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SHORT COMMUNICATION

Multidrug Resistance Protein Functionality: No Effect of Intracellular or Extracellular pH Changes

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ABSTRACT. A major problem in the treatment of cancer is cellular resistance to cytotoxic drugs. In tumor cells *in vitro*, the development of multidrug resistance is usually accompanied by increased expression of drug transporters, either P-glycoprotein (P-gp) or multidrug resistance-associated protein (MRP_1). Both proteins belong to the superfamily of ATP-binding cassette (ABC) transporter proteins and mediate the transport of a broad range of drugs. Altenberg *et al.* (*Proc Natl Acad Sci USA* **90:** 9735–9738, 1993) have shown that changes in intra- or extracellular pH do not mediate P-gp-dependent multidrug resistance. Therefore, we similarly studied whether changes in intra- or extracellular pH could mediate MRP_1-dependent multidrug resistance. In particular, we measured the MRP_1-mediated efflux of hydroxyrubicin from GLC4/ADR cells. Since hydroxyrubicin is a fully neutral anthracycline derivative that has no deprotonable function at pH lower than 10 and so cannot accumulate in non-nuclear compartments under the influence of pH or transmembrane gradients, we hypothesized that any modifications of its kinetics of efflux as a function of pH can be assigned to a modification of the transporter efficiency. However, as our data show, modifications of extra- and/or intracellular pH yielded no modification of the MRP_1-mediated efflux of hydroxyrubicin. BIOCHEM PHARMACOL **60**;10:1485–1489, 2000.

KEY WORDS. multidrug resistance; MRP_1; pH; anthracycline; doxorubicin

A major problem in the treatment of cancer is cellular resistance to cytotoxic drugs. In tumor cells *in vitro*, the development of MDR§ is usually accompanied by increased expression of the drug transporters P-gp or MRP_1 [1–6]. Since both proteins belong to the superfamily of ATP-binding cassette (ABC) transporter proteins [5, 6] and mediate the transport of a broad range of drugs, MDR produced by overexpression of MRP_1 seems to be phenotypically similar to that produced by Pgp. However, major differences exist with regard to the substrates handled by these proteins. Neutral molecule and organic cations are substrates for both transporters, but MRP_1 has been additionally identified as a transporter of organic anions [4, 7, 8]. An important difference between the transport properties of both proteins is that the efflux of typical MDR drugs, such as anthracycline by MRP_1, seems to be dependent on the intracellular glutathione levels [9–12]. Very recently, Cole *et al.* have demonstrated that MRP_1 can actively co-transport GSH and unmodified vincristine and that vincristine and GSH in combination behave as competitive inhibitors of MRP-mediated transport of its high-affinity substrate, leukotriene C4 [13], thus disproving the dogma that MRP_1 exclusively transports cytostatic drugs after metabolic conversion to conjugates. In addition, since some of our recent data obtained using viable cells have shown that the kinetics of anthracycline transport by MRP_1 are very similar to those for P-gp, we are therefore in favor of the idea that MRP_1 transports these molecules predominantly in their unmetabolized form [12].

To continue gaining insight into the mechanisms of drug transport in MDR cells [12, 14–16], we studied whether changes in intra- or extracellular pH can mediate MRP_1-dependent multidrug resistance. In particular, we measured the MRP_1-mediated efflux of hydroxyrubicin in GLC4/ADR cells. Since OH-DOX is a fully neutral anthracycline derivative that has no deprotonable function at pH lower than 10 [17] and so cannot accumulate in non-nuclear compartments under the influence of pH or transmembrane gradients, we hypothesized that any modifications of its kinetics of efflux as a function of pH can be assigned to a modification of the transporter. However, as our data show, modifications of extra- and/or intracellular pH yielded no modification of the MRP_1-mediated efflux of hydroxyrubicin.

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§ *Abbreviations:* MDR, multidrug resistance; P-gp, P-glycoprotein; MRP_1, multidrug resistance protein; OH-DOX, hydroxyrubicin; pH_i: intracellular pH; pH_e: extracellular pH; C_n: overall concentration of drug bound to the nucleus; and 6_i cytosolic free drug concentration.

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Hydroxyrubicin

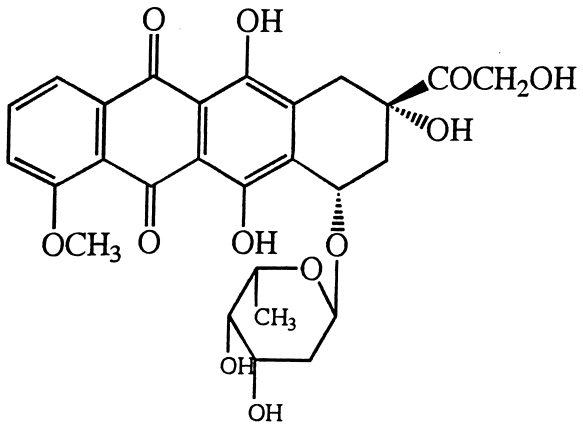

FIG. 1. Structure of hydroxyrubicin.

MATERIALS AND METHODS

Cell Culture and Cytotoxicity Assay

GLC4 and MRP_1-expressing GLC4/ADR cells [18] were cultured in RPMI-1640 medium (Sigma Chemical Co.) supplemented with 10% fetal bovine serum (Bio Media Co.) at 37° in a humidified incubator with 5% CO_2. The resistant GLC4/ADR cells were cultured with 1.2 μM doxorubicin (DOX) until 1 to 4 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 50% inhibitory drug concentrations (IC_{50}s) 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.

Drugs and Chemicals

Purified DOX was kindly provided by Laboratoire Pharmacia and Upjohn, OH-DOX (Fig. 1) was provided by author W.P. [17]. Concentrations were determined by diluting stock solutions to approximately 10^{-5} M with ϵ_{480} = 11,500 M^{-1} cm^{-1}. Stock solutions were prepared just before use. 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 $CaCl_2$, and 0.5 mM $MgCl_2$ at 6.8, 7.3, and 7.8 pH in either the presence or absence of 5 mM glucose. The acetoxymethyl-ester of SNARF-1 (seminaphtorhodafluor), a pH-sensitive dye, was obtained from Molecular Probes.

Determination of the MRP_1-Mediated Efflux of OH-DOX at Different pH_i and pH_e Levels

Our experimental set-up for measuring the kinetics of active transport of anthracyclines from tumor cells has been extensively described and discussed before [14, 16, 19–23]. It is based on the continuous spectrofluorometric monitoring (Perkin Elmer LS50B spectrofluorometer) of the decrease in fluorescence signal of the anthracycline at 590 nm (λ_{ex} = 480 nm) after incubation with cells in a 1-cm quartz cuvette. The decrease in fluorescence during incubation with cells is due to quenching of the fluorescence by intercalation of anthracycline between the base pairs of DNA. We have previously shown that this method allows accurate measurements of the free cytosolic concentration of anthracyclines in the steady state as well as rates of initial uptake and kinetics of active efflux [14, 16, 19–23].

The kinetics of anthracycline efflux were determined under the following experimental conditions. Cells (1.5×10^6/mL) were incubated with 2 μM OH-DOX in glucose-containing buffer at 37°. The decrease in fluorescence signal, F, was then followed until a steady state was reached. C_n was determined from the decrease in fluorescence signal: $C_n = C_T \cdot (F_0 - F_n)/F_0$, where C_T is the total drug concentration added to the cells, and F_0 and F_n are the fluorescence signals at $t = 0$ and at the steady state, respectively (Fig. 2a).

Cells in the steady state were centrifuged and very rapidly suspended in anthracycline-free buffer without modification of either C_n or $\mathcal{6}_i$. The kinetics of passive efflux when sensitive cells were used, and passive plus active efflux when resistant cells were used, were determined in terms of the fluorescence signal (Fig. 2b) under the following 6 conditions: (1) cells were incubated with hydroxyrubicin in buffer at pH_e 7.3 and resuspended in hydroxyrubicin-free buffer at pH_e 7.3 (under these conditions pH_i = 7.4); (2) cells were incubated with anthracycline in buffer at pH_e 7.3 and resuspended in buffer at pH_e 6.8 (under these conditions pH_i = 7.2); (3) cells were incubated in buffer at pH_e 7.8 and resuspended in buffer at pH_e 7.8 (under these condition pH_i = 7.7); (4) cells were incubated in buffer at pH_e 7.8 and resuspended in buffer at pH_e 7.8 in the presence of 25 mM NH_4Cl (under these conditions pH_i = 8.4); (5) cells were incubated with anthracycline in buffer at pH_e 7.3, supplemented with 25 mM NH_4Cl incubated for ~2 min, centrifuged, and suspended in buffer at pH_e 7.3 (under these conditions pH_i = 6.6; and (6) cells were incubated in buffer at pH_e 7.3 and resuspended in buffer at pH_e = 7.3 in the presence of 25 mM NH_4Cl (under these conditions pH_i = 7.6).

$\mathcal{6}_i$ was determined, in separate experiments, as follows. OH-DOX was incubated with cells until a steady state was reached. After the addition of 0.02% Triton X-100 to permeabilize the membrane, the fluorescence signal (F_N), the overall concentration of drug bound to the nucleus ($C_N = C_T \times (F_0 - F_n)/F_0$), and the free drug concentration ($C_E = C_T \times [F_N/F_0]$) were determined. From this, it

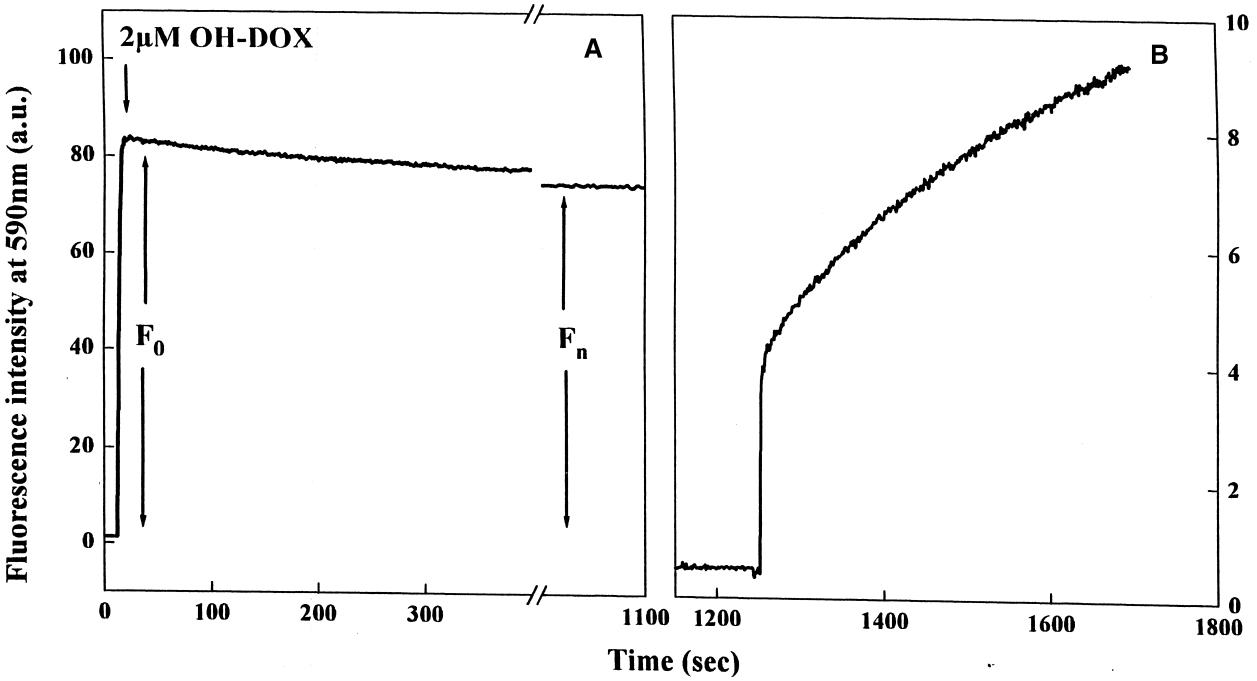

FIG. 2. Uptake and efflux of OH-DOX in GLC4/ADR cells. F, fluorescence intensity (arbitrary unit: a.u.) at 590 nm (λ_{ex} = 480 nm) was recorded as a function of time. Cells (1.5 × 10^6/mL) were suspended in a cuvette filled with 2 mL buffer at pH = 7.3 under vigorous stirring. At t = 0, a small volume of a stock anthracycline solution was added to the cells yielding a C_T = 2 μM OH-DOX solution. The fluorescence intensity was then F_0. Once the steady state was reached, the fluorescence, F_n, and $C_n = C_T (F_0 - F_n)/F_0$ (curve a) were determined. The cells were then centrifuged and rapidly suspended in anthracycline-free buffer (curve b). The appearance of the fluorescence signal indicating the efflux of the drug from the cells was recorded as a function of time.

follows that $\delta_i = C_n \times C_E/C_N$, i.e. $C_T \times (F_0 - F_n)/(F_0 - F_N)$ [19].

Intracellular pH_i Measurement

Intracellular pH was measured by laser microspectrofluorometry using the pH-sensitive dye SNARF-1 (seminaphtorhodafluor). This method, which allows accurate determination of pH in volumes as small as 0.25 μm^3, has been described previously at length [24].

RESULTS

Cell-Growth Inhibition

The IC_{50} values for DOX and OH-DOX in sensitive cells were 11 ± 2 and 26 ± 4 nM, respectively and in resistant cells 700 and 80 nM, respectively. Therefore, the resistance factors were 63 for DOX and 3 for OH-DOX.

Determination of δ_i in the Cytosol

Under our experimental conditions, i.e. 1.5 × 10^6 cells/mL and 2 μM OH-DOX, the value of C_E/C_N used to calculate δ_i was equal to 2.3. It also did not depend on the pH values, since the molecule was fully neutral within the pH range used.

Determination of Efflux Kinetics

The kinetics for the passive efflux of the drug were proportional to δ_i. In a first approximation, the kinetics of active efflux also were proportional to δ_i [12, 14]. Therefore, in both cases, the kinetics of efflux could be written as $V_{efflux} = k \cdot \delta_i$. In the case of sensitive cells, k stands for k_-, the passive efflux coefficient. In the case of resistant cells $k = k_- + k_a$, where k_a is the active efflux coefficient [12]. Table 1 shows the values of k_{efflux} obtained under the experimental conditions described in Materials and Methods. Given that the resistance of the GLC4/ADR cells is not perfectly constant, the results of experiments performed on one day were always compared with those of experiments performed on the same day at pH_e 7.3 and pH_i 7.4 (the standard conditions). Therefore, for each set of experiments we have calculated the ratio

$$r = (k_{efflux})_{pH}/(k_{efflux})_{st.}$$

where $(k_{efflux})_{st.}$ was the efflux constant determined under our standard conditions and $(k_{efflux})_{pH}$ the efflux constant determined at $pH_e \neq 7.3$ and/or $pH_i \neq 7.4$. Figure 3 shows the plot of r as a function of pH_i when pH_e was equal to 7.3, 7.8, or 6.8 (pH_e being the extracellular pH under the efflux conditions). As can be seen, there is no systematic variation of r with the pH (either pH_i and/or pH_e). Similar experiments performed with sensitive cells yielded a k_-

TABLE 1. Efflux kinetics of OH-DOX in GLC4/ADR cells

Experiment	pH_e	pH_i	$k_+ \times 10^{-12}$ l. cell^{-1}. s^{-1}
I	7.3 ± 0.05	7.4 ± 0.1	1.6 ± 0.3
	7.8 ± 0.05	8.3 ± 0.1	1.8 ± 0.3
	7.3 ± 0.05	7.6 ± 0.1	1.9 ± 0.3
II	7.3 ± 0.05	7.4 ± 0.1	1.3 ± 0.2
	7.3 ± 0.05	6.6 ± 0.2	1.3 ± 0.2
III	7.3 ± 0.05	7.4 ± 0.1	1.1 ± 0.2
	7.8 ± 0.05	8.3 ± 0.1	1.5 ± 0.3
IV	7.3 ± 0.05	7.4 ± 0.1	1.7 ± 0.3
	7.8 ± 0.05	7.7 ± 0.1	2.2 ± 0.3
	7.8 ± 0.05	8.3 ± 0.1	2.0 ± 0.3
	7.3 ± 0.05	7.6 ± 0.1	1.8 ± 0.3
V	7.3 ± 0.05	7.4 ± 0.1	2.1 ± 0.3
	7.8 ± 0.05	8.3 ± 0.1	1.6 ± 0.2
	7.3 ± 0.05	7.6 ± 0.1	2.6 ± 0.4
VI	7.3 ± 0.05	7.4 ± 0.1	1.2 ± 0.2
	7.8 ± 0.05	8.3 ± 0.1	0.9 ± 0.2
VII	7.3 ± 0.05	7.4 ± 0.1	1.2 ± 0.2
	7.3 ± 0.05	6.6 ± 0.2	1.2 ± 0.2
VIII	7.3 ± 0.05	7.4 ± 0.1	1.4 ± 0.2
	7.3 ± 0.05	6.6 ± 0.2	1.4 ± 0.2
IX	7.3 ± 0.05	7.4 ± 0.1	1.3 ± 0.2
	7.3 ± 0.05	7.6 ± 0.1	1.2 ± 0.2
X	7.3 ± 0.05	7.4 ± 0.1	1.0 ± 0.2
	6.8 ± 0.05	7.2 ± 0.1	1.3 ± 0.2

Each roman numeral corresponds to data of experiments performed the same day. Each value is the mean ± SD of 3–4 independent experiments.

value that, as expected, depended neither on pH_e nor on pH_i. k_ was equal to 0.7 ± 0.2 × 10^{-12} L. cells^{-1}. sec^{-1}. It follows that the active efflux coefficient, $k_a = k_{efflux} - k_-$, depended on neither pH_i nor pH_e.

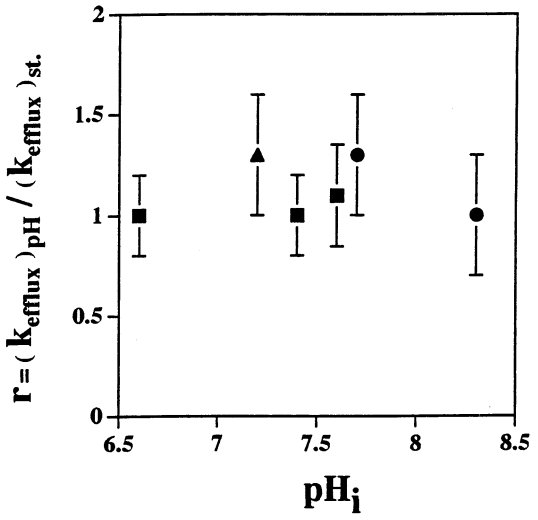

FIG. 3. Efflux kinetics of OH-DOX in GLC4/ADR cells. $r = (k_{efflux})_{pH}/(k_{efflux})_{st.}$ is plotted as a function of pH_i at pH_e = 7.3 (■), 7.8 (●), and 6.8 (▲); pH_e is the extracellular pH under the efflux conditions; $(k_{efflux})_{st.}$ is the efflux constant determined at pH_i = 7.4 and pH_e = 7.3; and $(k_{efflux})_{pH}$ is the efflux constant determined at $pH_i \neq 7.4$ and/or $pH_e \neq 7.3$. Each value is the mean ± SD of 3–4 independent experiments.

DISCUSSION

Gaining a better understanding of the molecular requirements for drug–protein interactions is a prerequisite for the rational design of new compounds that can overcome MDR. To obtain some insight into the problem, we have studied the efflux from resistant cells of the 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 (Fig. 1). At neutral pH, about 96% of the amine group of DOX is protonated and the molecule carries a net positive charge of +1 [16]. Substitution of a hydroxyl group for the amine group results in the loss of the positive charge [17].

We have already presented data that clearly demonstrate the existence of MRP_1-mediated efflux of the uncharged OH-DOX molecule [12]. Now, in our present work with OH-DOX, we have shown that the MRP_1 depends on neither intracellular nor extracellular pH levels. Using rhodamine 123 as substrate, Altenberg *et al.* demonstrated that changes in intra- or extracellular pH did not mediate P-gp-dependent MDR [25] and so concluded that P-gp-mediated MDR is not a consequence of changes in pH_i or pH_e. From our present data, we drew the same conclusion for MRP_1-mediated MDR. However, the transport of OH-DOX as well as that of other anthracyclines by MRP_1 apparently depends on GSH, since the depletion of GSH causes a very important decrease in anthracycline efflux [9–12]. Furthermore, since pH-sensitive enzymes intervene in the metabolism of GSH, it was conceivable to us that

acute changes in pH might have an effect on MRP_1-mediated anthracycline efflux. For instance, in one previous study, the GSH reductase activity decreased by a factor of 2 at pH ~6.5 [26], but this had no effect on the ability of MRP_1 to pump out OH-DOX. Thus, it follows that the lower amount of GSH that should be present at this pH was enough to sustain full MRP_1 activity. On the other hand, if GSH were co-transported with its substrate, its protonation state could influence the ability of MRP_1 to pump out drug. From the species distribution for different GSH protonation species [27], one can estimate that at pH 6.6 about 98% of the amine group of GSH is protonated and the molecule carries a net negative charge of −1, whereas at pH 8.5, only 40% of the amine group is protonated and the molecule carries a net negative charge of −1.6. Therefore, it appears that the value of the net negative charge on GSH does not influence MRP_1.

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