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P-glycoprotein-mediated efflux of hydroxyrubicin, a neutral anthracycline derivative, in resistant K562 cells

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Abstract Hydroxyrubin (OH-Dox), a neutral doxorubicin derivative that is only slightly cross-resistant to doxorubicin (Dox), can be actively pumped out of resistant K562 cells by P-glycoprotein (P-gp). This efflux is saturable and can be inhibited by verapamil. The Michaelis constant is equal to 2 ± 0.5 μM. However, the efficiency of P-gp in pumping out the drugs is 2.5 times less for OH-Dox than for Dox. This shows that in order to be pumped out by P-gp a molecule does not necessarily have to have a basic center. The mean influx coefficient for the drug is 5 times higher for OH-Dox than for Dox. In conclusion, the degree of resistance of analogs is related not only to their ability to be recognized and transported by P-gp but also, and probably essentially, to their kinetics of uptake. Both parameters have to be taken into account in the rational design of new compounds capable of overcoming multidrug resistance.

Key words: Multidrug resistance; P-Glycoprotein; Doxorubicin; Michaelis constant

1. Introduction

The multidrug resistance (MDR) cell phenotype reflects the capacity of some cell strains to display cross-resistance to certain cytotoxic drugs (typically anthracyclines, vinca alkaloids, colchicine, etc.) [1,2]. It is now well established that the MDR phenotype is strongly correlated with the over-expression of P-gp, a 170-kDa membrane glycoprotein encoded by *mdr* class genes, that actively pumps drugs out of resistant cells. This efflux is energy dependent.

It is now generally accepted that the uptake of drugs, such as anthracyclines, by cells occurs by passive diffusion of the neutral form of the drug [3–7]. However, the mechanism according to which the drugs are removed from the cells is still unknown. It has been reported that (i) a wide variety of drugs recognized by P-gp are positively charged [8]; (ii) an ideal modulator of P-gp would have at least two planar aromatic rings, a tertiary nitrogen that would be charged at physiological pH, and be relatively lipophilic [9]; and (iii) P-gp specifically pumps out the protonated form of daunorubicin [10].

A better comprehension of the molecular requirements for drug–protein interactions is a necessary prerequisite to the rational design of new compounds capable of overcoming MDR. To get some insight into that problem, we have studied the uptake and the efflux in reistant cells of a neutral molecule, OH-Dox, a synthetic analog of Dox in which the amine group of the sugar portion has been replaced by a hydroxyl group (Scheme 1) [11–13]. At neutral pH, about 96% of the amine group of Dox is protonated and the molecule carries a net positive charge of +1 [6]. Substitution of a hydroxyl group for the amine group results in the loss of the positive charge.

We present here data that clearly demonstrate (i) the existence of P-gp-mediated efflux of the uncharged molecule (OH-Dox), and (ii) the fact that the kinetics of uptake must be taken into account when searching for new non-cross-resistant compounds.

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Abbreviations: MDR, multidrug resistance; Dox, doxorubicin; OH-Dox, hydroxyrubicin; P-gp, P-glycoprotein.

2. Materials and methods

2.1. Cell culture and cytotoxicity assay

Dox-sensitive and -resistant erythroleukemia K562 cells were routinely cultured as described previously [6]. For the spectrofluorometric assays, in order to have cells in the exponential growth phase, culture was initiated at 5×10^5 cells/ml, and cells were used 24 h later, when the culture had grown to about 8×10^5 cells/ml. A 'resistance factor' was obtained by dividing the IC_{50} of resistant cells by the IC_{50} of the corresponding sensitive cells. The resistance factors thus obtained were 30 and 2.5 for Dox and OH-Dox, respectively.

2.2. Drugs and chemicals

Purified Dox (Scheme 1) was kindly provided by Laboratoire Roger Bellon (France), and OH-Dox was prepared according to the method of Horton et al. [11]. Concentrations were determined by diluting stock solutions to approximately 10^{-5} M and using $\varepsilon_{480} = 11{,}500$ $M^{-1} \cdot cm^{-1}$. Stock solutions were prepared just before use. Verapamil was obtained from Sigma. All other reagents were of the highest quality available. Deionized double-distilled water was used throughout the experiments. Experiments were performed in HEPES Na^+ buffer solutions containing 20 mM HEPES buffer plus 132 mM NaCl, 3.5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.25, in either the presence or absence of 5 mM glucose.

Absorption spectra were recorded on a Cary 219 spectrophotometer and fluorescence spectra on a Jobin Yvon JY 3CS spectrofluorometer.

2.3. Cellular drug accumulation

The uptake of anthracycline in cells was followed by monitoring the decrease of the fluorescence signal at 590 nm ($\lambda_{ex} = 480$ nm). This spectrofluorometric method has been previously described [6,14,15]. Using this method it is possible to accurately quantify the kinetics of uptake of the drug by the cells and the amount of anthracycline intercalated between the base pairs in the nucleus in the steady state, since incubation of the cells with the drug proceeds without compromising cell viability. All experiments were conducted in 1 cm quartz cuvettes containing 2 ml of buffer at 37°C.

2.4. Direct determination of the P-Gp-mediated efflux of OH-Dox and Dox

Cells (1×10^6/ml were incubated for 30 min in the presence of 10 mM NaN_3 and in the absence of glucose in HEPES buffer. The incorporation of OH-Dox in these energy-depleted resistant cells thus compared with that observed in sensitive cells. The addition, at steady state, of 5 mM glucose gave rise to ATP synthesis via the glycolysis pathway and to an increase in the fluorescent signal due to the release of drug from the cells. The rate of the active efflux was determined from the slope

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of the tangent to the curve $F = f(t)$ at the time point corresponding to the time of addition of glucose [16].

2.5. Cell nuclei preparation

Cell nuclei were prepared according to the method of Mellon and Bhorjee [17].

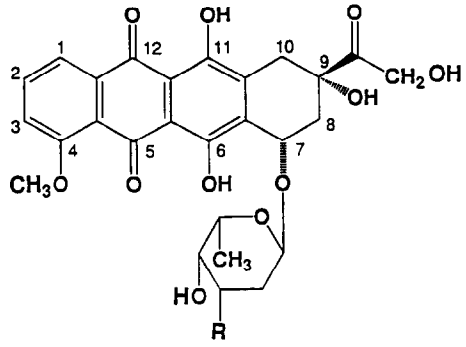
3. Results

3.1. Comparison of OH-Dox and Dox uptake by sensitive and resistant K562 cells

Cells (2×10^6/ml) were incubated, at 37°C and pH 7.2, with various concentrations of OH-Dox ranging from 0.5 to 3 μM. In every case, steady state was reached within about 30 min. Fig. 1 shows a typical experiment dealing with 1 μM drug. As can be seen, the overall concentration of drug intercalated between the base pairs in the nucleus, C_n, was 0.12 ± 0.02 μM for resistant cells and 0.35 ± 0.03 μM for sensitive cells. After the addition of 0.05% Triton X-100, which permeabilized the membrane, yielding the equilibrium state [12], the overall concentration of drug bound to the nuclei of both types of cells (C_N) became the same and equal to 0.35 ± 0.03 μM.

The initial rate of uptake, V_+, was determined from the slope of the tangent to the curves at $t = 0$ [6]. It was roughly proportional to the drug concentrations added to the cells at $t = 0$. V_+ can be written in two ways [6]: either as (i) $V_+ = k_+ \cdot n \cdot [D_e]$, where k_+ is the mean influx coefficient for the drug and depends on the extracellular pH value, $[D_e]$ is the extracellular drug concentration, and n is the number of cells/ml, or as (ii) $V_+ = k_+^\circ \cdot n \cdot [D_e^\circ]$, where k_+° is the influx coefficient for the neutral form of the molecule and $[D_e^\circ]$ is the extracellular concentration of drug in the neutral form. The relationship between the two coefficients is $k_+ = k_+^\circ / (1 + 10^{pK_a - pH})$. For OH-Dox, which can only be neutral, we have calculated that $k_+ = k_+^\circ = (2.0 \pm 0.5) \times 10^{-10}$ s^{-1}.

Analogous experiments were performed with Dox. It is well known that its uptake by cells is very slow. In fact as the first pK_a of deprotonation of Dox yielding the neutral species is equal to 8.4 [18], at pH 7.2, only 6% of the drug is in the neutral form and $[D_e^\circ] = 0.06 \times [D_e]$. We have calculated that $k_+^\circ = (6.0 \pm 1.0) \times 10^{-10}$ s^{-1} and that $k_+ = (0.4 \pm 0.1) \times 10^{-10}$ s^{-1}.



	R
Doxorubicin (Dox)	NH_2
Hydroxyrubicin (OH-Dox)	OH

Scheme 1.

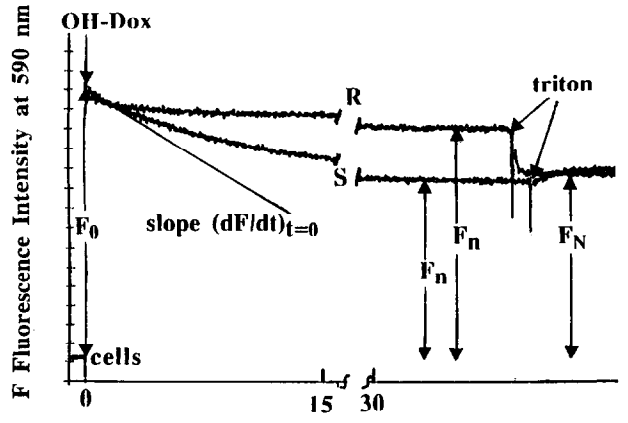


Fig. 1. Uptake of hydroxydoxorubicin (OH-Dox) by drug-sensitive (S) and drug-resistant (R) K562 cells. Fluorescence intensity at 590 nm (λ_{ex} = 480 nm) was recorded as a function of time. Cells (4×10^6) were suspended in a cuvette filled with 2 ml buffer at $pH_e = 7.25$ under vigorous stirring. At $t = 0$, 20 μl of a 100 μM stock OH-Dox solution was added to the cells, yielding a $C_T = 1$ μM OH-Dox solution. The fluorescence intensity was then F_0. The slope of the tangent to the curve. $F = f(t)$ at $t = 0$, was $(dF/dt)_{t=0}$, and the initial rate of uptake was $V_+ = (dF/dt)_{t=0} \cdot (C_T/F_0)$. Once the steady state was reached, the fluorescence was F_n, and the concentration of drug intercalated between the base pairs in the nucleus was $C_n = C_T(F_0 - F_n)/F_0$. The addition of 0.05% Triton X-100 yielded the equilibrium state. The overall concentration C_N of drug intercalated between the base pairs in the nucleus was then $C_N = C_T \cdot (F_0 - F_N)/F_0$.

3.2. Kinetics of the P-gp-mediated efflux of OH-Dox and Dox

Cells (2×10^6 cells/ml) were incubated for 30 min, in the absence of glucose and in the presence of NaN_3, with various concentrations of OH-Dox ranging from 0.5 to 7 μM. The accumulation of OH-Dox in these energy-deprived cells was the same as in sensitive cells. Fig. 2 shows a typical plot obtained with 1 μM drug. At the steady state, glucose was added, yielding ATP synthesis via the glycolysis pathway [16,19]. Under these conditions the active efflux of OH-Dox was observed. In every case, at steady state, and just before the addition of glucose, the free drug concentration in the cytosol, C_i', was equal to the free drug concentration in the extracellular medium. Active efflux, V_a, was plotted as a function of C_i', and a clear saturation of the active efflux was observed (Fig. 3). From these data we have estimated that the Michaelis constant, K_m, was equal to about 2 ± 0.5 μM (in the case of 4′-*O*-tetrahydropyranyl doxorubicin, we previously obtained $K_m \sim 0.6$ μM [16]). We checked that the active efflux of OH-Dox was completely inhibited by 10 μM verapamil.

To measure the active efflux of Dox, 1×10^6 cells/ml were incubated with 1 μM drug for 4 h in NaN_3-containing buffer in the absence of glucose. However, even after such a long time, the overall concentration of drug bound to the nuclei was only 0.28 μM. Under these conditions, in which the intra- and extracellular pH are the same, the overall concentration of drug bound to the nuclei in the steady state is expected to be 0.5 μM, i.e. the same as in the equilibrium state [15]. It was not possible to increase the incubation time without cell perturbation, and glucose was added after 4 h despite the fact that the steady state was not reached. However, we took into account that the free

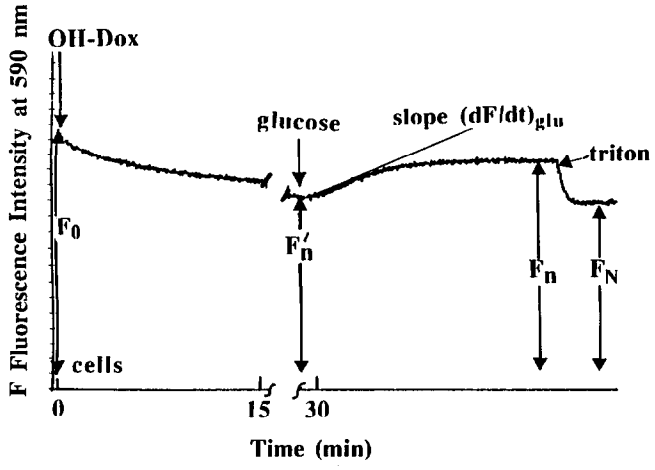


Fig. 2. Direct determination of the active kinetics of efflux, V_a, of OH-Dox under the effect of P-gp. Drug-resistant K562 cells (2×10^6/ml) were incubated with 1 μM OH-Dox in the presence of 10 mM N_3^- and in the absence of glucose. Fluorescence intensity at 590 nm (λ_{ex} = 480 nm) was recorded as a function of time. At steady state, the fluorescence intensity was F_n', and the concentration of drug intercalated between the base pairs was equal to $C_n' = C_T(F_0 - F_n')/F_0$. At $t = t_{glu}$, 5 mM glucose was added, yielding the release of OH-Dox. The slope of the tangent to the curve was $(dF/dt)_{t_{glu}}$, and the kinetics of release of OH-Dox was $V_a = (dF/dt)_{t_{glu}} \cdot (C_T/F_0)$. At the new steady state, the fluorescence intensity was F_n. The addition of 0.05% Triton X-100 yielded the equilibrium state. The fluorescence intensity was then F_N.

drug concentration, C_i', was not the same as in the extracellular medium and so calculated it using the relationship that we have previously demonstrated, $C_i' = C_E \cdot C_n'/C_N$, where C_E and C_N stand for the overall drug concentration in the extracellular medium and bound to the nucleus in the equilibrium state, respectively [15,16].

The active efflux, V_a, depends on the free drug concentration C_i' in the cytosol, and on the cell number, n. To compare the data obtained for both drugs, we calculated the parameter $k_a = V_a/C_i' \cdot n$, hereafter named the active efflux coefficient. k_a was equal to $10 \pm 2 \times 10^{-10}$ s^{-1} and $4 \pm 1 \times 10^{-10}$ s^{-1} for Dox and OH-Dox, respectively.

3.3. Interaction of OH-Dox and Dox with DNA and cell nuclei

The quenching of OH-Dox and Dox fluorescence emission through DNA interaction was followed by adding increasing amounts of calf thymus DNA to a 1 μM antibiotic HEPES buffer solution at 37°C. F/F_0 was plotted as a function of the base pair concentration (data not shown). The titration midpoints of these curves were equal to 3 ± 1 μM and 13 ± 2 μM for Dox and OH-Dox, respectively.

When 1×10^6 nuclei/ml were incubated with antibiotics of 1 μM concentration, the overall concentration of drug intercalated between the base pairs in the nuclei was 0.18 ± 0.02 and 0.5 ± 0.03 μM, respectively.

4. Discussion

4.1. OH-Dox is 2.5 times less effective than Dox in inhibiting the growth of sensitive cells

This can be perfectly correlated with the amount of drug present in the cell nuclei at steady state, with the amount of drug that binds isolated cell nuclei, and with the affinity of the two drugs for DNA. Thus, when 1×10^6 sensitive cells/ml are incubated with 1 μM drug, the overall concentration of drug bound to the nuclei is ~3-fold higher for Dox than for OH-Dox. The same ratio is obtained when drugs are incubated with cell nuclei. Moreover, the affinity for DNA is ~4-fold higher for Dox than for OH-Dox. This last point corroborates the recent data of Chaires et al. [20]. This clearly shows that the cytotoxicity of an anthracycline is mainly governed by the amount of drug present inside the cell nuclei, which is itself determined by the drug's affinity for DNA.

4.2. OH-Dox is 5 times more effective than Dox in inhibiting the growth of resistant cells

This indicates that the amount of drug bound to the cell nuclei is higher in the case of OH-Dox than in the case of Dox. One interpretation is that OH-Dox is not transported by P-gp. Our data clearly show that this neutral molecule is pumped out by P-gp, albeit less efficiently than Dox; that the active efflux of OH-Dox is saturable, and that it can be inhibited by verapamil. The efficiency of P-gp in pumping out the drugs is 2.5 times less for OH-Dox than for Dox. Results indicate that, to be pumped out by P-gp, a molecule does not necessarily have to have a basic center.

The second parameter that has to be taken into account is the kinetics of uptake of the drugs by the cells. The mean influx coefficient for the drug is 5 times higher for OH-Dox than for Dox. To reach a high intracellular drug concentration, the influx coefficient k_+ should be the highest and the active efflux coefficient k_a should be the lowest. These two conditions can be taken into account simultaneously by the ratio k_a/k_+, which should be as low as possible. This ratio is equal to 25 and 2 for Dox and OH-Dox, respectively, and corresponds well with the degree of resistance (30 and 2.5 for Dox and OH-Dox, respectively).

In conclusion, we can say that the degree of resistance of anthracyclines is related not only to their ability to be recognized and transported by P-gp but also to their kinetics of uptake. Both parameters must be taken into account in the rational design of new compounds capable of overcoming MDR.

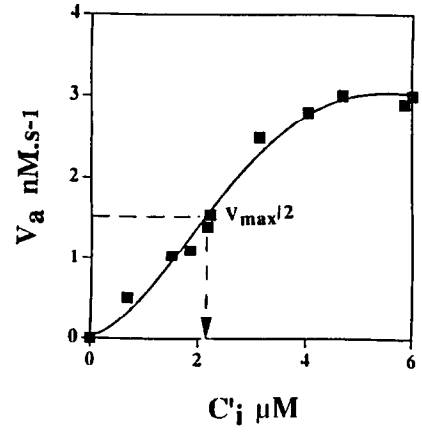


Fig. 3. Variation of kinetics of P-gp-mediated efflux of OH-Dox as a function of C_i', the cytosol free drug concentration. Resistant cells (2×10^6/ml) were incubated in the presence of various concentrations of OH-Dox ranging from 0.5 to 7 μM. V_a and C_i' were determined as described in section 2.

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