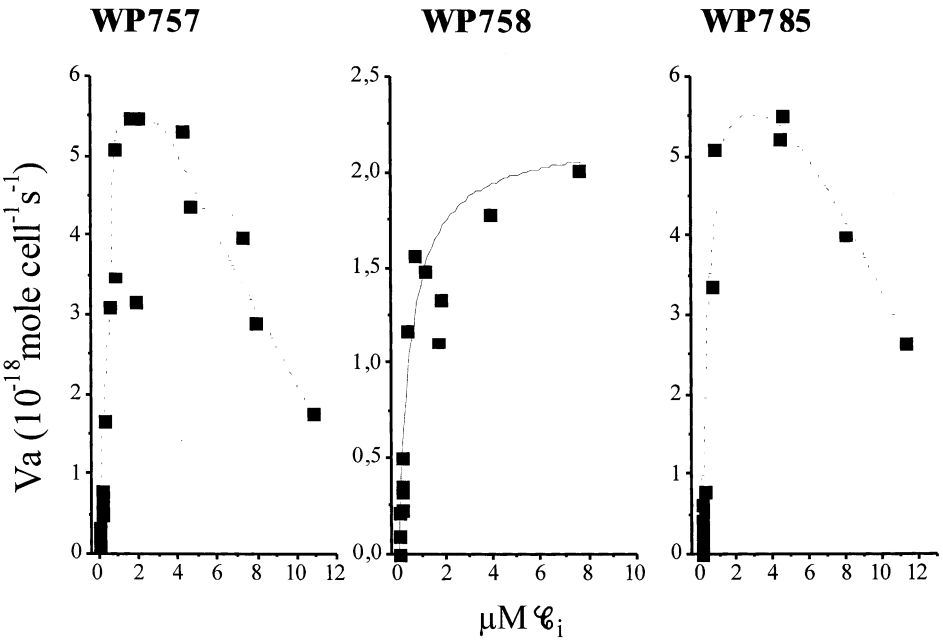


Fig. 3. Kinetics of the P-gp-mediated efflux of anthracycline derivatives plotted as a function of $\mathscr{C}_i$ the cytosol free drug concentration. Drugs are WP757, WP758 and WP785. K562/ADR cells (1×10^6/mL) were incubated in the presence of various concentrations of drug ranging from 0 to 15 μM. V_a and $\mathscr{C}_i$ were determined as described. Data points are from a representative experiment. The data for WP758 were fitted using Eq. (1).

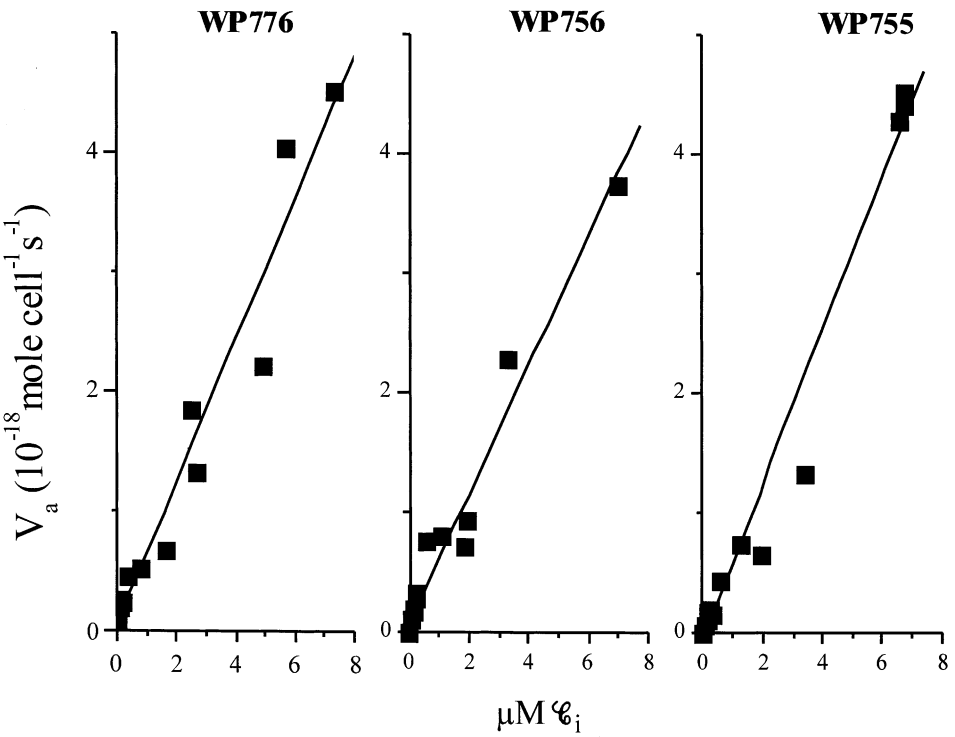


Fig. 4. Kinetics of the P-gp-mediated efflux of anthracycline derivatives plotted as a function of $\mathscr{C}_i$ the cytosol free drug concentration. The drugs are WP776, WP756, and WP755. K562/ADR cells (1×10^6/mL) were incubated in the presence of various concentrations of drug ranging from 0 to 12 μM. V_a and $\mathscr{C}_i$ were determined as described. Data points are from a representative experiment. The data were fitted using Eq. (2).

WP758, and one bearing a nitro group, WP756. The drug concentrations were varied from 0.1 to 5 μM and V_a, the rate of active efflux, was determined as described above. Here also DNR was used as a control. The values of the active parameters k_a was for the three compounds equal to $0.6 \pm 0.1 \times 10^{-12}$ L cell^{-1} s^{-1}. For a sake of comparison the k_a values for the different drugs and resistant cells lines are presented in Fig. 6.

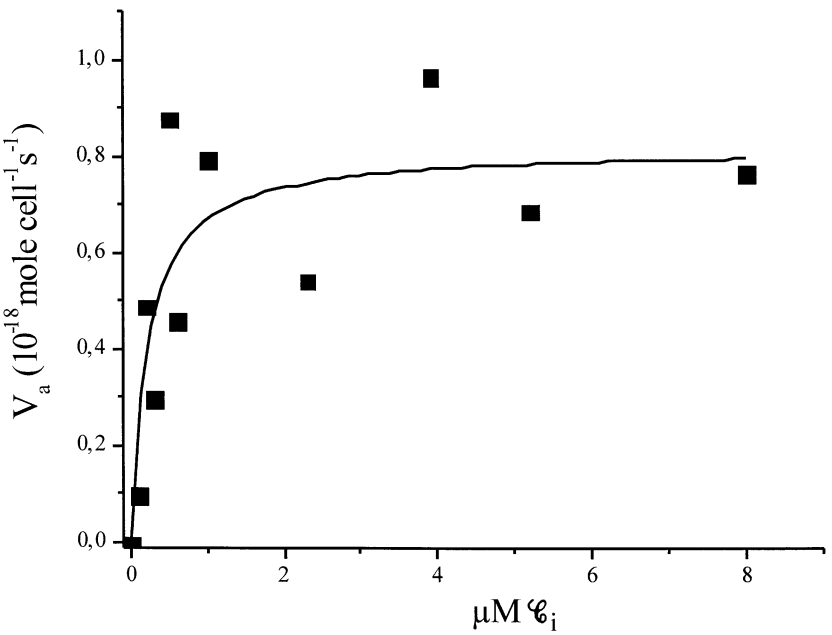


Fig. 5. Kinetics of the P-gp-mediated efflux of WP744 plotted as a function of $\mathscr{C}_i$ the cytosol free drug concentration. K562/ADR cells (1×10^6/mL) were incubated in the presence of various concentrations of drug ranging from 0 to 12 μM. V_a and $\mathscr{C}_i$ were determined as described. Data points are from a representative experiment. The data were fitted using Eq. (1).

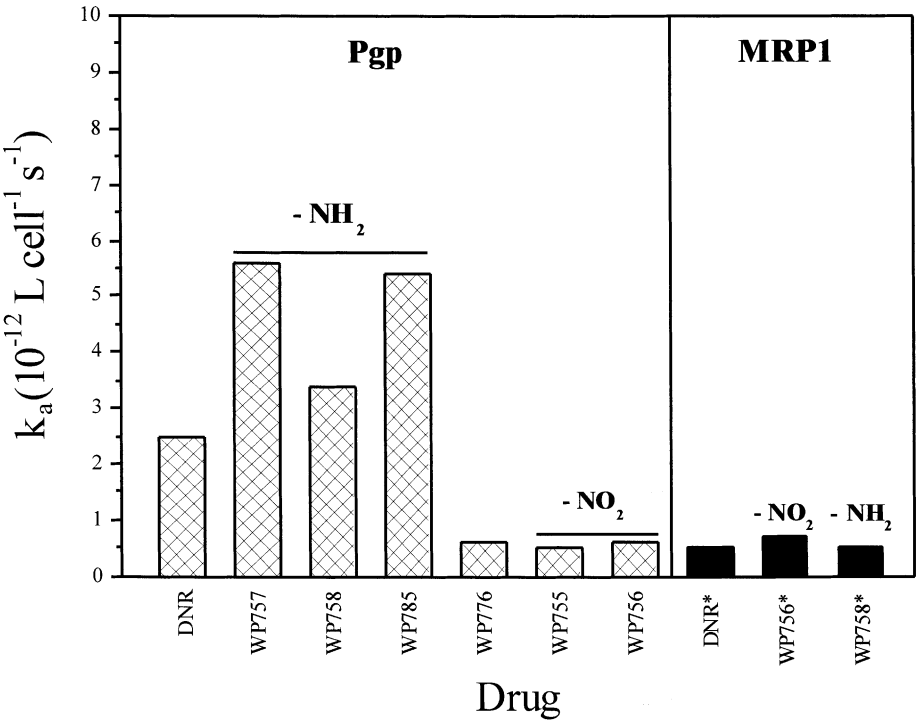


Fig. 6. Kinetic parameters of anthracycline derivatives. Active efflux coefficient k_a for K562/ADR (left) and GLC4/ADR (right).

4. Discussion

MRP1 and P-gp are very distantly related members of the superfamily of ATP binding cassette transmembrane (ABC) transporters which can be divided into two major clusters: the P-gp cluster and the MRP cluster. Both P-gp and MRP1 confer resistance to a similar but not identical spectrum of naturally derived chemotherapeutic agents. However, now several lines of evidence have shown that P-gp and MRP1 employ different mechanisms in conferring resistance to naturally derived chemotherapeutic agents [27–30]. Evidences have been provided that MRP1 exports GSH physiologically [27,30] and that drugs such as vincristine [28] and DNR [29,30] are co-transported with GSH.

Let us first consider the data obtained with MRP1. For all the compounds studied here, the efficiency of the efflux is rather low and the saturation of the efflux cannot be observed at the concentrations that can be used for *in vivo* experiments. Thus, only k_a value have been determined. The k_a values obtained for DNR and for the amino (WP758) and nitro derivatives (WP756) are very similar. Recently we have also found that the efficiency of the MRP1-mediated efflux of different anthracyclines was very similar [25]. This result is not surprising in relation with the observations, that MRP1 co-transports DNR with GSH and that, in addition, at any intracellular GSH concentration, the rates of GSH and DNR efflux were very similar. This strongly suggests that the stoichiometry between GSH and daunorubcin transport is 1/1. It seems that GSH is transported all the time and that DNR is just riding alone. In such situation the structure of the anthracycline has almost no impact on the efficiency (i.e. k_a) of its MRP1-mediated efflux.

Let us now consider the impact of anthracycline structure on its P-gp-mediated-efflux. For the two compounds, WP758 and WP744, as well as for DNR [21,25] it was possible to get the complete curve V_a vs. $\mathscr{C}_i$ and therefore to determine the maximum rate for the efflux and the Michaelis–Menten constant (see figure). The K_m values are 0.35 μM, 0.2 μM and 0.9 μM for WP758, WP744 and DNR, respectively. For both compounds, WP758 and WP744, the K_m value is lower than that of DNR showing clearly that these compounds have more affinity for P-gp than DNR. For two compounds, WP757 and WP785, the plot of V_a as a function of $\mathscr{C}_i$ results in a bell-shaped dependence on the cytosolic free drug concentration. (Fig. 3). In experiments performed with highly lipophilic molecules, such as aclacinomycin, such bell-shaped curve has been observed as well [14]. This type of bell-shaped curve indicates a self-inhibition of the efflux of the drug. This phenomenon is not surprising since it is well known that some inhibitors of P-gp-mediated efflux of drug have a detergent effect: they insert into the membrane and inhibit the P-gp functionality, probably by modifying the conformation of the protein [31]. This happens also in experiments with traditional inhibitors, which show that the stimulation of the P-gp ATPase activity by several MDR-reversing agents often results in a bell-shaped dependence on the reversing agent concentration [32].

For these compounds, only the k_a value can be determined and as it has been shown above the derivatives bearing an amino on the benzyl substituent are about 10-fold more efficiently pumped out (i.e. recognised) by P-gp than those having a nitro group or nothing on the phenyl ring. Also, the RF for the compounds having a free amino group is much more higher than for the others.

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, rhodamine 123, and the γ-emitting radiopharmaceutical ^{99m}Tc-MIBI. P-gp can transport neutral and positively but not negatively charged molecules. One recurring tenet in structure–activity relationship studies on P-gp modulators and substrates is the requirement of a basic nitrogen atom in the molecule. However, substances lacking a nitrogen atom have been described as being active modulators [33,34] and substrates [20]. Therefore, the role of basic nitrogen atom present in the most of MDR modulators, has been questioned.

A recent report by Seelig [35] compares 100 different substances previously tested as P-gp substrates. Substrate binding to P-gp is proposed to increase with the number of the hydrogen bonding acceptor units of the compounds. However, data are based on a count of the number of hydrogen bond acceptor units per molecule rather than on quantification of the hydrogen bond acceptor strength. Based on the hypothesis put forward in this report, Ecker *et al.* [36] have designed and synthesised a set of 12 analouges of the lead molecule propafenone and tested them for multidrug resistance-reverting activity. The sum of the hydrogen bond acceptors strengths was calculated and correlated with the EC_{50} values for P-gp inhibition using two different fluorochrome substrates: Rhodamine 123 and DNR. The results clearly demonstrated a strong correlation between hydrogen bond acceptor strength and pharmacological activity within this set of compounds. The nitrogen atom does not interact with P-gp in a charged form but functions as an electron donor group, which can be replaced by other hydrogen bond acceptor group.

In this context we have recently determined the efflux of anthracyclines in K562/ADR cells, at different intracellular and extracellular pH levels in order to have different amount of neutral and charged form of anthracycline [37]. Our data show that both forms are recognised by P-gp but the neutral form is pumped about three times more efficiently than the charged form. In both forms an amine nitrogen has a hydrogen donor bonding ability, however, only the neutral form has hydrogen bond acceptor ability. Let us consider now the series of compounds with the benzyl group. The compounds bearing a nitro groups on the benzyl ring are bad substrate for P-gp in comparison to those having an amine group. Nitro groups are considerably larger than the amines and the lack of transport of the nitro derivatives could be due to steric effect. However, the observation that WP776, which has no substituent on the benzyl ring, is also a bad substrate for P-gp rules out this possibility. At the opposite, the compounds which have a free amine group with hydrogen bond acceptor ability, on the benzyl ring are very good substrates. It is interesting to notice that in the case of the ortho derivative (WP758), the amine group on the benzyl ring can be hydrogen bonded to the secondary amine on the sugar. Such an interaction should decrease its ability to make H-bond with residues of P-gp and actually, the ortho derivative (WP758) is a less good substrate for P-gp than the meta and para derivatives (WP785 and WP757).The proposition that the aminoderivatives are good substrates for P-gp because they have hydrogen bond acceptor ability is in perfect agreement with: (i) the data of Ecker *et al.* [36] that show that the nitrogen atom, in P-gp modulators, does not interact with P-gp in a charged form but functions as an electron donor group and (ii) the high percentage of amino acids with hydrogen bonding donor side chains which are found in the transmembrane sequences (TM 4–6 and TM 11–12) of P-gp relevant for substrate interaction [38–40].

Acknowledgments

This research was supported by grants from l'Université Paris Nord, and CNRS.

References

[1] Alvarez M, Robey R, Sandor V, Nishiyama K, Matsumoto Y, Paul K, Bates S, Fojo T. Using the national cancer institute anticancer drug screen to assess the effect of MRP expression on drug sensitivity profiles. Mol Pharmacol 1998;54:802–14.
[2] Beck J, Handgretinger R, Dopfer R, Klingebiel T, Niethammer D, Gekeler V. Br J Haematol 1995;89:356–63.
[3] Brock I, Hipfner DR, Nielsen BS, Jensen PB, Deeley RG, Cole SPC, Sehested M. Sequential co-expression of the multidrug resistance genes MRP and MDR1 and their products in VP-16 (etoposide)-selected h69 small cell lung cancer cells. Cancer Res 1995;55:459–62.
[4] Hasegawa S, Abe T, Naito S, Kotoh S, Kumazawa J, Hipfner DR, Deeley RG, Cole SPC, Huwano M. Br J Cancer 1996;71:907–13.
[5] Cole SPC, Bhardwaj G, Gerlach JH, Mackie JE, Grant CE, Almquist KC, Stewart AJ, Kurz EU, Duncan AMV, Deeley RG. Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. Science 1992;258:1650–4.
[6] Zaman GJR, Flens MJ, Van Leusden MR, de Haas M, Mulder HS, Lankelma J, Pinedo HM, Scheper RJ, Baas F, Broxterman HJ, Borst P. The human multidrug resistance-associated protein MRP is a plasma membrane drug-efflux pump. Proc Natl Acad Sci USA 1994;91:8822–6.
[7] Cole SPC, Sparks KE, Fraser K, Loe DW, Grant CE, Wilson GM, Deeley RG. Cancer Res 1994;54:5902–10.
[8] Zijlstra JG, de Vries EGE, Mulder NH. Multifactorial drug resistance in an adriamycin-resistant human small cell lung carcinoma cell line. Cancer Res 1987;47:1780–4.
[9] VanLuyn MJA, Muller M, Renes J, Meijer C, Scheper RJ, Nienhuis EF, Mulder NH, Jansen PLM, de Vries EGE. Transport of glutathione

conjugates into secretory vesicles is mediated by the multidrug-resistance protein 1. Int J Cancer 1998;76:55–62.
[10] Cui YH, Konig J, Buchholz U, Spring H, Leier I, Keppler D. Drug resistance and ATP-dependent conjugate transport mediated by the apical multidrug resistance protein, MRP2, permanently expressed in human and canine cells. Mol Pharmacol 1999;55:929–37.
[11] Belinsky MG, Kruh GG. MOAT-E (ARA) is a full-length MRP cMOAT subfamily transporter expressed in kidney and liver. Br J Cancer 1999;80:1342–9.
[12] Kawabe T, Chen ZS, Wada M, Uchiumi T, Ono M, Akiyama S, Kuwano M. Enhanced transport of anticancer agents and leukotriene C-4 by the human canalicular multispecific organic anion transporter (cMOAT/MRP2). FEBS Lett 1999;456:327–31.
[13] Marbeuf-Gueye C, Ettori D, Priebe W, Kozlowski H, Garnier-Suillerot A. Correlation between the kinetics of anthracycline uptake and the resistance factor in cancer cells expressing the multidrug resistance protein or the P-glycoprotein. Biochim Biophys Acta 1999;1450: 374–84.
[14] Garnier-Suillerot A, Marbeuf-Gueye C, Salerno M, Loetchutinat C, Kokt I, Krawczyk M, Kowalczyk T, Priebe W. Analysis of drug transport kinetics in multidrug-resistant cells: implications for drug action. Curr Med Chem 2001;8:51–64.
[15] Wiese M, Pajeva IK. Structure–activity relationships of multidrug resistance reversers. Curr Med Chem 2001;8:685–713.
[16] Priebe W. Mechanism of action-governed design of anthracycline antibiotics: ''turn-off/turn-on'' approach. Curr Pharm Design 1995;1: 51–68.
[17] Priebe W, Van NT, Burke TG, Perez-Soler R. Removal of the basic center from doxorubicin partially overcomes multidrug resistance and decreases cardiotoxicity. Anti-Cancer Drugs 1993;4:37–48.
[18] Consoli U, Priebe W, Ling Y-H, Mahadevia R, Griffin M, Zhao S, Perez-Soler R, Andreeff M. The novel anthracycline annamycin is not affected by P-glycoprotein-related multidrug resistance: comparison with idarubicin and doxorubicin in HL-60 leukemia cell lines. Blood 1996;88:633–44.
[19] Ling Y-H, Priebe W, Yang LY, Burke TG, Pommier Y, Perez-Soler R. In vitro cytotoxicity, cellular pharmacology, and DNA lesions induced by annamycin, an anthracycline derivative with high affinity for lipid membranes. Cancer Res 1993;53:1583–9.
[20] Borrel M-N, Fiallo M, Priebe W, Garnier-Suillerot A. P-glycoprotein mediated efflux of hydroxyrubicin, a neutral anthracycline derivative, in resistant K562 cells. FEBS Lett 1994;356:287–90.
[21] Mankhetkorn S, Dubru F, Hesschenbrouck J, Fiallo M, Garnier-Suillerot A. Relation among the resistance factor, kinetics of uptake, and kinetics of the P-glycoprotein-mediated efflux of doxorubicin, daunorubicin, 8-(S)-fluoroidarubicin, and idarubicin in multidrug-resistant K562 cells. Mol Pharmacol 1996;49:532–9.
[22] Chaires JB, Leng F, Przewloka T, Fokt I, Ling Y-H, Perez-Soler R, Oriebe W. Structure-based design of a new bisintercalating anthracycline antibiotic. J Med Chem 1997;40:261–6.
[23] Frézard F, Garnier-Suillerot A. Determination of the osmotic active drug concentration in the cytoplasm of anthracycline-resistant and-sensitive K562 cells. Biochim Biophys Acta 1991;1091:29–35.
[24] Frézard F, Garnier-Suillerot A. Comparison of the membrane transport of anthracycline derivatives in drug-resistant and drug-sensitive K562 cells. Eur J Biochem 1991;196:483–91.
[25] Marbeuf-Gueye C, Broxterman HJ, Dubru F, Priebe W, Garnier-Suillerot A. Kinetics of anthracycline efflux from multidrug resistance protein-expressing cancer cells compared with P-glycoprotein-expressing cancer cells. Mol Pharamacol 1998;53:141–7.
[26] Hill T. Co-operative theory in biochemistry. Berlin: Springer, 1985. p. 63–7.
[27] Rappa G, Lorico A, Flavell RA, Sartorelli AC. Evidence that the multidrug resistance protein (MRP) functions as a co-transporter of glutathione and natural produce toxins. Cancer Res 1997;57: 15232–7.
[28] Loe DW, Deeley RG, Cole SPC. Characterization of vincristine transport by the M-r 190,000 multidrug resistance protein (MRP): evidence for co-transport with reduced glutathione. Cancer Res 1998;58:5130–6.
[29] Renes J, de Vries EGE, Nuenhuis EF, Jansen PLM, Müller M. ATP- and glutathione-dependent transport of chemotherapeutic drugs by the multidrug resistance protein MRP1. Br J Pharmacol 1999;126: 681–8.
[30] Salerno M, Garnier-Suillerot A. Kinetics of glutathione and daunorubicin efflux from MRP1 overexpressing small cell lung cancer cells. Eur J Pharmacol 2001;421:1–9.
[31] Borrel M-N, Fiallo M, Veress I, Garnier-Suillerot A. The effect of crown ethers, tetraalkylammonium salts, and polyoxyethylene amphiphiles on pirarubicin incorporation in K562 resistant cells. Biochem Pharmacol 1995;50:2069–76.
[32] Pascaud C, Garrigos M, Orlowski S. Multidrug resistance transporter P-glycoprotein has distinct but interacting binding sites for cytotoxic drugs and reversing agents. Biochem J 1998;333:351–8.
[33] Ueda K, Okamura N, Hirai M, Tanigawa Y, Saeki T, Kioka N, Komano T, Hori R. Human P-glycoprotein transports cortisol, aldosterone, and dexamethasone, but not progesterone. J Biol Chem 1992;267:24248–52.
[34] Borrel M-N, Pereira E, Fiallo M, Garnier-Suillerot A. Mobile ionophores are a novel class of P-glycoprotein inhibitors. The effects of ionophores on 4′-O-tetrahydropyranyl-adriamycin incorporation in K562 drug-resistant cells. Eur J Biochem 1994;223:125–33.
[35] Seelig A. A general pattern for substrate recognition by P-glycoprotein. Eur J Biochem 1998;251:252–61.
[36] Ecker G, Huber M, Schmid D, Chiba P. The importance of a nitrogen atom in modulators of multidrug resistance. Mol Pharmacol 1999;56: 791–6.
[37] Frezard F, Pereira E, Quidu P, Priebe W, Garnier-Suillerot A. P-glycoprotein preferentially effluxes anthracyclines containing free basic versus charged amine. Eur J Biochem 2001;268:1561–7.
[38] Hsu SI-H, Cohen D, Kirschner LS, Lothstein L, Hartstein M, Horowitz SB. Structural analysis of the mouse MDR1a (P-glycoprotein) promoter reveals the basis for the differential transcript heterogeneity in multidrug resistant J774.2 cells. Mol Cell Biol 1990;10:3596–606.
[39] Gottesman MM, Pastan I. Biochemistry of multidrug resistance mediated by the multidrug transporter. Ann Rev Biochem 1993;62: 385–427.
[40] Zhang X, Collins KI, Greenberger LM. Functional evidence that transmembrane 12 and the loop between transmembrane 11 and 12 form part of the drug-binding domain in P-glycoprotein encoded by MDR1. J Biol Chem 1995;53:5994–6000.