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Inhibition of the *P*-glycoprotein- and multidrug resistance protein-mediated efflux of anthracyclines and calceinacetoxymethyl ester by PAK-104P

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Abstract

Multidrug resistance phenotype in mammalian cells is often correlated with overexpression of *P*-glycoprotein or Multidrug Resistance-Associated protein (MRP_1). Both proteins are energy-dependent drug efflux pumps that efficiently reduce the intracellular accumulation and hence the cytotoxicity of many natural cytotoxins. Overexpression of these transporters by tumor 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 that can inhibit the proliferation of tumors cells despite the expression of these transporters. *P*-glycoprotein-mediated multidrug resistance is reversed by a variety of compounds, but surprisingly, few agents reverse the MRP_1-mediated multidrug resistance. However, it has recently been shown that 2-[4-(diphenylmethyl)-1-piperazinyl]ethyl-5-(*trans*-4,6-dimethyl-1,3,2-dioxaphosphorinan-2-yl)-2,6-dimethyl-4-(3-nitrophenyl)-3-pyridinecarboxylate P oxide (PAK-104P) was able to inhibit the *P*-glycoprotein and MRP_1-mediated efflux of several compounds. Understanding of the interactions between transporters and multidrug resistance reversing agents is important in the design of more effective multidrug resistance modulators. We now examined the effect of PAK-104P on Pgp-and MRP1-mediated efflux of three anthracyclines, daunorubicin, pirarubicin, hydroxydoxorubicin and of calcein acetoxymethyl ester and calcein. Our data show that PAK-104P non-competitively inhibits the *P*-glycoprotein-mediated efflux of anthracycline derivatives and calcein acetoxymethyl ester with an inhibitory constant $K_I = 0.25 \pm 0.05$ μM. PAK-104P also non-competitively inhibits the MRP_1-mediated efflux of daunorubicin, pirarubicin, hydroxyrubicin, calcein acetoxymethyl ester and calcein. However, surprisingly, in this case the K_I values obtained were very different ranging from 0.06 for hydroxyrubicin to 10 μM for calcein. These data strongly suggested the existence of two different mechanisms for the inhibition by PAK-104P of the MRP_1-mediated efflux of molecules: a first mechanism, involving a low-affinity site for PAK-104P, and which would concern molecules such as calcein, cysteinyl leukotriene LCT_4 etc. whose efflux do not depend on glutathione. A second mechanism involving a high-affinity site for PAK-104P and which would concern molecules such as anthracyclines, calcein acetoxymethyl ester whose efflux depends on the presence of glutathione.

Keywords: Multidrug resistance; *P*-glycoprotein; MRP_1 (multidrug resistance-associated protein); Daunorubicin; Calcein-AM; Inhibitor; PAK-104P

1. Introduction

Drug resistance mediated by active efflux pumps is widespread from prokaryotes to eukaryotes. Multidrug resistance phenotype in mammalian cells is often correlated with overexpression of *P*-glycoprotein or Multidrug Resistance-Associated protein (MRP_1). Both proteins are energy-dependent drug efflux pumps that efficiently reduce the intracellular accumulation and hence the cytotoxicity of many natural cytotoxins (Bradley et al., 1988; Zaman et al., 1994; Broxterman et al., 1995; Loe et al., 1996). Clinical experience and laboratory studies have demonstrated that most currently used natural product anti cancer drugs are excellent substrates for *P*-glycoprotein and/or MRP_1. Overexpression of these transporters by tumor cells is thought to be a significant factor in both intrinsic and acquired resistance to anticancer drugs. Consequently a

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great deal of interest is focused on identifying chemical agents that can either antagonise drug transport by these proteins or that can inhibit the proliferation of tumors cells despite the expression of these transporters (Marbeuf-Gueye et al., 1999).

P-glycoprotein-mediated multidrug resistance is reversed by a variety of compounds, including Ca^{2+} channel blockers and their analogs (Stein, 1997). Surprisingly, few agents reverse the MRP_1-mediated multidrug resistance and almost all agents that reverse *P*-glycoprotein-mediated multidrug resistance cannot reverse MRP_1-mediated multidrug resistance. However, some new compounds were recently shown to reverse the MRP_1-mediated multidrug resistance and, among them, the most efficient seems to be 2-[4-(diphenylmethyl)-1-piperazinyl]ethyl-5-(*trans*-4,6-dimethyl-1,3,2-dioxaphosphorinan-2-yl)-2, 6-dimethyl-4-(3-nitrophenyl)-3-pyridinecarboxylate P oxide (PAK-104P). Thus, Sumizawa et al. (1997) have shown that PAK-104P completely inhibits ATP-dependent $[^3H]LTC_4$ transport in membrane vesicles isolated from C-A120 cells overexpressing MRP_1, that it inhibits the ATP-dependent efflux of antimony potassium tartrate in KB carcinoma resistant cells expressing MRP_1 (Chen et al., 1997) that it can completely reverse the resistance to vincristine in both *P*-glycoprotein- and MRP_1-mediated multidrug resistant cells and that it can completely reverse the resistance to doxorubicin in *P*-glycoprotein cells but only partially in MRP_1 cells (Shudo et al., 1990).

The present study examined the effect of PAK-104P on *P*-glycoprotein- and MRP1-mediated efflux of three anthracyclines, daunorubicin, pirarubicin, hydroxydoxorubicin and of calcein acetoxymethyl ester and calcein.

2. Materials and methods

2.1. Cell culture and cytotoxicity assay

K562 leukemia cells and the *P*-glycoprotein expressing K562/ADR cells (Mankhetkorn et al., 1996), as well as GLC4 and the MRP_1-expressing GLC4/ADR cells (Zijlstra et al., 1987) were cultured in RPMI 1640 (Sigma) medium supplemented with 10% fetal calf serum (Bio media) at 37°C 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 resp. until 1 to 4 weeks before the experiments. Cell cultures used for experiments were split 1:2 one day before use in order to ensure logarithmic growth.

The cytotoxicity of the anthracyclines was determined by incubating cells (10^5) with six different concentrations of anthracylines for 72 h in standard six-well plates. Then the 50% inhibitory drug concentrations (IC_{50}s) were determined by counting the cells, using a Coulter counter. The resistance factor was defined as the IC_{50} for the resistant cells divided by the IC_{50} for the corresponding sensitive cells. The resistance factor was equal to 20 (daunorubicin), 8 (pirarubicin) and 3 (hydroxyrubicin) for K562/Adr cells, and to 23 (daunorubicin), 5 (pirarubicin) and 3 (hydroxyrubicin) for GLC4/ADR cells. The cytotoxicity of PAK-104 P for sensitive and resistant K562 and GLC4 cells was comparable. Therefore, the cells were not resistant to PAK-104P.

2.2. Drugs and chemicals

Purified doxorubicin, daunorubicin and pirarubicin were kindly provided by Laboratoire Pharmacia-Upjohn, Hydroxyrubicin was provided by W. Priebe (Priebe, 1995). Concentrations were determined by diluting stock solutions to approximately 10^{-5} M with $\varepsilon_{480} = 11500$ $M^{-1}cm^{-1}$. Stock solutions were prepared just before use. Calcein acetoxy methyl ester was from Molecular Probes (Eugene, OR) and was dissolved in dimethylsulfoxide as a stock solution of 1 mM and stored at −20°C. According to the supplier, the lot of calcein acetoxymethyl ester used has been determined by High Performance Liquid Chromatography to be 95% pure. $CoCl_2$, analytical grade, was from Aldrich and was dissolved in water. PAK-104P (Fig. 1), was a gift from Drs. Shudo, Iwasaki and Akiyama, Nissan, Chemical Industries. Triton X-100 was from Sigma and was dissolved in water. Before the experiments, the cells were counted, centrifuged and resuspended in HEPES/Na buffer solutions containing 20 mM HEPES plus 132 mM NaCl, 3.5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 5 mM glucose at pH = 7.3. All other reagents were of the highest quality available. Deionized double-distilled water was used throughout the experiments.

2.3. Cellular anthracycline accumulation

All experiments were conducted in 1-cm quartz cuvettes containing 2 ml of buffer at 37°C. The temperature was maintained at 37° using a circulating thermostated water bath. In a typical experiment, 2×10^6 cells were suspended in 2 ml of glucose containing HEPES buffer at pH 7.3, under vigorous stirring; 20 μl of the stock anthracycline solution was quickly added to this suspension, yielding an anthracycline concentration C_T.

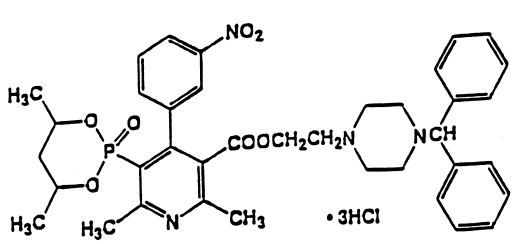

Fig. 1. Structure of PAK-104P.

The ability of PAK-104P to inhibit the Pgp- and MRP1-mediated efflux of anthracyclines was evaluated by measuring their uptake in resistant cells. This was done by continuous spectrofluorometric monitoring of the decrease of the fluorescence signal of the anthracycline at 590 nm ($\lambda_{ex} = 480$ nm) (Perkin Elmer LS50B) after incubation with cells. The decrease of the fluorescence signal occurring during incubation with cells is due to quenching after intercalation of anthracycline between the base pairs of DNA. We have previously shown that this methodology allows accurate measurement of the nuclear concentration of anthracycline in the steady state, their initial rates of uptake, and kinetics of active efflux (Frézard and Garnier-Suillerot, 1991a,b; Mankhetkorn et al., 1996; Marbeuf-Gueye et al., 1998).

The overall nuclear concentration C_n of anthracycline in drug-resistant cells was determined, at steady state, in the presence of PAK-104P at different concentrations. [i] In all cases, C_n increased as the concentration of inhibitor increased, and this can be quantified using the following equation:

$$\alpha = \left(C_n^i - C_n\right)/\left(C_N - C_n\right), \quad (1)$$

where C_N is the overall concentrations of drug intercalated between the base pairs in the nucleus after the addition of Triton X-100 (more details are given in Appendix A and Fig. 2). α represents the fold increase in the nuclear concentration of anthracycline in the presence of PAK-104P; α varies between 0 (in the absence of PAK-104P) and 1 (when the amount of anthracycline in resistant cell is the same as in sensitive cells), in other words, α is the fraction of blockage of the pump by PAK-104P.

2.4. P-glycoprotein- or MRP1-mediated efflux of anthracycline derivatives at steady state

In order to characterise the efflux, we defined a mean active efflux coefficient (k_a) (Marbeuf-Gueye et al., 1998) according to the equation: $V_a = k_a \mathcal{C}_i$, where V_a is the kinetics of the transporter-mediated efflux of anthracycline and $\mathcal{C}_i$, the intracellular free drug concentration. In the presence of PAK-104P the rate of active efflux becomes $V_a^i = k_a^i \mathcal{C}_i$. k_a is a good measure of V_M/K_m, the ratio of the maximum rate of efflux to the Michaelis constant. The ability of PAK-104 P to inhibit the transporter-mediated efflux of drug can be calculated using the ratio, $r = k_a^i/k_a$, which is equal to 1 when there is no inhibition of the active efflux and to 0 when the *P*-glycoprotein-mediated active efflux is completely blocked (more details are given in Appendix A).

2.5. Transport studies of calceinacetoxymethyl ester and calcein

The methodology for the determination of the kinetics of calcein formation and of the transport parameters for calceinacetoxymethyl ester and calcein has been described and validated previously (Essodaïgui et al., 1998, 1999). Basically, the fluorescence signal was monitored continuously during the incubation of cells with calceinace-

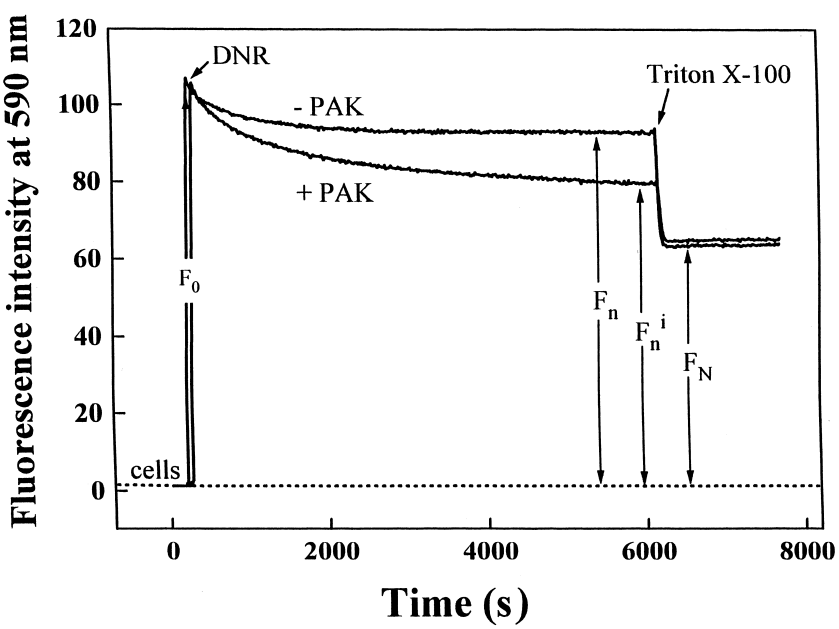

Fig. 2. Time course of uptake of daunorubicin in GLC4/Adr cells. Cells (10^6/ml) were incubated with 1 μM daunorubicin (DNR) either in the absence (−PAK) or presence (+PAK) of 1 μM PAK-104P. The fluorescence intensity (F) at 590 nm was recorded as a function of the time of incubation of cells with drug. At steady state the fluorescence intensity was F_n (F_n^i in the presence of PAK). The addition of Triton X-100 yielded a decrease of the fluorescence signal which became equal to F_N in both cases.

toxymethyl ester. Since calceinacetoxymethyl ester itself is a non-fluorescent molecule, the fluorescence measured was only due to calcein, which is formed inside cells by cytoplasmic esterases. To distinguish intracellular from extracellular calcein, 2 μM Co^{2+} was added to the cells, which completely quenched the fluorescence of calcein present in the extracellular medium. This concentration of Co^{2+} was selected in preliminary experiments and was not toxic to the cells during the experiments. In order to relate the fluorescence signals measured to calcein concentrations, a solution of calceinacetoxymethyl ester of known concentration was completely hydrolysed and its fluorescence signal was recorded (F_{CAL}). The initial rates of calcein formation ($d[CAL]/dt$) were determined in the initial linear part of the kinetics, typically 400 s after the addition of calceinacetoxymethyl ester.

Cells were always incubated in a quartz cuvette as a suspension of 1×10^6 cells/ml in HEPES buffer with/without 10-mM sodium azide or 5-mM glucose, as indicated. Fluorescence was recorded at 515 nm ($\lambda_{ex} = 493$ nm). Before the addition of calceinacetoxymethyl ester, a 30-min pre-incubation with 10-mM azide in a glucose-free buffer was used in some of the experiments to deplete cellular ATP, which blocks energy-dependent transport activities. Other details of the experimental setups are given in Section 3. When the effect of PAK-104P on calcein and calceinacetoxymethyl ester transport was evaluated, it was added together with calceinacetoxymethyl ester.

The value for the parameter k_a, characteristic of the pump-mediated efflux of calceinacetoxymethyl ester, was calculated from the fluorescence data (see Fig. 3 and Appendix B).

3. Results

Fig. 1 shows the structure of the inhibitor, PAK-104P, used in this study.

3.1. Accumulation of daunorubicin, hydroxyrubicin and pirarubicin in GLC4/Adr and K562/Adr cells in the presence of PAK-104P

The effect of PAK on daunorubicin, hydroxyrubicin and pirarubicin retention in GLC4/Adr and K562/Adr cells was characterised by the parameter $\alpha = (C_n^i - C_n^0)/(C_N - C_n^0)$ which should vary between 0 in the absence of PAK and 1 when the pump is completely blocked. Fig. 4 shows the variation of α as a function of PAK when K562/Adr cells were incubated with (i) 1, 3 or 6 μM daunorubicin, (ii) 1, 2 and 3 μM hydroxyrubicin, (iii) 1 μM pirarubicin. For the three drugs, the PAK-104P concentration (0.5 ± 0.2 μM) required to obtain $\alpha = 0.5$ did not depend on the anthracycline concentrations used. These data already strongly suggested that PAK-104P inhibited non-competitively the *P*-glycoprotein-mediated efflux of these anthracyclines. Similar experiments were performed with GLC4/Adr cells. Here also, for the three anthracyclines, the PAK-104P concentration required to get $\alpha = 0.5$ did not depend on the anthracycline concentrations used. However, surprisingly, it depends on the nature of anthra-

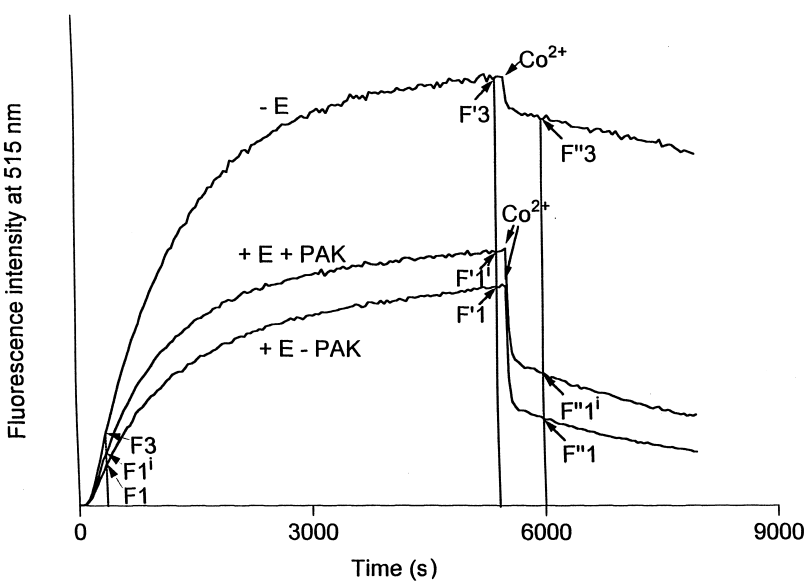

Fig. 3. Time course of calceinacetoxymethyl ester uptake by GLC4/Adr cells. The fluorescence of calcein was recorded at 515 nm as a function of time in ATP-rich cells in the presence of 1 μM PAK-104P (E + PAK) or in its absence (E − PAK) and in energy-deprived cells (−E). Calceinacetoxymethyl ester was added at $t = 0$, at 0.5 μM final concentration. Arrows indicate the addition of 2 μM Co^{2+}.

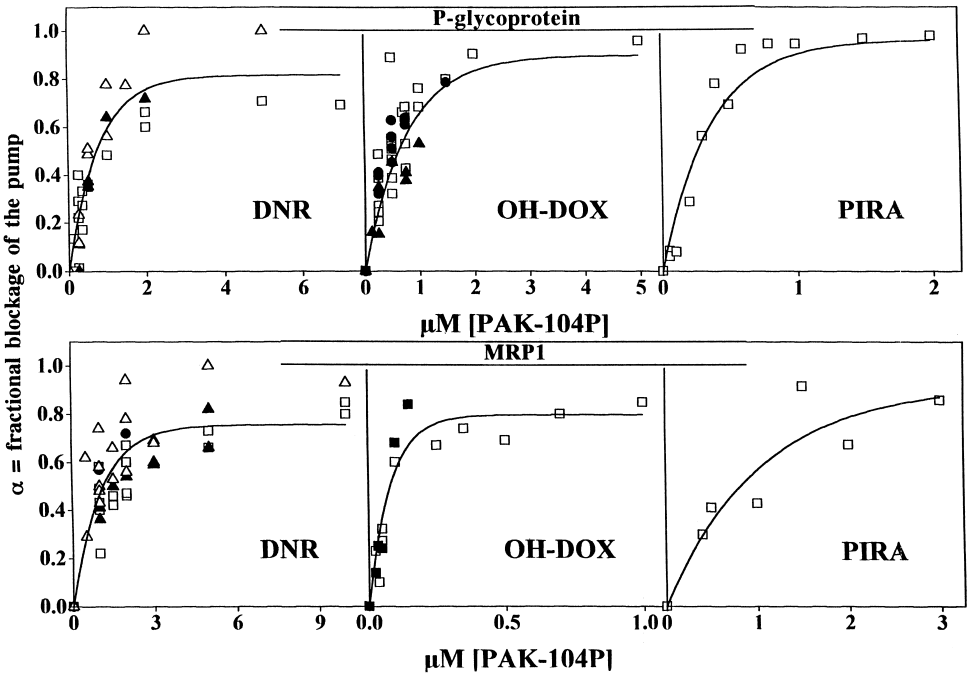

Fig. 4. Accumulation of daunorubicin (DNR), hydroxyrubicin (OH-DOX) and pirarubicin (PIRA) in K562/Adr (upper) and GLC4/Adr (lower) cells in the presence of PAK-104P. α, the fractional blockage of the pump (alternatively the fold increase in the nuclear concentration of anthracycline) was plotted as a function of the PAK-104P concentration.

cycline, being equal to 1.0 ± 0.2 μM for daunorubicin and pirarubicin, and 0.10 ± 0.2 for hydroxyrubicin.

3.2. Ability of PAK-104P to inhibit the P-glycoprotein- and MPR_1-mediated efflux of daunorubicin, hydroxyrubicin and pirarubicin

The rate of the pump-mediated efflux was calculated according to the experimental section and Appendix A. As an example, Fig. 5 shows the plot of V_a as a function of $\mathcal{C}_i$ for daunorubicin and K562/Adr and for daunorubicin and GLC4/Adr, respectively, in the presence of various PAK-104P concentrations.

The inhibition produced may be expressed in various ways. One way is to calculate the fractional activity $r = V_a^1/V_a$ where V_a and V_a^1 stand for the rate of active efflux in the absence and presence of inhibitor, respectively. At low substrate concentrations V_a can be written as k_a. $\mathcal{C}_i$

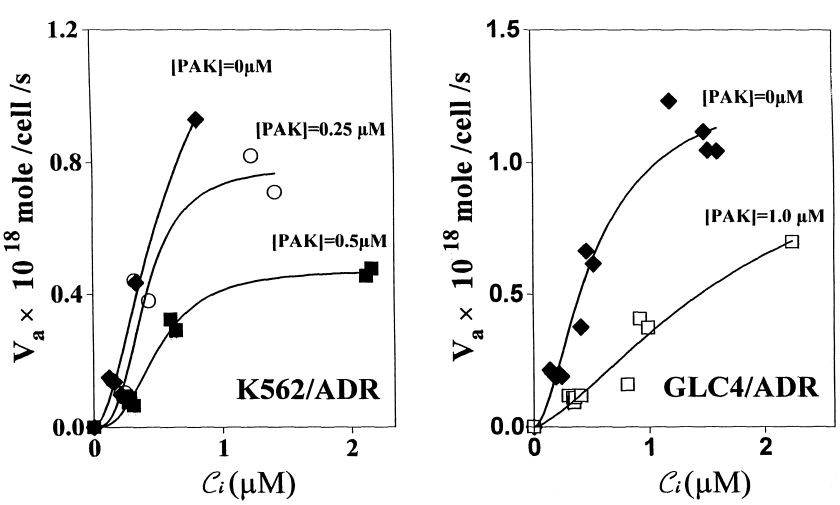

Fig. 5. Kinetics of the *P*-glycoprotein-and MRP_1-mediated efflux of daunorubicin (DNR) plotted as a function of $\mathcal{C}_i$, the cytosol free drug concentration. K562/Adr or GLC4/Adr cells (1×10^6/ml) were incubated in the presence of various concentrations of daunorubicin, ranging from 0 to 6 μM and various concentrations of PAK-104P. V_a and $\mathcal{C}_i$ were determined as described. Data points are from 3–5 independent experiments on different days.

and therefore $r = k_a^i/k_a$ represents fractional activity. A simple method to distinguish between non-competitive and competitive inhibition for enzymes was suggested (Wigler, 1999). This is based on the following equation

$$I_{50} = K'_I(1 + [S]/K'_s),$$

where I_{50} is the inhibitor concentration at half inhibition of efflux through the MDR pump, i.e., $V_a^i = V_a/2$ or $k_a^i = k_a/2$.

This equation was obtained from the rate equation for competitive inhibition of the MDR pump and the Michaelis equation with no assumptions about the value of the $[S]/K'_s$ratio (Wigler, 1999; Wigler and Patterson, 1993). For competitive inhibition the $[I]$ at half inhibition of efflux through the pump is $[I]_{50}$, the apparent inhibition constant is K'_I, the substrate concentration is $[S]$ and the apparent Michaelis constant for anthracycline is K'_s. It follows that for competitive inhibition

$$r = [K_i(1 + S/K_s)]/[I + K_i(1 + S/K_s)],$$

and for non-competitive

$$r = K_i/(I + K_i),$$

In the six cases, the r values were plotted as a function of I = [PAK-104P] (Fig. 6). Data were fitted using the above equation, yielding in each case a K_i value, which does not depend on the substrate concentration. This corroborates what was expected from the plot of $\alpha = f(I)$: PAK-104P non-competitively inhibits the *P*-glycoprotein- and the MRP_1-mediated efflux of anthracyclines. The values of the inhibitory constant are shown in Table 1. As can be seen

Table 1
Kinetic parameter for PAK-104P acting on the multidrug resistance pump of K562/Adr (*P*-glycoprotein) and GLC4/Adr (MRP_1)
K_I is the inhibition constant calculated using relation (19).

Substrate	K562/Adr K_I (μM)	GLC4/Adr K_I (μM)
Hydroxyrubicin	0.21 ± 0.05	0.06 ± 0.01
Daunorubicin	0.33 ± 0.08	0.35 ± 0.05
Pirarubicin	0.19 ± 0.05	0.36 ± 0.5
Calceinacetoxymethyl ester	0.15 ± 0.04	0.53 ± 0.04
Calcein	–	10 ± 3

for *P*-glycoprotein the K_I value does not depend on the nature of anthracycline, which is what could be expected in non-competitive inhibition. However, for MRP_1 the K_I values depend on the anthracycline nature.

3.3. Ability of PAK-104P to inhibit the P-glycoprotein-mediated efflux of calceinacetoxymethyl ester and the MRP_1-mediated efflux of calceinacetoxymethyl ester and calcein

The effect of PAK-104P on the calcein accumulation ([CAL-AM] = 0.5 or 0.1 μM) in K562/Adr and GLC4/Adr was measured as shown in Fig. 3. The efflux of calcein from GLC4/Adr cells was determined by the addition of Co^{2+}, which quenched the fluorescence of calcein in the extracellular medium. Almost no quenching was observed when Co^{2+} was added to K562/Adr cells,

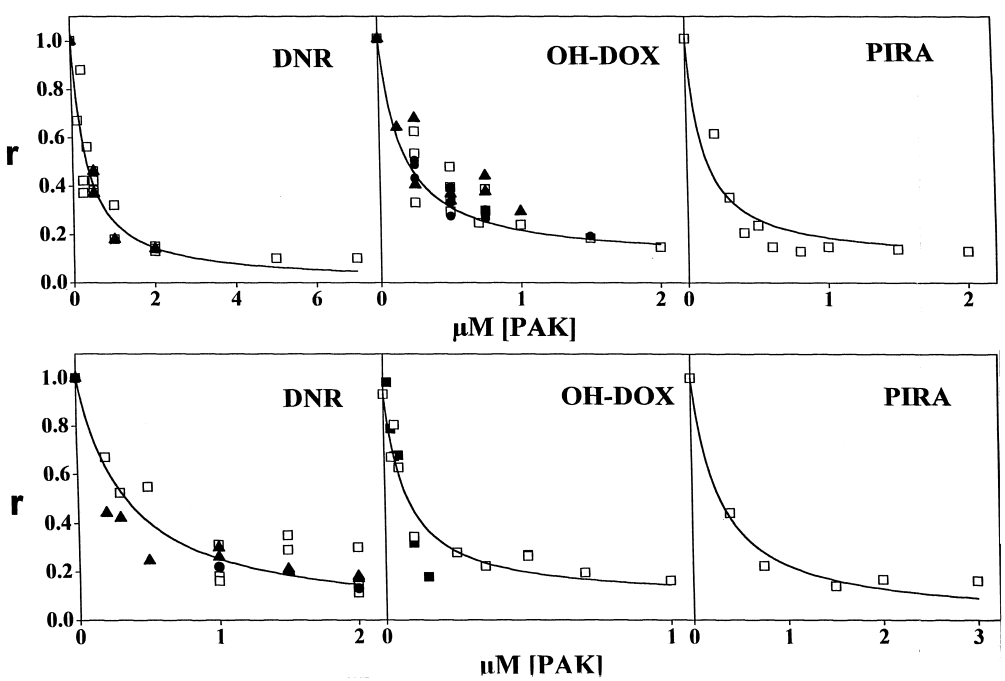

Fig. 6. Inhibition of (upper) the *P*-glycoprotein-and (lower) the MRP_1-mediated efflux of daunorubicin (DNR), hydroxyrubicin (OH-DOX) and pirarubicin (PIRA) by PAK-104P. $r = k_a^i/k_a$ was plotted as a function of PAK-104P concentration ($[I]$). Anthracycline concentrations used were 0.5 μM (■), 1 μM (□), 2 μM (●), 3 μM (▲), 6 μM (△). The data were fitted using equation $r = K_i/([I] + K_i)$.

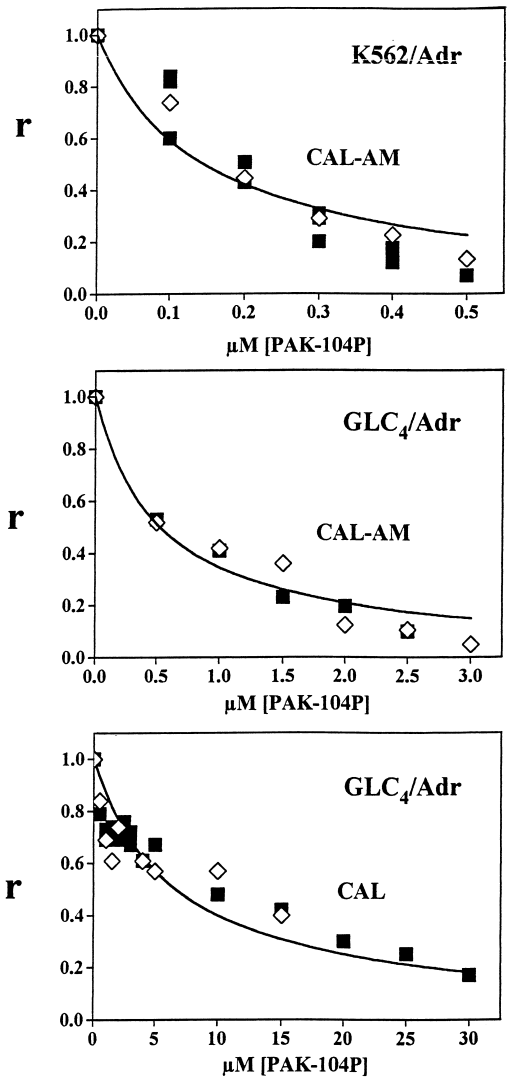

Fig. 7. Inhibition of the *P*-glycoprotein-and MRP_1-mediated efflux of calceinacetoxymethyl ester and of MRP_1-mediated efflux of calcein by PAK-104P. $r = k_a^i / k_a$ ($r_{CAL\text{-}AM}$ and r_{CAL}) was plotted as a function of PAK-104P concentration ($[I]$). Calceinacetoxymethyl ester concentrations used were 0.1 μM (◇), 0.5 μM (■). The data were fitted using equation $r = K_i/([I]+K_i)$.

P-glycoprotein being unable to pump out calcein. The *r* values for the calceinacetoxymethyl ester and calcein efflux were calculated (see Appendix B). From their plots as a function of the PAK-104P concentration (Fig. 7) the K_I values were calculated which, here also, did not depend on the substrate concentration. These values are reported in Table 1.

4. Discussion

In the present study, we have determined the kinetic parameters for inhibition by PAK-104P of the two multidrug resistance pumps, *P*-glycoprotein and MRP_1. Very few compounds are really efficient at low concentrations to inhibit the MRP_1-mediated efflux of drugs and, especially, very few compounds are able to inhibit both *P*-glycoprotein and MRP_1. Therefore, in this context, PAK-104P which is able to inhibit the efflux of both pumps at low concentrations is a very interesting molecule (Shudo et al., 1990; Chen et al., 1997; Sumizawa et al., 1997).

It is not known if PAK-104P is transported or not by MRP1 and *P*-glycoprotein. Our data show that neither K562/Adr nor GLC4/Adr is resistant to PAK-104P. This can be either because PAK-104P is not transported by *P*-glycoprotein and MRP_1 or because the rate of membrane equilibration is so rapid that flipping via the pump cannot keep pace with it. Some chemosensitizers (verapamil, cyclosporin A, etc.) appear to be transported by *P*-glycoprotein (the majority have not been tested), but multidrug resistant cells are not resistant to these compounds. For chemosensitizers, the rate of membrane equilibration is so rapid that the transporter essentially operates in a futile cycle; transport turnover is high, with high rates of ATP hydrolysis, but no gradient is generated, and cells will not, therefore, be resistant to chemosensitizers (Sharom, 1997). From this we infer that the PAK-104P concentrations were the same in the extra and intracellular media and equal to that added to the cells.

Let us first consider the data obtained with *P*-glycoprotein-expressing cells. Lan et al. (1996) have determined kinetic parameters for several reversers acting on the multidrug resistance pump (*P*-glycoprotein) of drug-resistant P388 cells measured with vinblastine and daunorubicin. They found that, for a reverser, the intrinsic K_I does not depend on the nature of the substrate, i.e., similar values were obtained using either daunorubicin or vinblastine. Such a result is what can be expected in the case of non-competitive inhibition. This is also what we observed for PAK-104P and *P*-glycoprotein-overexpressing K562/Adr cells: the intrinsic K_I values determined using daunorubicin, pirarubicin, hydroxyrubicin or calceinacetoxymethyl ester were very similar, i.e., 0.25 ± 0.1 μM, and the inhibition is non-competitive. The comparison of these data to those previously obtained with verapamil or verapamil derivatives, shows that verapamil (Mankhetkorn and Garnier-Suillerot, 1998; Mankhetkorn et al., 1999) and most verapamil derivatives (Teodori et al., 1999) are unable to completely block the *P*-glycoprotein-mediated efflux of anthracyclines, about 15% of the *P*-glycoprotein-functionality is still present even in the presence of a very large excess of these compounds. This is also what is observed with PAK-104P. It follows that these compounds are unable to restore, in multidrug-resistant cells, the same level of drug as in sensitive cells. However, PAK-104P is more efficient to restore the intracellular drug level in resistant cells than is verapamil, the K_I value being ~ 1 μM for verapamil and 0.25 μM for PAK-104P.

Concerning the data obtained with GLC4/Adr cells, the most striking regard the fact that the ability of PAK-104P

to increase intracellular drug concentration apparently depends on the drug used (see Table 1). From the observation that K_I did not depend on the substrate concentration, we have inferred that the inhibition was non-competitive. However, how to explain that K_I depends on the nature of the substrate? Actually the most striking difference is between calcein and anthracyclines (at least daunorubicin and pirarubicin, the case of hydroxyrubicin deserves more comment and will be discussed later) the K_I being about 30 times higher in the case of calcein than in the case of daunorubicin and pirarubicin. We have compared our data to those available from the literature. LTC_4, a substrate for the GS-X pump is transported by MRP_1 (Leier et al., 1994) and the ATP-dependent [^{3}H]LTC_4 uptake in membrane vesicles from C-A120 cells has been shown to be almost completely inhibited by 100 μM PAK-104P (Sumizawa et al., 1997). Using the data of Fig. 8 from Sumizawa et al. (1997), we have estimated an intrinsic inhibitory constant equal to 50 μM. So, it follows that the K_I value is very high for the two negatively charged compounds, LTC_4 and calcein, whose efflux does not depend on the presence of glutathione (Feller et al., 1995). It should be noticed that these two compounds have, however, very different K_m values, very high for calcein (Essodaïgui et al., 1998) and very low for LTC_4 (Gao et al., 1998).

If we consider the case of anthracyclines, the K_I value determined for the inhibition of hydroxyrubicin efflux by PAK-104P was about five times lower than those determined for the inhibition of the efflux of daunorubicin and pirarubicin. However, we have already observed that the MRP_1-mediated efflux of hydroxyrubicin was special as compared to that of the other anthracyclines, with a V_{max} value about 10 times lower than for the latter (Marbeuf-Gueye et al., 1998). Preliminary results obtained by our group suggested that this could be related to an intracellular level of glutathione insufficient to sustain full MRP_1-mediated efflux of hydroxyrubicin and in that case the K_I value would just be an apparent, underestimated value.

We therefore propose the existence of two different mechanisms for the inhibition by PAK-104P of the MRP_1-mediated efflux of molecules: a first mechanism, involving a low affinity site for PAK-104P, and which would concern molecules such as calcein, LCT_4 etc. whose efflux do not depend on glutathione. A second mechanism involving a high affinity site for PAK-104P and which would concern molecules such as anthracyclines, calceinacetoxymethyl ester whose efflux depend on the presence of glutathione.

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Appendix A. Determination of the *P*-glycoprotein- and MRP1-mediated efflux of anthracycline at steady state

At steady state, according to the parameters described in Fig. 2, the overall concentration of anthracycline intercalated between the base pairs in the nucleus is $C_n = C_T.(F_0 - F_n)/F_0$ and the extracellular free drug concentration is $C_e = C_T - C_n$. In the presence of PAK-104P at concentration [i], at steady state the overall concentration of drug intercalated between the base pairs in the nucleus is $C_n^i = C_T\ (F_0 - F_n^i)/F_0$. After the addition of 0.05% Triton X-100, which yields the equilibrium state, the overall concentration of drug intercalated between the base pairs in the nucleus is

$$C_N = C_T(F_0 - F_N)/F_0.$$

The intracellular free drug concentration, is

$$\mathcal{C}_i = C_n(C_E/C_N) \quad (A1).$$

At steady state the kinetics of the *P*-glycoprotein-mediated efflux of drug is

$$V_a = V_+ - V_-. \quad (A2)$$

V_+ and V_- are the kinetics of passive influx and efflux of the drug, respectively and V_a the kinetics of active efflux. In this expression, $V_+ = k_+ C_e$, $V_- = k_-\ \mathcal{C}_i$ and $V_a = k_a\ \mathcal{C}_i$, C_e and $\mathcal{C}_i$ being the extra and intracellular free drug concentrations at steady state, respectively, k_+ and k_- are the passive coefficients of influx, and efflux respectively and k_a the active efflux coefficient.

$$V_a = k_+ C_e - k_- \mathcal{C}_i. \quad (A3)$$

The relation $V_a = k_a \mathcal{C}_i$ is valid when the efflux is not saturated, i.e., when the kinetics of active efflux are linearly dependent on the intracellular free drug concentration. According to our previous work this condition is fulfilled here (Mankhetkorn et al., 1996).

Using the above equations, k_a can be written as

$$k_a = (k_+ C_e - k_- \mathcal{C}_i)/\mathcal{C}_i. \quad (A4)$$

Making the reasonable assumption that $k_+ = k_-$, it follows that

$$V_a = k_+(C_e - \mathcal{C}_i), \quad (A5)$$

and

$$k_a = k_+(C_e - \mathcal{C}_i)/\mathcal{C}_i. \quad (A6)$$

In the following, k_a and k_a^i will stand for the transporter-mediated active efflux coefficient of anthracycline in the

absence and in the presence of PAK-104P, respectively. We have checked that the passive influx coefficient, k_+ that characterises the passive diffusion of anthracycline, does not depend on the presence of PAK-104P.

r can be written as a simple function of the fluorescence parameters defined above:

$$r = \left[\left(F_N - F_n^i\right)\left(F_0 - F_n^i\right)\right] / \left[\left(F_N - F_n\right)\left(F_0 - F_n\right)\right]. \quad \text{(A7)}$$

Appendix B. Determination of the *P*-glycoprotein- and MRP1-mediated efflux of calceinacetoxymethyl ester and of the MRP1-mediated efflux of calcein at steady state

k_a characteristic of the pump-mediated efflux of calceinacetoxymethyl ester was defined as

$$d[\text{CAL-AM}]/dt = k_a[\text{CAL-AM}]_i, \quad \text{(A8)}$$

where $[\text{CAL-AM}]_i$ is the free intracellular calceinacetoxymethyl ester concentration.

$$d[\text{CAL-AM}]/dt = \left[C_T(dF_3/dt - dF_1 dt)\right] / \left[F_{CAL}\right], \quad \text{(A9)}$$

at short time incubation (i.e., less than 500 s) $dF_3/dt - dF_1/dt$ is equal to $(F_3 - F_1)/t$ were F_3 and F_1 are the fluorescence intensity at 400s in ATP-depleted and ATP-rich cells, respectively. C_T is the concentration of calceinacetoxymethyl ester added to cells. All these parameters are shown in Fig. 3.

In energy-depleted cells, the extra-and intracellular concentrations of calceinacetoxymethyl ester are equal

$$[\text{CAL-AM}]_i = [\text{CAL-AM}]_e = C_T[1 - F_3/F_{CAL}]. \quad \text{(A10)}$$

We have previously shown (Essodaïgui et al., 1998) that the limiting step for calcein formation is not the passage of calceinacetoxymethyl ester through the plasma membrane, but the rate of calceinacetoxymethyl ester hydrolysis characterised by the constant K_{hyd}. Therefore the kinetic for calcein formation is

$$\begin{aligned} d[\text{CAL}]_i/dt &= K_{hyd}[\text{CAL-AM}]_i \\ &= K_{hyd}C_T\left[(F_{CAL} - F_3)/F_{CAL}\right] \\ &= C_T(dF_3/dt), \end{aligned}$$

it follows that

$$K_{hyd} = (dF_3/dt)\left[F_{CAL}/(F_{CAL} - F_3)\right], \quad \text{(A11)}$$

This relation can be used to determine $[\text{CAL-AM}]_i$ in ATP-rich cells.

$$[\text{CAL-AM}]_i = C_T\left[(dF_1/dt)/(dF_3/dt)\right]\left[(F_{CAL} - F_3)/F_{CAL}\right],$$

and therefore,

$$k_a = (F_3 - F_1)/(F_{CAL} - F_3)(F_3/F_1)(1/t), \quad \text{(A12)}$$

and $r_{CAL\text{-}AM} = k_a^i/k_a$ can be simply written as a function of the fluorescence parameters

$$r_{CAL\text{-}AM} = \left[\left(F_3 - F_1^i\right)F_1\right] / \left[\left(F_3 - F_1\right)F_1^i\right]. \quad \text{(A13)}$$

Similarly, the parameter r_{CAL} can be calculated, which characterised the MRP_1-mediated efflux of calcein.

$$r_{CAL} = \left[\left(F_1'^i - F_1''^i\right)F_1'\right] / \left[\left(F_1' - F_1''\right)F_1'^i\right]. \quad \text{(A14)}$$

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