Nucleotide-induced conformational changes in the human multidrug resistance protein MRP1 are related to the capacity of chemotherapeutic drugs to accumulate or not in resistant cells

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Abstract Intracellular accumulation of anthracycline derivatives was measured in a human embryonic kidney cell line (HEK) and a resistant subline (HEK/multidrug resistance protein (MRP1)) overexpressing MRP1 at the plasma membrane surface. Two compounds (daunorubicin and doxorubicin) were rejected outside the multidrug-resistant cells. On the contrary, three compounds (4′-deoxy-4′-iodo-doxorubicin, 4-demethoxy-daunorubicin and 3′-(3-methoxymorpholino)doxorubicin) accumulated equally within sensitive HEK cells and resistant HEK/MRP1 cells. Our main objective here was to characterize the MRP1 conformational changes mediated by the binding of these anthracycline derivatives and to determine whether these conformational changes are related to MRP1-mediated drug transport. MRP1 was reconstituted in lipid vesicles as previously described [Manciu, L., Chang, X.B., Riordan, J.R. and Ruysschaert, J.-M. (2000) Biochemistry 39, 13026–13033]. The reconstituted protein was shown to conserve its ATPase and drug transport activity. Acrylamide quenching of Trp fluorescence was used to monitor drug-dependent conformational changes. Binding of drugs (4-demethoxy-daunorubicin and 3′-(3-methoxymorpholino)doxorubicin) which accumulate in resistant cells immobilizes MRP1 in a conformational state that is insensitive to ATP binding whereas drugs rejected outside the resistant cells (daunorubicin, doxorubicin) favor a conformational change which may be a required step in the transport process.

Key words: Multidrug resistance; Multidrug resistance protein 1; Anthracycline; Quenching of tryptophan fluorescence; Flow cytometry

1. Introduction

Multidrug resistance (MDR) is one of the major obstacles encountered during cancer chemotherapy. This resistance is frequently associated with the overexpression of P-glycoprotein (Pgp) [1] or MDR protein 1 (MRP1) [2]. According to their sequence, these two proteins belong to the large ATP binding cassette (ABC) superfamily of membrane transport proteins that includes yeast, bacteria, and mammalian transporters [3]. Pgp and MRP1 function as ATP-dependent efflux pumps that extrude cytotoxic drugs from cells before they can reach their intracellular targets, thus conferring cell resistance to many structurally and functionally unrelated anti-cancer drugs including the *Vinca* alkaloids, anthracyclines, etoposide, taxol, colchicine and actinomycin D [4–6]. Experiments with inside-out membrane vesicles indicated that MRP1 transports a range of substrates that are conjugated to glutathione (GSH), glucuronide or sulfate [7–13] but also unconjugated compounds (daunorubicin (DNR), vincristine and etoposide) provided that GSH is also present [14–16]. The mechanism by which MRP1 transports conjugated organic anions and unmodified xenobiotics has yet to be elucidated. However, it is likely that, like Pgp, MRP1 is a ATP-dependent drug efflux pump and that ATP hydrolysis is coupled to drug transport. Distinct Pgp conformations induced by drugs and ATP binding have been characterized [17–21]. Recently, purified MRP1 reconstituted into lipid vesicles has been shown to adopt different conformations during its catalytic cycle [22].

The aim of the present study is to characterize the nucleotide-induced MRP1 conformational changes in the presence of a series of anthracycline derivatives and to determine whether these conformational changes are related to MRP1-mediated drug transport.

Intracellular accumulation of DNR, doxorubicin (DOX) and several derivatives (4′-deoxy-4′-iodo-doxorubicin (iodoDOX), 4-demethoxy-daunorubicin (demethoxyDNR) and 3′-(3-methoxymorpholino)doxorubicin (FCE)) was measured by flow cytometry in human embryonic kidney cell line (HEK) and its resistant, (MRP1)-overexpressing subline (HEK/MRP1). IodoDOX, demethoxyDNR and FCE accumulated equally into sensitive HEK cells and resistant HEK/MRP1 cells. On the contrary, accumulation of DNR and doxorubicin was lower in resistant HEK/MRP1 cells compared to sensitive HEK cells. Quenching of tryptophan (Trp) fluorescence by acrylamide revealed that MRP1 adopts different conformations upon addition of MgATP and anthracycline derivatives and that the coupling between the drug binding site and the

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Abbreviations: MDR, multidrug resistance; MRP1, multidrug resistance protein 1; Pgp, P-glycoprotein; DNR, daunorubicin; DOX, doxorubicin; iodoDOX, 4′-deoxy-4′-iodo-doxorubicin; demethoxy-DNR, 4-demethoxy-daunorubicin; FCE, 3′-(3-methoxymorpholino)-doxorubicin; GSH, glutathione; ATPγS, adenosine-5′-*O*-(3-thiotriphosphate); Trp, tryptophan

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catalytic cycle is differently affected by drugs which accumulate or do not accumulate in resistant cells overexpressing MRP1.

2. Materials and methods

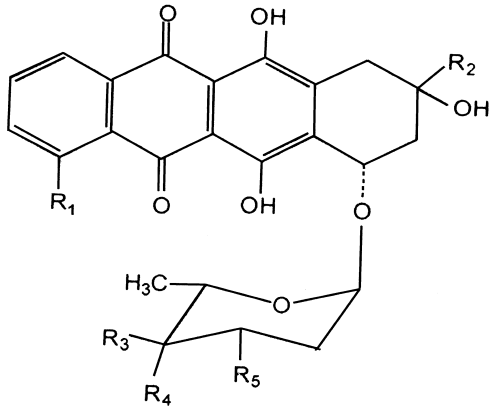
2.1. Materials

IodoDOX, demethoxyDNR and FCE were obtained from Pharmacia-Farmitalia (Milan, Italy). Daunorobucin (DNR), doxorubicin (DOX), ATP and adenosine-5′-*O*-(3-thiotriphosphate) (ATPγS) were purchased from Sigma. Hygromycin B was a Boehringer product. Dulbecco's modified Eagle medium (DMEM), fetal calf serum, L-glutamine and penicillin-streptomycin solution were supplied by Gibco BRL. Asolectin (Sigma) was purified according to the method of Kagawa and Racker [23] and stored at −20°C in chloroform. FITC-conjugated secondary antibody was a Coulter product.

2.2. Cell lines and cell culture

The cell lines used were HEK and its drug-resistant subline HEK/MRP1. The HEK/MRP1 cells overexpressing MRP1 were obtained by transfection with a pCEBV7 expression vector containing the full-length MRP1 cDNA (pCEBV7-MRP1). The parental cell line (HEK) was transfected with pCEBV7 vector alone. The pCEBV7 vector was derived from pREP7 expression vector by replacing the Rous sarcoma viral promoter with a cytomegalovirus promoter. This vector contains also the *hph* gene from *Escherichia coli* that confers resistance to hygromycin B and allows maintenance of the vector in transfected cells. Cells were cultured in DMEM medium supplemented with 10% fetal calf serum, 2% L-glutamine and 1% penicillin-streptomycin solution at 37°C in a humidified atmosphere of 5% CO_2.

It has been established by flow cytometry measurements on cells incubated with the antiMRP1 monoclonal antibody (QCRL-1) (kindly provided by Dr. R.G. Deeley (Queen's University, Kingston, ON, Canada)) coupled to a FITC-labeled secondary antibody that, in contrast to sensitive cells, the HEK/MRP1 resistant cell line expresses MRP1 at the membrane surface. No Pgp was detected using an antiPgp monoclonal antibody (MRK16) (Research Diagnostics, Inc.).



Compound	R_1	R_2	R_3	R_4	R_5
Daunorubicin	OCH_3	$COCH_3$	H	OH	NH_2
Doxorubicin	OCH_3	COCH2OH	H	OH	NH_2
4'-deoxy-4'-iodo-doxorubicin	OCH_3	COCH2OH	H	I	NH_2
FCE 23762	OCH_3	COCH2OH	H	OH	
4-demethoxy-daunorubicin	H	$COCH_3$	H	OH	NH_2

Fig. 1. Anthracycline structures.

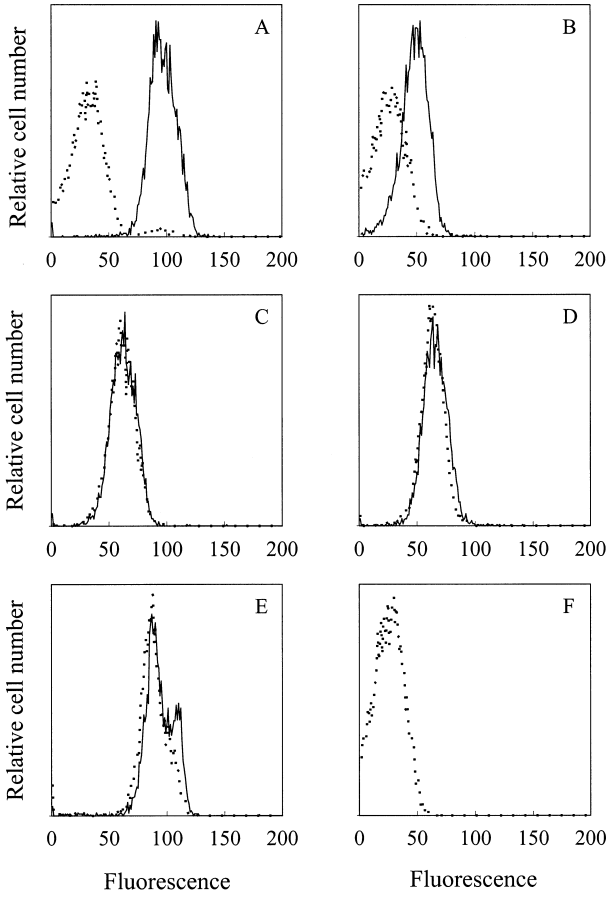


Fig. 2. Cellular accumulation of anthracyclines. Fluorescence intensity histograms were obtained by flow cytometry after incubation of HEK (—) and HEK/MRP1 (····) cells (10^6 cells/ml) with different anthracyclines (1 μM) at 37°C. A, DNR; B, DOX; C, iodoDOX; D, FCE 23762; E, demethoxyDNR; F, HEK/MRP1 resistant cells without drugs (profile was the same for the sensible cells). Fluorescence intensity is displayed on a log scale. A total of 10 000 cells were counted for each histogram. Experiments were repeated three times and gave essentially the same profiles as the ones shown here.

2.3. Purification and reconstitution of MRP1

The purification and reconstitution of MRP1 was carried out as described previously [22,24]. Proteoliposomes were prepared by incubation of asolectin liposomes with purified protein dissolved in 0.1% DDM. The detergent/protein/phospholipid mixture was gently stirred for 20 min at 4°C and the detergent was removed by adding SM_2 Bio-Beads.

2.4. Drug accumulation

Cellular accumulation of anthracycline derivatives was measured as described [25]. Briefly, 10^6 cells/ml were incubated for 3 h with 1 μM of different anthracyclines in DMEM complete medium at 37°C. They were then washed twice in PBS buffer (containing 135 mM NaCl, 2.5 mM NaH_2PO_4 and 9 mM Na_2HPO_4) and resuspended in 500 μl PBS. Cell fluorescence was measured by flow cytometry on a EPICS®-PROFILE II cytometer. 5×10^3 cells were counted in each case. Each experiment was repeated at least three times.

2.5. ATPase activity

The ATPase activity of proteoliposomes was measured according to the procedure described by Shapiro and Ling [26]. The protein content of the samples was determined by the method of Peterson [27].

2.6. Fluorescence quenching

Acrylamide quenching experiments were performed on a SLM Aminco 8000 spectrofluorimeter at room temperature as described

previously [22]. The excitation wavelength was set at 290 nm and fluorescence was monitored at 334 nm. Increasing amounts of acrylamide were added from a 3 M stock solution to the proteoliposomes suspension (1 ml in water) containing 7 μg of reconstituted MRP1 and various ligands. The final concentrations for the nucleotides and GSH were 3 and 2.5 mM respectively. Anthracycline derivatives were added to a final concentration of 10 μM. The acrylamide concentration was from 0 to 0.08 M. Above this concentration, the static quenching by acrylamide is responsible for the deviation from linearity in Stern–Volmer plots. The quenching data were plotted according to the Stern–Volmer equation [28]:

$$F_0/F = 1 + K_{sv}[Q] \quad (1)$$

where F_0 and F are the fluorescence intensities in the absence and presence of the acrylamide, [Q] is the concentration of acrylamide, and K_{sv} is the Stern–Volmer quenching constant.

3. Results

3.1. Cellular accumulation of drugs at the steady state

Cellular accumulation of several anthracyclines (Fig. 1) in HEK and HEK/MRP1 cells was measured at the steady state by flow cytometry [25]. The fluorescence histograms of cells incubated during 3 h with the anthracyclines are shown in Fig. 2A–E. The fluorescence of cells incubated without any drug was also recorded and the fluorescence histogram is displayed in Fig. 2F. Fluorescence distribution of DNR and DOX was significantly shifted to lower values for HEK/MRP1 cells overexpressing MRP1. This means lower intracellular concentrations of these anthracyclines as compared to sensitive HEK cells (Fig. 2A,B). In contrast, iodoDOX, demethoxyDNR and FCE fluorescence histograms for HEK/MRP1 resistant cells were identical to those obtained for HEK sensitive cells (Fig. 2C–E) indicating that those drugs accumulate at the same extent in both cell lines.

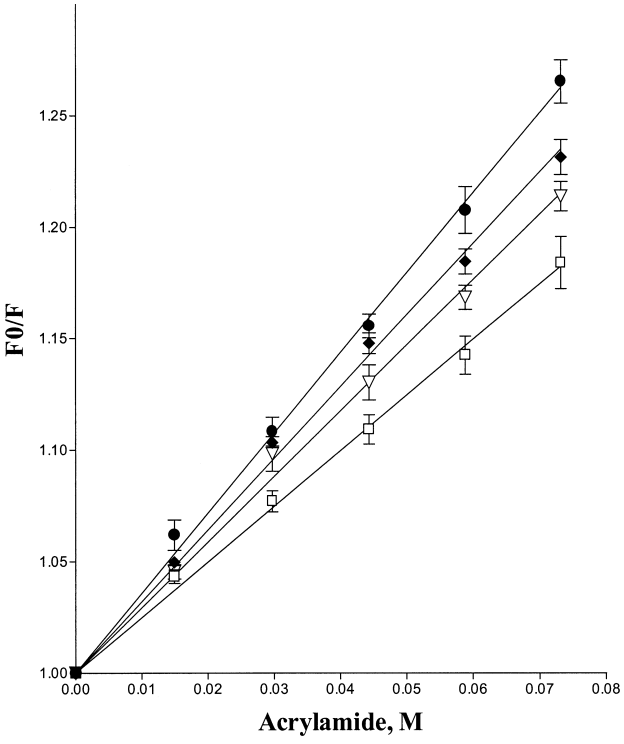


Fig. 3. Stern–Volmer plots of MRP1 Trp quenching by acrylamide upon co-addition of DNR, GSH and nucleotides. Addition of doxorubicin to MRP1 gives identical Stern–Volmer plots (data not shown). F is the measured fluorescence intensity and F_0 is the initial fluorescence intensity in the absence of acrylamide. ●, 3 mM MgATP; □, 10 μM anthracycline derivative and 2.5 mM GSH; ▽, 10 μM DNR, 2.5 mM GSH and 3 mM MgATPγS; ◆, 10 μM DNR, 2.5 mM GSH and 3 mM MgATP. The results are the means of three experiments. The error bars represent the standard deviation.

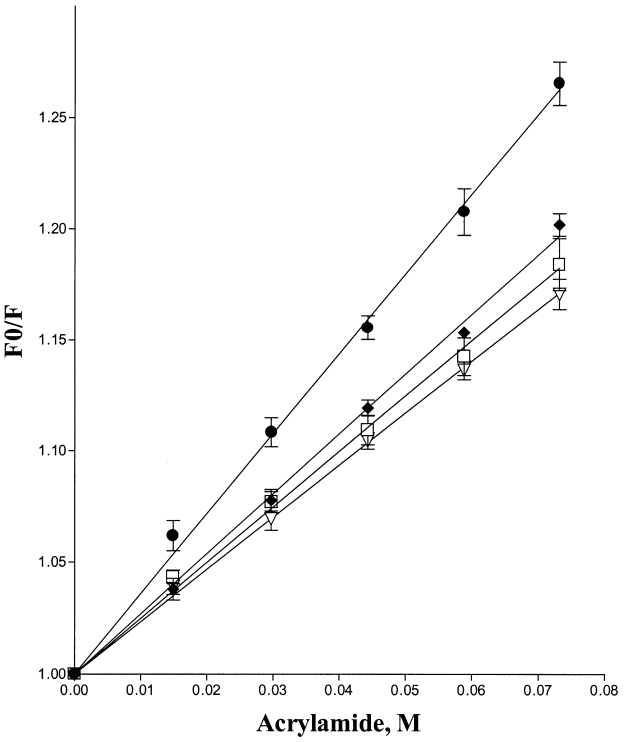


Fig. 4. Stern–Volmer plots of MRP1 Trp quenching by acrylamide upon co-addition of demethoxyDNR, GSH and nucleotides. Addition of FCE to MRP1 gives identical Stern–Volmer plots (data not shown). F is the measured fluorescence intensity and F_0 is the initial fluorescence intensity in the absence of acrylamide. ●, 3 mM MgATP; □, 10 μM anthracycline derivative and 2.5 mM GSH; ▽, 10 μM demethoxyDNR, 2.5 mM GSH and 3 mM MgATPγS; ◆, 10 μM demethoxyDNR, 2.5 mM GSH and 3 mM MgATP. The results are the means of three experiments. The error bars represent the standard deviation.

3.2. Fluorescence experiments

Fluorescence experiments were conducted in the presence of specific ligands of MRP1 in order to detect changes of accessibility of MRP1 domains to the aqueous environment occurring upon binding of drugs and nucleotides. Five anthracycline derivatives (DNR, DOX, iodoDOX, demethoxyDNR and FCE) (Fig. 1) were used and added at a final concentration of 10 μM to MRP1-containing proteoliposomes in the presence of MgATP (3 mM) or MgATPγS (3 mM) in order to discriminate between nucleotide binding and nucleotide hydrolysis. All experiments were carried out at 2.5 mM GSH [14–16,29,30]. Upon co-addition of MgATP or MgATPγS and anthracycline derivatives, the quenching efficiency was quite dependent on the nature of the substrate added.

In the presence of drugs (DNR and DOX) which do not accumulate in resistant cells, addition of MgATP led to the highest level of fluorescence quenching (Fig. 3) and therefore to the highest Trp exposure to the water environment. The fact that MgATPγS did not significantly modify this exposure,

indicates that MgATP binding is mainly responsible for these changes in accessibility (Fig. 3).

In the presence of drugs which accumulate both in sensitive and resistant cells, two distinct situations were observed:

1. In the presence of FCE or demethoxyDNR, addition of MgATP or MgATPγS did not affect significantly the fluorescence quenching (Fig. 4).
2. In the presence of iodoDOX, addition of MgATPγS and MgATP caused an increase in fluorescence quenching similar to that observed for transported drugs (DNR and DOX) (Fig. 5).

3.3. ATPase activity of MRP1

ATP was shown to be unable to modify the fluorescence of Trps after binding of FCE or demethoxyDNR. A possible explanation can be that MRP1 is not able to bind and hydrolyze the nucleotide in the presence of these anthracycline derivatives. MRP1 ATPase activity was therefore measured in the absence and in the presence of each of the anthracycline derivatives and GSH.

In the absence of drugs and GSH, the reconstituted MRP1 showed a basal ATPase activity of about 10 ± 2 nmol/mg/min. This ATPase activity was 1.5–2-fold stimulated in the presence of 20 μM anthracycline derivatives and 2.5 mM GSH (Fig. 6).

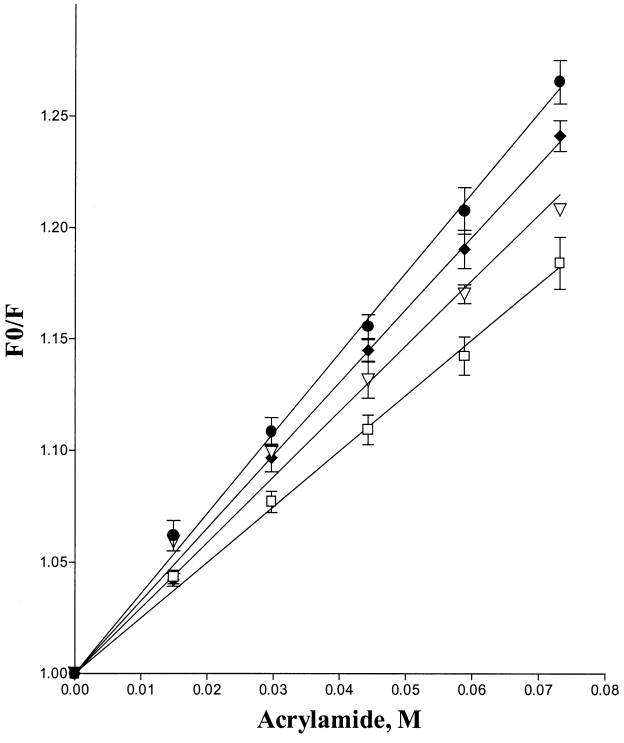


Fig. 5. Stern–Volmer plots of MRP1 Trp quenching by acrylamide upon co-addition of iodoDOX, GSH and nucleotides. F is the measured fluorescence intensity and F_0 is the initial fluorescence intensity in the absence of acrylamide. ●, 3 mM MgATP; □, 10 μM anthracycline derivative and 2.5 mM GSH; ▽, 10 μM iodoDOX, 2.5 mM GSH and 3 mM MgATPγS; ♦, 10 μM iodoDOX, 2.5 mM GSH and 3 mM MgATP. The results are the means of three experiments. The error bars represent the standard deviation. Absence of error bar means that the error bar is within the symbol.

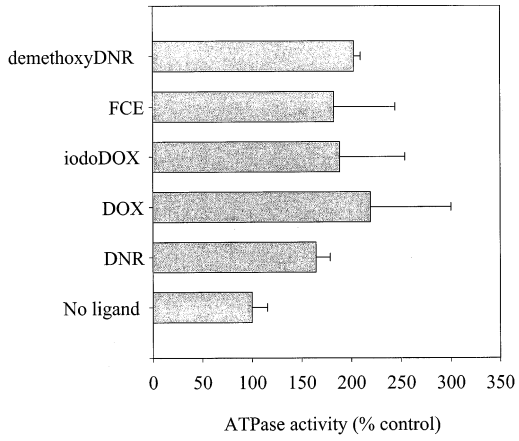


Fig. 6. Stimulation of MRP1 ATPase activity by anthracycline derivatives. ATPase activity of MRP1 was measured in the presence of 20 μM anthracyclines and 2.5 mM GSH. Data are means ± S.D. ($n = 3$). All the ATPase activity values measured in the presence of anthracycline derivatives and GSH were statistically different from the ATPase value of MRP1 in the absence of ligand, as calculated with Student's t-test ($P < 0.05$, $n = 3$).

The increase in the MRP1 ATPase activity was similar in magnitude to that reported in membrane vesicles [31].

4. Discussion

The experiments reported here demonstrate that ATP binding is necessary for the protein to undergo the conformational change leading to the transport of DNR and DOX outside the cell. Binding of drugs (FCE or demethoxyDNR) which accumulate in the resistant cell immobilizes MRP1 in a conformational state insensitive to ATP binding and ATP hydrolysis even though ATPase activity measurements confirmed that ATP binds to MRP1 and is hydrolyzed in the presence of the five anthracyclines tested. These observations strongly suggest that, in the presence of drugs rejected outside resistant cells, the protein undergoes after binding and hydrolysis of MgATP, conformational changes which are crucial steps involved in the catalytic cycle of drug transport. In contrast, drugs like FCE and demethoxyDNR probably inhibit one or several steps involved in the catalytic cycle. It is likely that the structural states associated to these steps do not allow the transport of drugs across the membrane. It has been recently suggested that Lmr A, a bacterial resistance protein, functions by an alternating two-sites mechanism [32]. Indeed, it seems that it passes through two configurations, one containing a high-affinity, inside-facing, drug binding site and one containing a low-affinity, outside-facing, drug release site. Moreover, the interconversion of these two configurations is ATP-dependent. Our data suggest that non-transported drug could be irreversibly associated to the high-affinity binding site; addition of ATP would inhibit the conformational change required to transport the drug to the low affinity binding site and to bring the drug in contact with the water phase. Non-transported molecules would be locked in their high membrane affinity binding site. They are not transported and accumulate into resistant cells.

IodoDOX accumulates at the same extent in sensitive and resistant cells although its binding to MRP1 causes the same structural changes as those described for transported drugs. It

is likely that its high lipophilicity favors a rapid and passive rediffusion into the plasma membrane and its accumulation into resistant cells overexpressing MRP1 [33].

In conclusion, we demonstrate here that the coupling between the drug binding site(s) and the catalytic sites is differently affected by binding of anthracycline derivatives which accumulate or do not accumulate in resistant cells. It is quite obvious that a knowledge of the tertiary structure of the MRP1-ligand complexes will open the way to a molecular understanding of the resistance process associated to MRP1. A preliminary but crucial step was to identify these structural intermediates involved in the transport process.

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