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Short communication

The absence of stereoselective P-glycoprotein- and multidrug resistance-associated protein-mediated transport of daunorubicin

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Abstract

Multidrug resistance phenotype in mammalian cells is often correlated with overexpression of P-glycoprotein (P-gp) or multidrug resistance-associated protein (MRP1). Both proteins are energy-dependent drug efflux pumps that efficiently reduce the intracellular accumulation and hence the cytotoxicity of many natural cytotoxins. Thus, both P-gp and MRP1 proteins are able to transport anthracycline but the role of chirality has not, up to now, been addressed. In this study, we compared the P-gp- and MRP1-mediated efflux of daunorubicin and its enantiomer WP900 in multidrug-resistant cells overexpressing either P-gp (K562/ADR cells) or MRP1 (GLC4/ADR cells). Using fluorescence techniques, we showed that in both cell lines the presence of the pump yielded a gradient of drug concentration: the intracellular free drug concentration in the cytosol was lower than the extracellular free drug concentration. Our data showed that the gradient of concentration generated by the pump was the same whether DNR or WP900 was used. This means that P-gp on the one hand and MRP1 on the other recognise WP900 as well as DNR and that the chirality of the molecule plays no role.

Keywords: P-glycoprotein; MRP1; Daunorubicin; Enantiomer

1. Introduction

The multidrug resistance phenotype in mammalian cells is often correlated with overexpression of P-glycoprotein (P-gp) or multidrug resistance-associated protein (MRP_1) [1,2]. Both proteins are energy-dependent drug efflux pumps that efficiently reduce the intracellular accumulation and hence the cytotoxicity of many natural cytotoxins [3–6]. Overexpression of these transporters by tumor cells is thought to be a significant factor in both intrinsic and acquired resistance to anticancer drugs.

A thorough understanding of P-gp and MRP_1 transport characteristics is necessary for the development of novel chemotherapeutics, chemomodulators, and treatment regimes that could be used clinically to counteract the resulting accumulation deficit. Consequently, a great deal of interest is focused on identifying: (i) new non-cross-resistant drugs having physicochemical properties enhancing the uptake of the drug by the resistant cells [7–11]; and (ii) chemical agents (reverting agents) that can antagonise drug transport by these proteins [12–14]. P-gp-mediated multidrug resistance is reversed by a variety of compounds, including Ca^{2+} channel blockers such as verapamil and their analogs [15, 16]. The (−)-isomer of verapamil is 10-fold more potent as a calcium antagonist than the (+)-isomer. However, both enantiomers have been reported to similarly reverse multidrug resistance [17–21], suggesting that their recognition by P-gp is non-chiral. Surprisingly, few agents reverse the MRP_1-mediated multidrug resistance, and almost all agents that reverse P-gp-mediated multidrug resistance cannot reverse MRP_1-mediated multi-

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Abbreviations: P-gp, P-glycoprotein; MRP1, multidrug resistance-associated protein; DNR, daunorubicin; WP900, daunorubicin enantiomer; C_i, intracellular free drug concentration in the cytosol; C_e: extracellular free drug concentration; C_{n1}, overall concentration of drug accumulated inside the cell (in the nucleus and in the acidic compartment); C_n, overall concentration of drug bound to the nucleus; C_{n1} (or C_n), intracellular drug concentration; CCA, concanamycin A; and RF, resistance factor.

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Table 1
Cross-resistance pattern of K562/ADR and GLC4/ADR cells

cell line	K562/ADR		GLC4	GLC4/ADR	
K562					
6 ± 1 nM	380 ± 50 nM	RF 63	6 ± 1 nM	50 ± 5 nM	RF = 8
2.2 ± 0.5 μM	60 ± 10 μM	RF 28	2.2 ± 0.5 μM	9 ± 1 μM	RF = 4

IC_{50} is the drug concentration required to inhibit 50% of cell growth. Resistance factor value was calculated as resistant cell IC_{50}/sensitive cell IC_{50}. The values represent means ± SD of triplicate determinations.

drug resistance, while very little is known about the role of chirality [22].

It has been shown recently that both P-gp and MRP_1 proteins were able to transport anthracycline. However, the role of chirality has not, up to now, been addressed. As anthracycline and verapamil do not have the same binding site on P-gp [23–25] the data obtained with verapamil cannot be extrapolated to anthracycline. In this study, we compared the P-gp- and MRP_1-mediated efflux of the anthracycline DNR and of its enantiomer (WP900). For this purpose, we determined the gradient of anthracycline concentrations generated by the presence of the pump in erythroleukemia cells (K562) resistant to doxorubicin and overexpressing P-gp. This gradient is the same for both anthracyclines indicating that they are pumped out by P-gp with the same efficiency. Analogous data were obtained with small lung cancer cells (GLC4) overexpressing MRP_1: daunorubicin and its enantiomer are pumped out with the same efficiency.

2. Materials and methods

2.1. Cell culture and cytotoxicity assays

GLC4, small lung cancer cells, and the MRP_1-expressing GLC4/ADR cells [26], K562 leukemia cells and the P-glycoprotein-expressing K562/ADR cells [8] were cultured in RPMI-1640 (Sigma Chemical Co.) medium supplemented with 10% foetal bovine serum (Biomedia) 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 one to four weeks before experiments. Cell cultures used for experiments were split 1:2 one day before use in order to assure logarithmic growth.

The cytotoxicity of the anthracyclines was determined by incubating cells (10^5) with 6 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 using 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. The values obtained are shown in Table 1. As can be seen, the cytotoxicity of WP900 towards sensitive cells is about 300–400 times less than that of DNR.

2.2. Drugs and chemicals

Purified doxorubicin and daunorubicin (DNR) were kindly provided by Pharmacia-Upjohn Laboratory. Daunorubicin enantiomer (WP900) (Fig. 1) was provided by the author W. Priebe. Concentrations were determined by diluting stock solutions to approximately 10^{-5} M with ε_{480} = 11500 M^{-1} cm^{-1}. Stock solutions were prepared just before use. Concanamycin A (CCA) and Triton X-100 were from Sigma. Before the experiments, the cells were counted, centrifuged and resuspended in Na^+-HEPES buffer solution containing 20 mM HEPES plus 132 mM NaCl, 3.5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ at pH = 7.3. All other reagents were of the highest quality available. Deionized doubled-distilled water was used throughout the experiments.

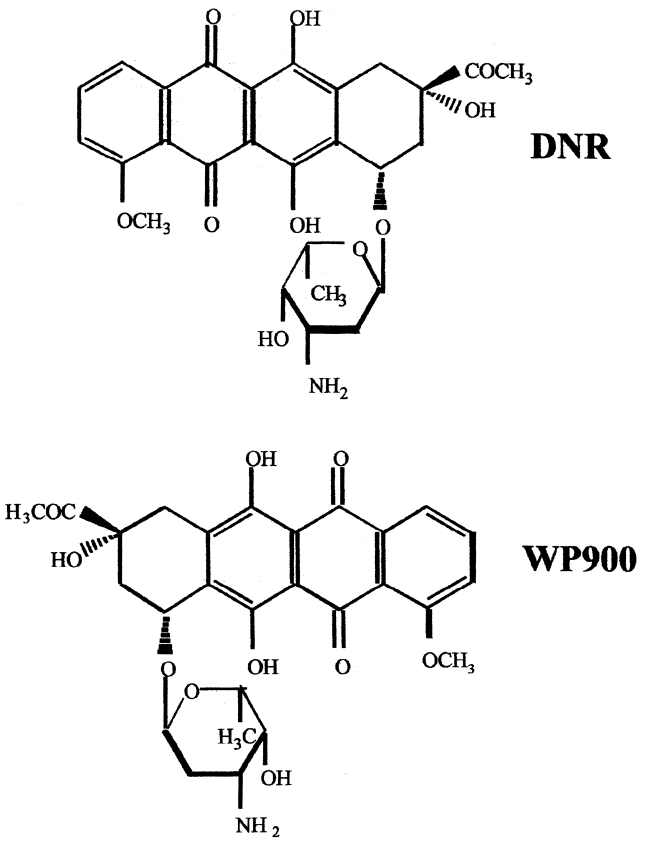

Fig. 1. Structure of daunorubicin and WP900.

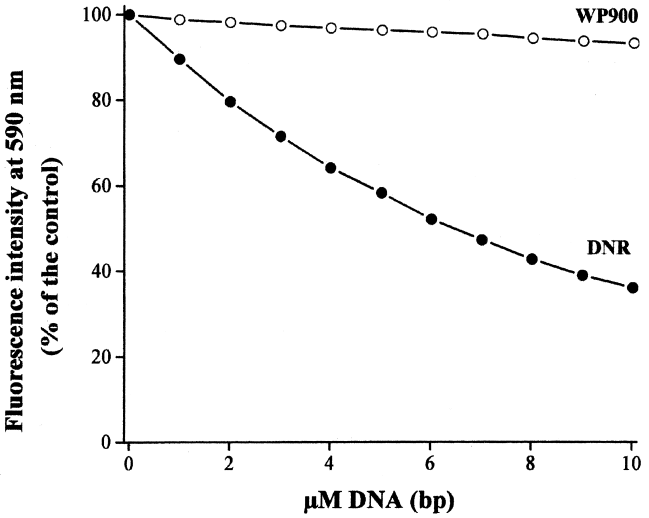

Fig. 2. Interaction of DNR and WP900 with DNA. The fluorescence emission at 590 nm (λ_{ex} = 480 nm) was recorded as a function of the DNA concentrations (bp) added to 1 μm anthracycline in buffer solution at pH 7.3 and 37°.

3. Results

3.1. Comparison of the interaction of daunorubicin and WP900 with DNA

It is well known that anthracyclines intercalate between the base pairs in DNA yielding a quenching of the fluorescence of the drug. Fig. 2 shows the decrease in the fluorescence signal of DNR (1 μM) when DNA was stepwise added (Na^+-HEPES buffer at pH 7.3, 37°). Fifty percent of quenching was observed at a molar ratio of DNA (base pairs) to DNR equal to ~6. When a similar experiment was performed with WP900, no quenching was observed, showing that this molecular was unable to intercalate between the base pairs in DNA.

3.2. Cellular anthracycline accumulation

The rationale and validation of our experimental set-up for measuring the accumulation of anthracyclines in tumor cells has been extensively described and discussed before [8,14,27–30]. It is based on a continuous spectrofluorometric monitoring (Perkin Elmer LS50B spectrofluorometer) of the decrease in the fluorescence signal of the anthracycline at 590 nm (λ_{ex} = 480 nm) after incubation with cells in a 1-cm quartz cuvette. This method will hereafter be named macrospectrofluorescence. The decrease in fluorescence occurring during incubation with cells is due to quenching of the fluorescence after intercalation of anthracycline between the base-pairs of DNA and/or to its accumulation into intracellular compartments. We have previously shown that this methodology allows one to measure accurately the overall concentration of anthracycline accumulated inside the cell and that intercalated between the base pairs in the nucleus in steady-state [8,14,27–30]. The accumulation of DNR and WP900 inside the cells was also monitored using flowcytometry (Becton-Dickinson).

3.3. Uptake of daunorubicin and WP900 by sensitive cells

Inside the cells daunorubicin can interact with many cellular components. It is well known that a quenching of its fluorescence signal is observed when the drug intercalates between the base pairs in nucleus and also when it accumulates, because it is a weak base, into acidic vacuolar compartments such as lysosomes. We verified that the quantum yield of fluorescence was the same for DNR and W900 and that the fluorescence of both molecules was subject to the same degree of quenching.

In a first set of experiments, the uptake of DNR and WP900 in sensitive cells was followed using macrospectrofluorescence. In these experiments cells, 10^6/mL, were incubated for 2 hr with various concentrations of drug ranging from 1 to 15 μM. Fig. 3 shows the decrease in the fluorescence signal when DNR 1 (A) or 10 μM (B), and WP900 1 (C) or 10 μM (D) were added to the cells suspended either in Na^+-HEPES buffer in the absence (a) or presence (b) of 20 nM CCA, which is a specific and potent inhibitor of vacuolar H^+-translocating ATPases [31,32]. As can be seen, at 1 μM DNR the accumulation was not dependent on the presence of CCA and, in both cases, the addition of Triton X-100 to permeabilise the membrane did not give rise to significant modification of the signal (Fig. 3A). This indicates that the nucleus binding sites were mainly occupied. However, things were different when high drug concentration was used (Fig. 3B). When cells were incubated in the presence of CCA, the fluorescence signal decreased as a function of the time of incubation and then plateaued after about 60 min. The addition of 5 μL Triton 4% did not yield any modification in the fluorescence signal, indicating that DNR was accumulated in the nucleus only and not accumulated in non-nuclear compartments (similar data were obtained using ATP-depleted cells, i.e. cells which were incubated in the presence of N_3^- and the absence of glucose). However, when cells were incubated without CCA, the accumulation of drug inside the cells was higher as attested by the strong decrease in the fluorescent signal. Moreover, after the addition of Triton X-100, the signal became the same as that observed in the presence of CCA.

When cells were incubated with any concentration of WP900, in the presence of CCA, no modification in the fluorescence signal was observed, in agreement with the previous observation that WP900 was unable to intercalate between the base pairs in DNA and with the fact that there was no gradient of pH in the intracellular vesicles (Fig. 3, C and D). However, when WP900 was incubated with cells in the absence of CCA, the decrease in the fluorescence signal was strong. After the addition of Triton X-100, the fluorescence signal became equal to that observed in the presence of CCA.

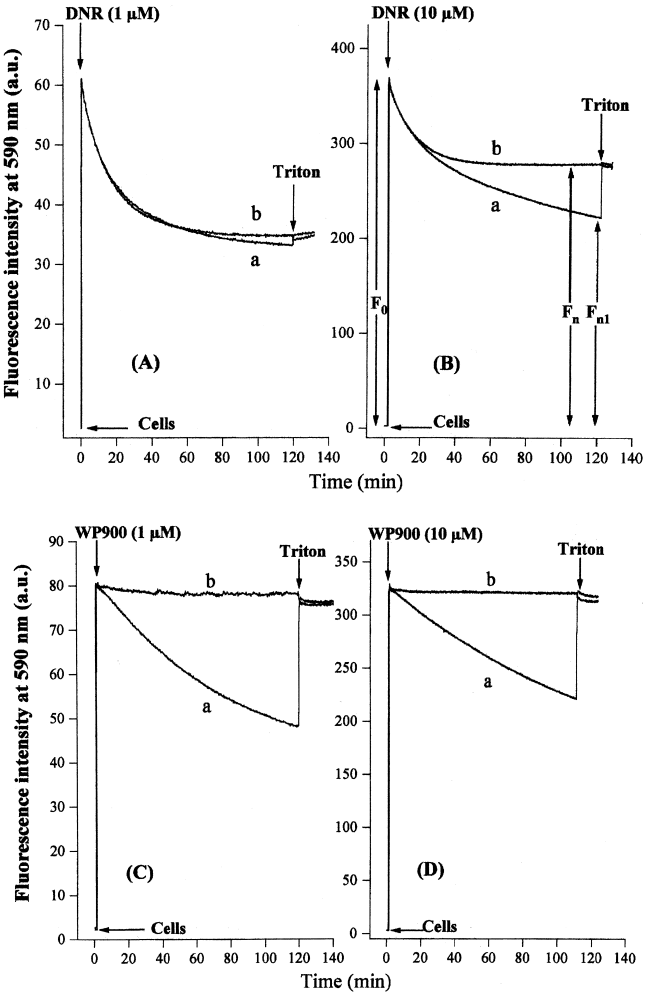

Fig. 3. Uptake of DNR and WP900 by sensitive K562 cells. The fluorescence signal at 590 nm was recorded as a function of time. Cells, 10^6/mL, were incubated with daunorubicin 1 μM (A) or 10 μM (B), with WP900 1 μM (C) or 10 μM (D), in HEPES buffer in the absence (a) or in the presence (b) of 20 nM CCA. At t = 0, the intensity of the fluorescence signal was F_0. After 2 hr, the intensity was F_{n1} (−CCA) or F_n (+CCA) and 5 μM Triton X-100 was added.

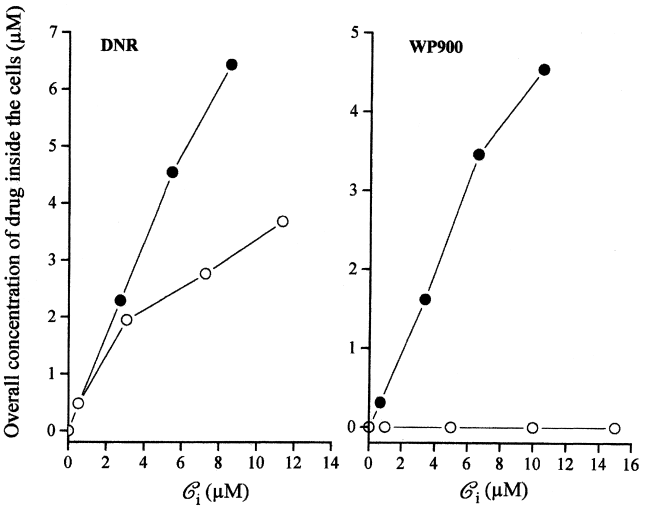

Fig. 4. Drug accumulation into sensitive K562 cells. The overall concentration of DNR (left) and WP900 (right) accumulated into sensitive cells after a 2-hr incubation was plotted as a function of the free drug concentration, $\mathscr{C}_i$, in the cytosol (here $\mathscr{C}_i = C_e$). Cells were incubated in HEPES buffer, in the absence (full circle, C_{n1}) or presence of 20 nM CCA (empty circle, C_n).

The observation that the decrease in the fluorescence signal is a measure of the drug accumulated inside the cell can be verified by the following simple experiment: after 2 hr, the interval of time after which most of our measurements were done, cells were centrifuged and the fluorescence signal of the supernatant recorded. In every case, the fluorescence intensity was equal to that observed in the presence of cells, indicating that this signal can be assigned to the drug free in the extracellular medium. It follows that the overall concentration of drug accumulated inside the cell (in the nucleus and/or in the acidic compartment) is directly proportional to the decrease in the fluorescence signal (the overall concentration is an apparent concentration corresponding to the drug intercalated between the base-pairs of DNA referring to the total volume of solution in which cells are suspended). The overall concentration will hereafter be labelled C_{n1} ($C_{n1} = C_T.\ [(F_0 - F_{n1})/F_0]$ where C_T is the total drug concentration added to the cells). In the same manner, the overall concentration of drug bound to the nucleus can be determined from the decrease in the fluorescence signal measured in the presence of CCA. This concentration will hereafter be labelled C_n ($C_n = C_T.\ [(F_0 - F_n)/F_0]$).

We have previously determined that the intracellular pH, in both sensitive and resistant K562 or GLC4 cells, was very close to 7.3 [27, 33]. The extracellular pH being equal to 7.3, it follows that for sensitive cells, in steady state, the concentration of drug free in the cytosol, $\mathscr{C}_i$, and in the extracellular medium, C_e, are the same ($\mathscr{C}_i$ is the local concentration of drug free in the cytosol, referring to the cell volume). C_e is proportional to the fluorescence signal of the drug incubated with cells: $C_e = (F_n/F_0)$. C_T in the presence of CCA and $C_e = (F_{n1}/F_0)$. C_T in the absence of CCA. Fig. 4 shows the plot of the overall concentration of DNR (left) or WP900 (right) accumulated inside the cell as a function of $\mathscr{C}_i$; the measurements were performed after 2 hr of incubation either in the absence or in the presence of CCA.

In a second set of experiments, the accumulation of the drug was followed using flow cytometry and the signal was recorded after a 2-hr incubation under the conditions described above. Surprisingly, the signal was more intense in the case of cells incubated with drug in the presence of CCA than in its absence, although, according to the previous experiments, the intracellular drug accumulation was lower in the first case than in the second. Actually, due to the quenching of the drug fluorescence when it is either bound to the nucleus and/or accumulated inside acidic compartment, the fluorescence signal recorded via flow cytometry is not at all proportional to the drug concentration inside the cells. This is clearly seen in Fig. 5, where the flow cytom-

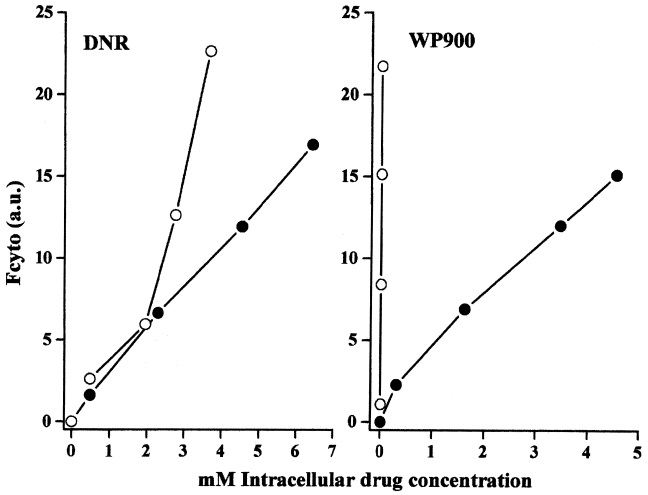

Fig. 5. Flow cytometry and intracellular drug concentration. The intensity of the fluorescence signal (F_{cyto}) recorded via flow cytometry was plotted as a function of the intracellular DNR (left) or WP900 (right) concentration determined in HEPES buffer in the absence (full circle, C_{n1}) or in the presence (empty circle, C_n) of 20 nM CCA.

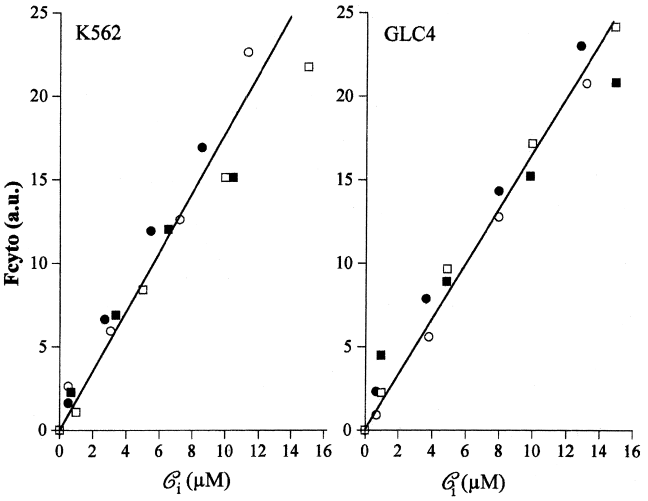

Fig. 6. Flow cytometry and the cytosolic free drug concentration. The intensity of the fluorescence signal (F_{cyto}) recorded via flow cyometry was plotted as a function of the cytosolic free drug concentration, $\mathscr{C}_i$, determined in HEPES buffer in the absence or in the presence of 20 nM CCA. K562 cells (left) and GLC4 cells (right); WP900 (circle) and daunorubicin (square) in the absence (full symbol) or presence (empty symbol) of 20 nM CCA.

etry signal was plotted as a function of the intracellular drug concentration either in the absence, $\mathscr{C}_{n1}$, or in the presence, $\mathscr{C}_n$, of CCA ($\mathscr{C}_n$ and $\mathscr{C}_{n1}$ are the intracellular concentrations of drug referring to the cell volume; using the estimation that the cell volume is 10^{-12} L, it follows that when 10^9 cells/L are used, $\mathscr{C}_n \sim C_n \times 10^3$). No correlation was observed and this is especially striking for WP900. However, when the flow cytometry signal was plotted as a function of the free drug concentration, $\mathscr{C}_i$, in the cytosol, a very good linear correlation was observed whether the cells were incubated or not with CCA. Fig. 6 shows the data for K562 and GLC4 cells.

From these experiments, we can conclude that the flow cytometry signal is strictly proportional to the amount of drug free in the cytosol. The signal arising from the nucleus is not detectable under these conditions, as the values obtained for DNR as well as for WP900 can be fitted with the same linear correlation (Fig. 6). It should be emphasised that when cells are incubated with anthracycline (especially at high concentration), many small brightly stained organelles are visible by fluorescent microscopy. Incubation of cells with CCA prevented the appearance of the brightly fluorescent bodies, indicating that the vacuoles could not be acidified. However, the signal detected by flow cytometry is lower in the absence of CCA, showing that the fluorescence signal from these compartments cannot be detected by this technique.

3.4. Uptake of daunorubicin and WP900 by resistant cells

Experiments similar to those described above were performed with resistant cells. The accumulation of drug in these cells was very low (DNR) and even undetectable (WP900) as was attested by the very small decrease in the fluorescence signal determined using macrofluorescence technique (Fig. 7). These experiments allowed the determination of the extracellular free drug concentration C_e. In the case of DNR, we have previously shown that it was also possible to calculate the cytosolic free drug concentration [28]. However, by this technique, it was quite impossible to determine the cytosolic free WP900 concentration. To solve this problem, and be able to calculate $\mathscr{C}_i$, we utilised the previous observation that the flow cytometry signal was proportional to $\mathscr{C}_i$. Therefore, the flow cytometry signals of resistant cells incubated for 2 hr with various concentrations of DNR or WP900 ranging from 1 to 15 μM were recorded, and the curve of Fig. 6 used as a calibration curve to determine $\mathscr{C}_i$.

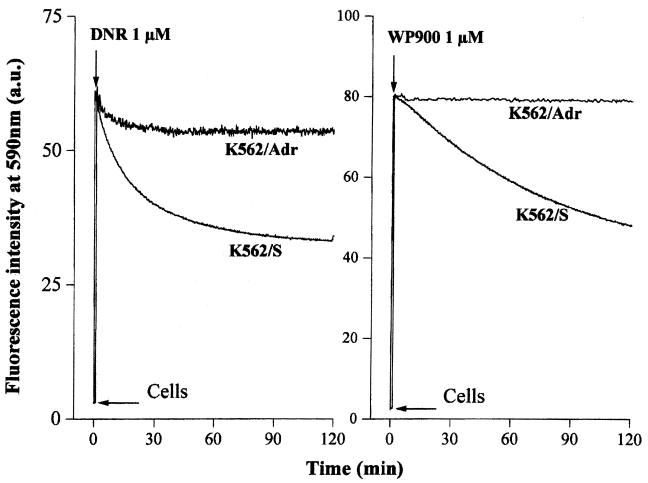

Fig. 7. Comparison of uptake of daunorubicin and WP900 by sensitive and resistant K562 cells. The fluorescence signal at 590 nm was recorded as a function of time. Cells, 10^6/mL, were incubated with 1 μM DNR (left) or 1 μM WP900 (right).

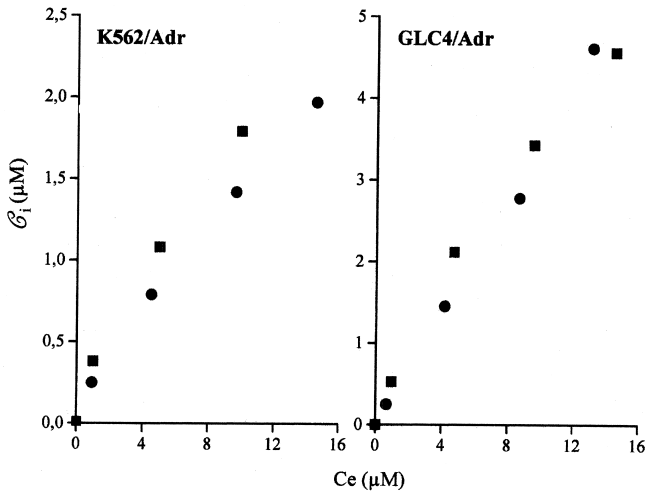

Fig. 8. Gradient of concentration generated by P-gp in K562/Adr and MRP_1 in GLC4/Adr cells. Cells were incubated for 2 hr either with DNR or WP900. The cytosolic free drug concentration $\mathscr{C}_i$ was plotted as a function of the extracellular drug concentration C_e for WP900 (circle) and DNR (square).

3.5. Free cytosolic drug concentration, $\mathscr{C}_i$, and extracellular drug concentration, C_e, in resistant cells

Fig. 8 shows the plot of the cytosolic free DNR or WP900 concentration, $\mathscr{C}_i$, as a function of the extracellular drug concentration for K562/ADR. Strictly similar experiments were performed with GLC4/ADR cells overexpressing MRP_1 pump. The data for MRP_1, which are very similar to those observed for K562 cells, are not shown except those related to the cytosolic free DNR or WP900 concentration $\mathscr{C}_i$, and the extracellular drug concentration C_e (Fig. 8).

As can be seen (Fig. 8), the points for DNR and WP900 are on the same line for K562/ADR and GLC4/ADR cells, respectively, indicating that the gradient of concentration generated by the pump, either P-gp or MRP_1, is the same whether the substrate is DNR or WP900.

4. Discussion

The aim of this study was to determine whether the P-gp- and MRP_1-mediated transport of anthracycline was stereoselective or not. The lack of stereoselectivity in the P-gp-mediated transport of verapamil has been previously demonstrated [17–21]. However, from these data it is not possible to draw any conclusion for anthracyclines as far as these molecules have a binding site different from that of verapamil [23–25].

The acute determination of the parameters characteristic of the transporter-mediated efflux of any molecule requires the determination of its rate of efflux and of its concentration, in the cytosol, which is likely to be substrate of the transporter. The efficiency of a transporter can also be characterised by the gradient of concentration, i.e. C_e versus $\mathscr{C}_i$, which is generated by the presence of the pump. A problem inherent to almost all studies on cellular efflux and specifically the study of P-gp and MRP_1 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. We have previously developed a fluorometric method to determine not only the rate of efflux of DNR (and other anthracyclines) but also the concentration of free drug in the cytosol which is likely to be pumped out [8,14,27–30]. This method is based on the observation that the DNR fluorescence is quenched when the molecule moves from the extra- to the intracellular medium where it intercalates between the base pairs of DNA in the nucleus. The drug bound to the nucleus being in thermodynamic equilibrium with that free in the cytosol, the measure of the concentration of drug bound to the nucleus yielded that of the drug free in the cytosol. Therefore, $\mathscr{C}_i$ can be determined in resistant cells.

DNR has two asymmetric centres at C(7) and C(9), both having the S configuration. In WP900, the two asymmetric centres at C(7) and C(9) have the R configuration, preventing its intercalation between the base pairs. It follows that no modification of the fluorescent signal is observed when this molecule moves from the extra- to the intracellular medium, in the presence of CCA. Therefore, the determination of $\mathscr{C}_i$ is not possible using the above methodology. One important piece of data of 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 drug concentration in the cytosol. Actually, our data clearly show that the cytofluorometric signal is proportional to the amount of drug free in the cytosol and that the participation of the drug bound to the nucleus or entrapped into intracellular organelles to this signal in undetectable.

We have been able to determine $\mathscr{C}_i$ for WP900 in resistant cells. Our data show that when C_e for DNR and WP900 are the same, $\mathscr{C}_i$ for DNR and WP900 are also the same. In other words, the gradient of concentration generated by the pump is the same whether DNR or WP900 is used. These data are observed for P-gp and for MRP_1. This means that P-gp on the one hand and MRP_1 on the other recognise WP900 as well as DNR and that the chirality of the molecule plays no role.

Acknowledgments

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