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Transport of new non-cross-resistant antitumor compounds of the benzoperimidine family in multidrug resistant cells

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Abstract

Multidrug resistance (MDR) phenotype in mammalian cells is often correlated with overexpression of P-glycoprotein 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. The influx and efflux of drugs across the cell membrane are in large part responsible for their intracellular concentrations, and in the search for new compounds able to overcome MDR, it is of prime importance to determine the molecular parameters whose modification would lead to an increase in the kinetics of uptake and/or to a decrease in the pump-mediated efflux. Here, we studied three members of a new family of benzoperimidine antitumor compounds which exhibit comparable cytotoxicity towards resistant cells expressing P-glycoprotein, or MRP1, and sensitive cells. We used spectrofluorometric methods to determine the kinetics of the uptake and release of these three drugs in different cell lines: the erythroleukemia cell line K562 and the resistant K562/Adr expressing P-glycoprotein, the small-cell lung cancer cell line GLC4 and resistant GLC4/Adr expressing MRP1. We also studied, using confocal microscopy, the intracellular distribution of these drugs in NIH/3T3 cells. Our data show that (i) the kinetics for the uptake of these drugs is very rapid, higher than 2×10^{-17} mole cell^{-1} s^{-1}, (ii) the drugs are strongly accumulated in the nucleus and lysosomes, (iii) the three drugs are recognized and pumped out by both transporters, as shown by the inhibition of P-glycoprotein- and MRP1-mediated efflux of pirarubicin by benzoperimidine, with inhibitory constants of 1.5 and 2.1 μM for P-glycoprotein and MRP1, respectively, suggesting that benzoperimidine is transported by the two transporters with $K_m \sim 2$ μM. In conclusion, the fast uptake kinetics of the benzoperimidines counterbalance their efflux by P-glycoprotein and MRP1.

Keywords: Multidrug resistance; P-glycoprotein; MRP1 (multidrug resistance-associated protein); Benzoperimidine

1. Introduction

Multidrug resistance (MDR) is frequently expressed by neoplastic cell types and results in a broad spectrum of resistance to drugs that show little structural similarity. MDR is a well-characterized phenomenon that is frequently associated with decreased drug accumulation due to enhanced drug efflux (Bradley et al., 1988; Gottesman and Pastan, 1993; Loe et al., 1996; Broxterman et al., 1995; Zaman et al., 1994; Sharom, 1997). This is correlated with the presence of membrane proteins, the P-glycoprotein, the multidrug resistance-associated protein (MRP1) and the canalicular multispecific organic anion transporter (c-MOAT). These proteins (Cui et al., 1999; Kool et al., 1997) belong to the ATP-binding cassette protein family.

The search for new compounds able to overcome MDR is of prime importance in the clinic, and for this reason, it is important to know the mechanism of this protein-mediated efflux of drug. Because the activity of a drug depends on its concentration in the compartment where its cellular target is located (Pereira and Garnier-Suillerot, 1994), and because the influx and efflux of drugs across the cell membrane is in large part responsible for their intracellular concentration, the mechanism of transport of these agents into the cell, and in particular, the kinetics of this transport

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are of crucial importance (Marbeuf-Gueye et al., 1998, 1999; Mankhetkorn et al., 1996).

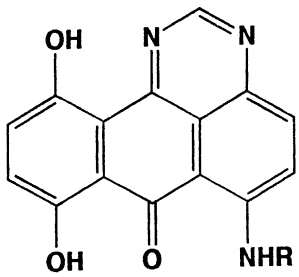
In order to increase the intracellular drug concentration in resistant cells, two main parameters must be taken into account: the kinetics of transporter-mediated efflux, which may be decreased, and/or the kinetics of drug uptake by cells, which may be increased (Stein, 1997; Marbeuf-Gueye et al., 1999; Garnier-Suillerot, 1995). To decrease the kinetics of the transporter-mediated efflux of the drug presupposes knowledge of the mechanisms of this transporter, which is far from the case for the three proteins involved in the MDR phenomenon. However, it is easier to increase the passive kinetics of uptake, for instance by increasing the lipophilicity of the molecules. Previous studies by our group have been directed at the question of the substrate specificity of P-glycoprotein- and MRP_1-mediated drug efflux in order to contribute to a rational design of drugs for the treatment of drug-resistant tumors. With this purpose, we have studied the ability of various chemical classes of molecules to be recognized by P-glycoprotein and MRP1, and the kinetics of P-glycoprotein- and MRP1-mediated efflux of a series of anthracyclines (Mankhetkorn et al., 1996; Mankhetkorn and Garnier-Suillerot, 1998; Marbeuf-Gueye et al., 1998; Essodaïgui et al., 1998; Vergote et al., 1998). We have recently shown that, at least in the case of one class of compounds, the anthracyclines, an increase in molecular parameters leading to an increase in uptake kinetics leads to a very important decrease in resistance (Garnier-Suillerot, 1995; Marbeuf-Gueye et al., 1999).

Recently, we synthesized a new family of benzoperimidine antitumor compounds, and here we studied three of them, BP1, BP2 and BP3, which are cytotoxic to sensitive cells and to resistant cells, which express P-glycoprotein or MRP1 (Stefanska et al., 1999). The aim of the present study was to determine the reason for this effect, and for this purpose, we studied the kinetics of the uptake and release of these three drugs in different cell lines: the erythroleukemia cell line K562 and resistant K562/Adr expressing P-glycoprotein, the small-cell lung cancer cell line GLC4 and resistant GLC4/Adr expressing MRP1. In addition, using confocal microscopy, we studied the intracellular distribution of these drugs in NIH/3T3 cells. Our data show that (i) the kinetics for the uptake of these drugs is very rapid, (ii) the drugs are strongly accumulated in the nucleus and lysosomes (iii) the three drugs are recognized and pumped out by both transporters, but this is very well counterbalanced by the very rapid uptake kinetics.

2. Materials and methods

2.1. Drugs and chemicals

Purified BP1, BP2 and BP3 (Fig. 1) were prepared according to Stefanska et al. (1999). Stock solutions were prepared just before use. Pirarubicin and doxorubicin were kindly provided by Pharmacia-Upjohn (Milano, Italy). 3′-deamino-3′-hydroxy-doxorubicin (hydroxyrubicin) was provided by W. Priebe (Priebe, 1995). LysoTracker™, Green DND-26 and 6-((*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino) caproyl) sphingosine (C_5-NBD-ceramide) were purchased from Molecular Probes. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), calf thymus DNA and defatted bovine serum albumin were 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 $MgCl_2$ at 7.3, in either the presence or the absence of 5 mM glucose.



$R = (CH_2)_2N(CH_3)_2$ **BP1**

$R = (CH_2)_2NH_2$ **BP2**

$R = (CH_2)_2NH(CH_2)_2OH$ **BP3**

Fig. 1. Structures of the benzoperimidine derivatives used.

2.2. DNA preparation

High-molecular weight calf thymus DNA was dissolved in phosphate-buffered saline (PBS) for 3 h under vigorous stirring. An absorption coefficient of 13200 M^{-1} cm^{-1} (bp) was used to calculate DNA concentrations from absorbance measurements at 260 nm.

2.3. Materials

Absorption spectra were recorded on a Cary 219 spectrophotometer and fluorescence spectra on a Perkin-Elmer LS 50B spectrofluorometer. Fluorescence microscopy was carried out on a Nikon Optiphot-2 epifluorescence microscope equipped with a Nipkow wheel coaxial-confocal attachment (Technical Instruments, San Francisco, CA). Cells were mounted in a perfusion chamber and viewed with a Nikon Plan-Apo X60 oil immersion objective (numerical aperture, 1.4). Confocal fluorescence images were detected with a cooled CCD camera (Micromax; Princeton Instruments, Evry, France) with a 12-bit

detector (RTEA-1317 K; Kodak). C_6-NBD-ceramide or LysoTracker™ Green DND-26 and BP1 were visualized with standard fluorescein and rhodamine filter sets, respectively. Image pairs were acquired (exposure time, 500 ms) for the same field containing one or more cells. Analysis and display were performed using IPLab software (Scanalytics, Fairfax, VA).

Fluorescence emission spectra of samples of about 10 μl were recorded with a UV-visible microspectrofluorometer prototype developed in our laboratory and which has been extensively described elsewhere (Sureau et al., 1990). This microspectrofluorometer was used, rather than a traditional spectrophotometer, to record fluorescence spectra of highly concentrated solutions, because the reabsorption of emitted light is negligible due to the low thickness of the sample even when very high concentrations of anthracyclines are used.

2.4. Cell lines and cultures

K562 leukemia cells and the P-gp expressing K562/ADR cells (Mankhetkorn et al., 1996), as well as GLC4 and the MRP1-expressing GLC4/ADR cells (Zijlstra et al., 1987) were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum 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 1–4 weeks before experiments. Cell cultures used for experiments were split 1:2 one day before use in order to ensure logarithmic growth. They were counted with a Coulter counter immediately before use in the assay. Cell viability was assessed by trypan blue exclusion and was higher than 95% under the various experimental conditions used. NIH/3T3 cells were obtained from the American Type Culture Collection (Manassas, VA). They were grown in Dubecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum in a humidified incubator with 5% CO_2. Before each experiment, cells were plated on 18-mm diameter glass coverslips and allowed to grow overnight to reach 75% confluence.

The cytotoxicity of the compounds was determined by incubating cells (10^5) with six different concentrations of the compound for 72 h in standard six-well plates. Then the IC_{50} values (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.

2.5. Acidic organelles and trans-Golgi labelling

Acidic organelles were stained with LysoTracker after incubation with 1 μM BP1 for 5 min at 37°C: cells were incubated with 50 nM of the probe (from 1 mM stock solution in dimethyl sulfoxide (DMSO)) in complemented DMEM medium for 30 min at 37°C. They were then washed with PBS prior to observation. The trans-Golgi membrane was stained according to Panago et al. (1989): 50 nmol of C_6-NBD-ceramide was dissolved in 200 ml of ethanol and injected into 10 ml of 10 mM HEPES-buffered minimal essential medium containing 0.34 mg of defatted bovine serum albumin. The solution was dialysed overnight at 4°C against HEPES-buffered minimal essential medium and aliquoted. After incubation with 1 μM BP1, cells were incubated with the C_6-NBD-ceramide-bovine serum albumin complex for 5 min at 37°C and washed with BPS prior to observation.

2.6. Cellular drug accumulation

The compounds used in this study are fluorescent and their addition to cells suspended in buffer resulted in a decrease in the fluorescence signal of the drugs comparable to that observed during the uptake of anthracycline by cells (Tarasiuk et al., 1989). This was used as an indication (and will be demonstrated hereafter) of the uptake of these drugs by cells and we have therefore monitored the decrease in the fluorescence signal at 660 nm ($\lambda_{ex} = 516$ nm) during incubation of the cells with drugs (this method as previously described for anthracycline) (Tarasiuk et al., 1989; Frézard and Garnier-Suillerot, 1991a,b). All experiments were conducted in 1-cm quartz cuvettes containing 2 ml of buffer at 37°C.

For some experiments, cells were depleted of ATP (energy-deprived) through incubation for 30 min in the presence of N_3^- and in the absence of glucose.

The uptake of the anthracycline derivative, pirarubicin, was monitored by following the decrease in the fluorescence signal at 590 nm (λ_{ex} at 480 nm) according to the procedure largely described previously (Tarasiuk et al., 1989; Frézard and Garnier-Suillerot, 1991a,b; Mankhetkorn et al., 1996; Marbeuf-Gueye et al., 1998).

2.7. Statistical analysis

Data were analysed by ANOVA test and the criterion of the differences between means ($\pm$SEM) was $P < 0.05$.

3. Results

The structures of the three new compounds used in the present study are shown in Fig. 1.

3.1. Cell-growth inhibition

The IC_{50} values obtained for the three drugs for sensitive and resistant K562 and GLC4 cells are shown in Table 1, together with the RF factor. For comparison, the values obtained for pirarubicin and doxorubicin have been added. As can be seen for the three compounds, the RF values

Table 1
Cross-resistance of doxorubicin-resistant K562 and GLC4 cells

Drug	K562 IC_{50} nM	K562/Adr IC_{50} nM	K562 RF	GLC4 IC_{50} nM	GLC4/Adr IC_{50} nM	GLC4 RF
BP1	4.4 ± 1.6	7 ± 2	1.5	10 ± 2	15 ± 1	1.5
BP2	18 ± 2	20 ± 1	1.1	114 ± 10	98 ± 8	1.2
BP3	5 ± 1	7 ± 1	1.5	9 ± 1	15 ± 2	1.7
DOX	10 ± 2	340 ± 30	34	9 ± 2	670 ± 70	74
PIRA	6 ± 1	50 ± 3	8	5 ± 1	25 ± 2	5

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 mean $\pm$ SD of triplicate determinations.

were lower than 2 in both cell lines. BP1 and BP3 were highly cytotoxic, the IC_{50} values being comparable to those of pirarubicin and doxorubicin. BP2 was less effective, its IC_{50} value being 4 and 10 times higher than that of the two other drugs in K562 and GLC4 cells, respectively.

3.2. Physicochemical properties of BP1, BP2 and BP3

The absorption and fluorescence spectra of BP1, BP2 and BP3 in aqueous solution were very similar, and as an example, those of BP1 recorded at pH 7.3 are shown in Fig. 2. The fluorescence spectra were recorded at different pH values, in NaCl 0.1 M, at 37°C. In the three cases, no modification of the spectra was observed from pH 2–5. However, when the pH was increased from 5 to 12, there was a strong decrease in the fluorescence signal, probably due to deprotonation of the molecule (Fig. 2, inset). The pK_a values determined from the plot of the fluorescence intensity at 660 nm as a function of the pH values was equal to 7.7 ± 0.1 for the three compounds.

Using microspectrofluorometry, we determined that auto quenching of drug fluorescence occurred at a very low concentration: the intensity of the fluorescence signal was proportional to the drug concentration up to about 20 μM and then plateaued (the experiments were performed at pH 7.3 and 5).

3.3. Accumulation of BP1, BP2 and BP3 in cells

Fig. 3 shows the typical fluorescence signal observed when GLC4 and K562 cells (10^6/ml), either sensitive or resistant, were incubated with 1 μM BP1. Because the fluorescence of the compounds was pH dependent, the pH of the extracellular medium was chosen to be equal to that of the cytosol, i.e. pH 7.3 (Frézar and Garnier-Suillerot, 1991a). Very similar curves (data not shown) were obtained using either BP2 or BP3. In all cases, a similar very rapid and large decrease in the fluorescence signal was observed. A steady state was obtained within about 10 min. A very similar decrease in the signal was observed with K562 or GLC4 cell lines, either sensitive or resistant. At steady state, the addition of 0.02% Triton X-100, which permeabilizes the cell membrane, yielded a partial recovery of the fluorescence signal. These first data strongly

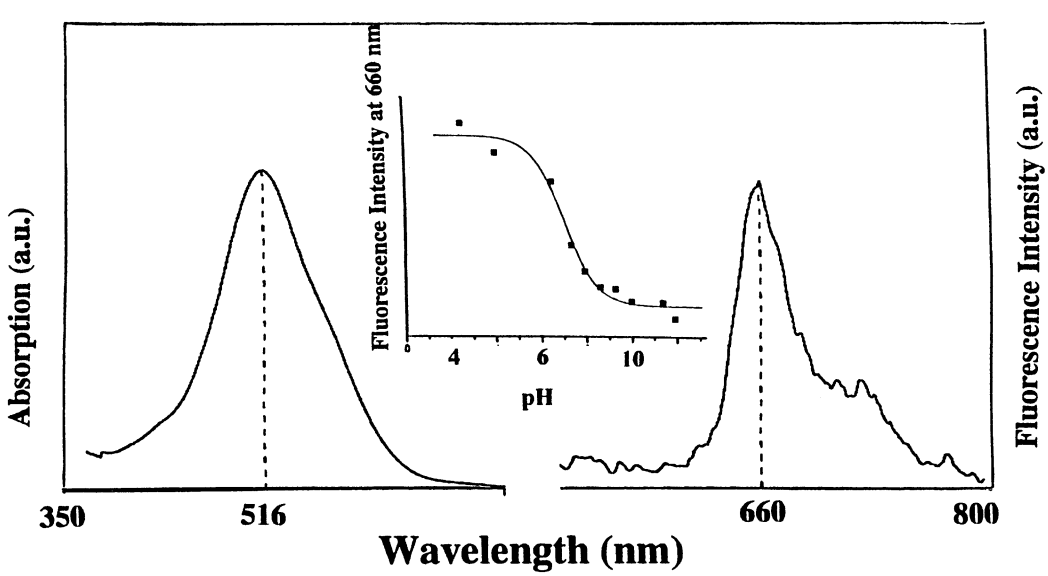


Fig. 2. Absorption (left) and fluorescence (right) spectra of BP1 in aqueous solution at pH 7.3. Inset: variation of the fluorescence signal at 660 nm (λ_{ex} = 590 nm) as a function of the pH. Experimental conditions: 0.1 M NaCl, 37°C.

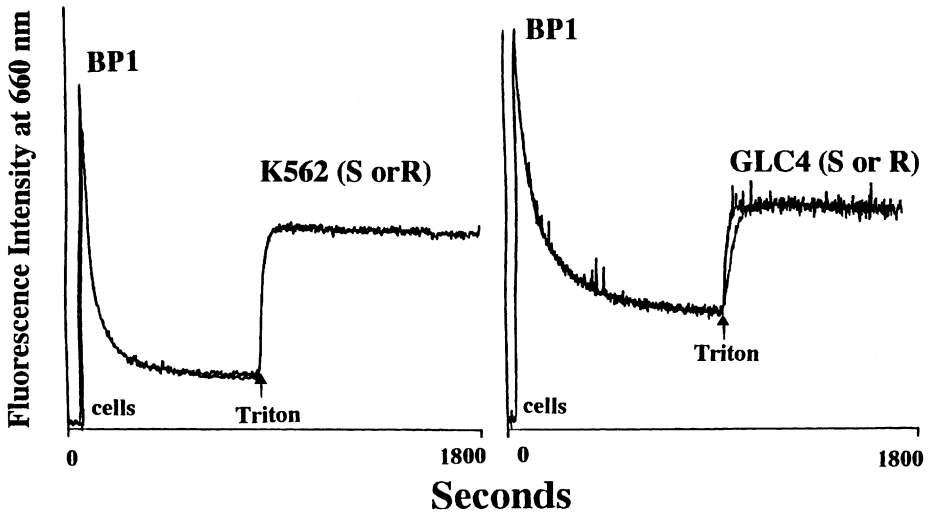


Fig. 3. Drug accumulation of BP1 in cells. Fluorescence intensity at 660 nm was recorded as a function of the time of incubation of cells (10^6/ml) with 1 μM drug. (Left) K562 cells sensitive and resistant; (right) GLC4 cells sensitive and resistant. At steady state, 0.02% Triton X-100 was added to permeabilize the membrane. Each experiment was repeated four times and this figure is a representative example.

suggest that the drugs accumulate in different compartments inside the cells. In order to determine the origin of the decrease of the fluorescence signal and, therefore, the nature of the compartments, the following experiments were performed.

3.4. Interaction of BP1, BP2 and BP3 with DNA

The addition of DNA to either BP1, BP2 or BP3 led to quenching of the fluorescence signal. The fluorometric titration of drugs (1 μM) with DNA was performed at 37°C in buffer at pH 7.3. In the presence of a very large excess of DNA ([base pairs]/[drug] ~ 200), quenching of fluorescence was maximum and equal to 71%, 59% and 60% for BP1, BP2 and BP3, respectively. 50% of the fluorescence quenching was observed at a base pair concentration equal to 5 ± 1, 5 ± 1 and 12 ± 2 μM for BP1, BP2 and BP3, respectively.

3.5. Interaction of BP1, BP2 and BP3 with cell nuclei

The addition of cell nuclei to the drug solution gave rise to a rapid decrease in the fluorescence signal. The kinetics for this decrease in signal were comparable to those observed with intact cells. Moreover, the quenching at steady state was similar to that observed with cells whose membrane had been permeabilized with 0.02% Triton X-100 (we previously checked that the addition of 0.02% Triton X-100 to the cells caused permeabilization of the plasma and intracellular membrane, but had no effect on the drug–DNA interaction). From these data, we can infer that the fluorescence quenching observed when the drugs interacted with cells whose membrane had been permeabilized was due to the drug–DNA interaction.

3.6. Modulation of BP1, BP2 and BP3 accumulation in cells

Now that the main site of drug accumulation inside the cells had been identified, the following experiments were designed to identify the other sites. In the following, data obtained with GLC4 cells and BP1 are reported, but strictly analogous data were obtained with the three other lines and the two other compounds.

First, energy-deprived cells were incubated with 1 μM BP1. The decrease in the fluorescence signal was smaller than in the case of ATP-rich cells (Fig. 4(A)).

Second, energy-deprived cells were incubated with 1 μM BP1. At steady state, 5 mM glucose was added, yielding a very slow decrease in the fluorescence signal (Fig. 4(B)).

Third, cells were incubated with 1 μM BP1 and at steady state, 10 μM FCCP was added, yielding a very fast increase in the fluorescence signal up to the value obtained with energy-deprived cells (Fig. 4(C)). To check that the effect of FCCP was not a result of ATP depletion because of uncoupling of oxidative phosphorylation, we measured the hydroxyrubicin accumulation in MDR cells and found that it was not modified by the presence of FCCP (hydroxyrubicin is a permanently neutral anthracycline derivative, which, therefore, cannot accumulate in acidic compartments and which is a P-glycoprotein and MRP1 substrate (Borrel et al., 1994).

Fourth, ATP-rich GLC4/Adr cells (10^6/ml) were incubated with 1 μM BP1. At steady state, we permeabilized the cells with increasing concentrations of Triton X-100. The addition of 0.01% Triton X-100 did not modify the fluorescence signal, however, the addition of 0.02% Triton X-100 yielded an important increase of the signal (Fig. 5). As a control for plasma membrane permeabilization by

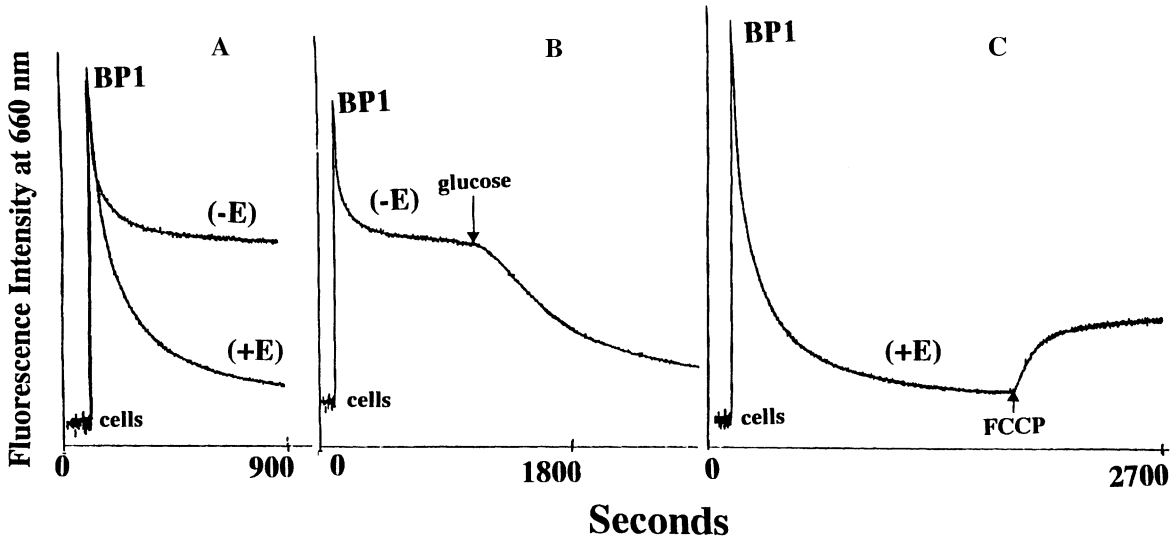


Fig. 4. Modulation of BP1 accumulation in GLC4 cells as a function of different parameters. Fluorescence intensity at 660 nm was recorded as a function of the time of incubation of cells (10^6/ml) with 1 μM drug. The cells were either energy-deprived (−E, A and B) or contained ATP (+E, A and C). At steady state, either glucose (B) or FCCP (C) was added. Each experiment was repeated three times and this figure is a representative example.

Triton X-100, we measured the uptake of hydroxyrubicin in GLC4/Adr cells. Hydroxyrubicin is a fully neutral derivative of doxorubicin that does not exhibit non-nuclear accumulation. The amount of hydroxyrubicin incorporated at steady state increased (decrease in the fluorescence signal) after the first addition of 0.01% Triton X-100. No further modification was observed at higher concentrations indicating that 0.01% Triton X-100 was sufficient to permeabilize the plasma membrane (Fig. 5).

The data obtained for the three compounds and the four cell lines under the conditions described above are summarized in Fig. 6, where the percentage of quenching of the fluorescence signal can be used as a good estimation of the percentage of drug accumulated inside the cells. The amount of drug incorporated inside both sensitive and resistant cells was comparable whatever the compound considered ($P > 0.05$). The accumulation of drugs in cells, either sensitive or resistant, when the pH was eliminated, i.e. in energy-deprived cells or in cells incubated with FCCP, was significantly less than in ATP-rich cells ($P < 0.05$). These data show that an energy-dependent drug accumulation occurred in non-nuclear compartments.

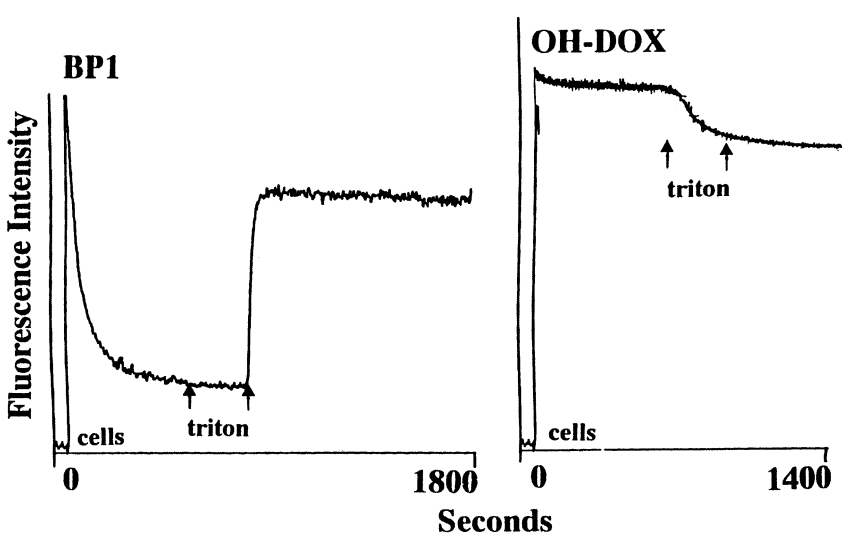


Fig. 5. Accumulation of drug in GLC4/Adr cells. Cells (10^6/ml) were incubated with 1 μM drug. The drug was BP1 and the fluorescence intensity was recorded at 660 nm (left), the drug was OH-DOX and the fluorescence intensity was recorded at 590 nm (right). At steady state, permeabilization of the membranes was achieved by the progressive addition of Triton X-100. Each experiment was repeated three times and this figure is a representative example.

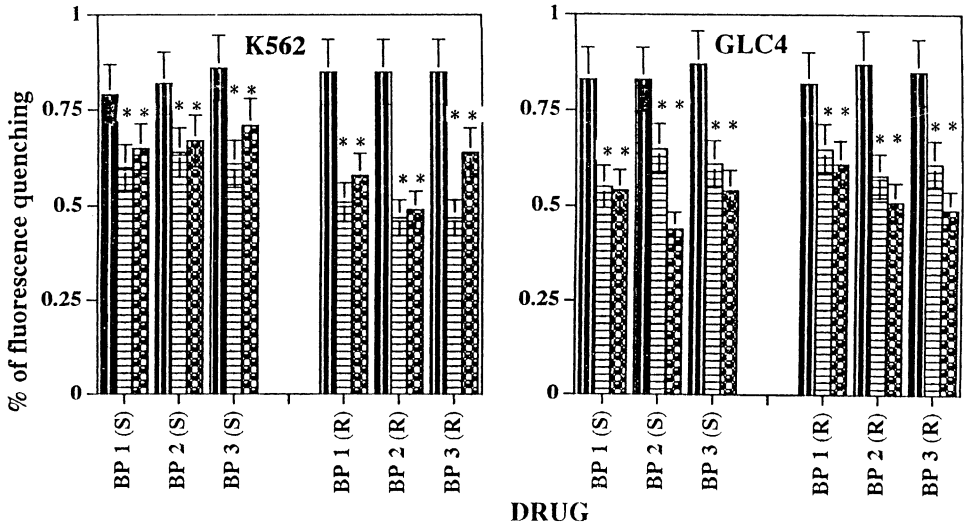


Fig. 6. Accumulation of BP1, BP2 and BP3 in sensitive and resistant K562 and GLC4 cells. The diagram shows the percentage of quenching of the fluorescence signal at steady state when the drugs (1 μM BP1, BP2 or BP3) were incubated with cells (10^6/ml). ( ) ATP-rich cells, ( ) ATP-rich cells plus FCCP, ( ) energy-deprived cells. S and R stand for sensitive and resistant cells, respectively. Each value is the mean ± SD of five independent experiments. Asterisks indicate values significantly different ($P < 0.05$) from respective controls (ANOVA test).

Fifth, NIH/3T3 cells were incubated with BP1 and then with either LysoTracker, to stain acidic organelles, or with C_6-NBD-ceramide, to stain trans-Golgi. These cells were used, in addition to the cell lines in which the transport studies were done, because their intracellular organelles are easily visualized. Confocal microscopy, showed punctuated zones of strong fluorescence which corresponded to the acidic organelles (Fig. 7).

From all these data, we can infer that acid compartments with an energy-dependent pH gradient (i.e. lysosomes) are a second site of drug accumulation.

3.7. Ability of BP1, BP2 and BP 3 to inhibit the P-glycoprotein- and MRP1-mediated efflux of pirarubicin

All the previous experiments showed a very similar behaviour of the four lines with the three drugs, and apparently the net uptake of benzoperimidines did not depend on whether the cells were sensitive or resistant, expressing either P-glycoprotein or MRP1.

We have previously developed a method to directly measure the rate of P-glycoprotein- and MRP1-mediated efflux of fluorescent molecules such as anthracyclines (Mankhetkorn et al., 1996; Marbeuf-Gueye et al., 1998): energy-depleted cells are incubated with anthracycline, and at steady state, glucose is added to promote ATP synthesis. A modification of the fluorescence signal is observed which is related to the occurrence of two simultaneous energy-dependent processes: (1) drug accumulation in acidic compartments which gives rise to a decrease in the fluorescence signal, and (2) pump-mediated efflux of drug, which gives rise to an increase in the signal. In the case of most anthracyclines, and especially when using a low concentration (~ 1 μM), drug accumulation can be neglected and the rate of pump-mediated efflux of anthracycline can be determined directly. However, for benzoperimidines, there is a strong accumulation of the drug and this prevents the direct determination of pump-mediated efflux of benzoperimidines. For this reason, we used indirect evidence and show that these drugs were able to inhibit the pumping of pirarubicin, a well-known substrate of P-glycoprotein and MRP1 (Frac > ézard and Garnier-Suillerot, 1991b; Marbeuf-Gueye et al., 1998). For this purpose, energy-deprived cells (10^6/ml) were incubated with 1 μM pirarubicin and the decrease in the fluorescence signal was followed at 590 nm (λ_{ex} = 480 nm). At steady state, BP1 was added at a concentration ranging from 0 to 10 μM. In control experiments, we checked (i) that BP1 did not yield a fluorescence signal when excited at 480 nm, (ii) that the first immediate decrease in the pirarubicin signal observed after BP1 addition was due to a screening effect of BP1; this was taken into account in the further calculations. Glucose was added, 5 min after the addition of BP1, causing ATP synthesis via glycolysis. An increase in the fluorescence signal was observed, indicating that pirarubicin was pumped out. Its intensity was dependent on the amount of BP1 added (Fig. 8). The following parameters were calculated according to a procedure used previously (Mankhetkorn et al., 1996; Marbeuf-Gueye et al., 1998): (i) V_a, the kinetics for the active efflux of pirarubicin, from the slope of the fluorescence signal; (ii) C_n^{BP1} the amount of pirarubicin incorporated in the nucleus at the new steady state, from the intensity of the fluorescence signal; (iii) the concentration of BP1 free in the cytosol from the decrease of the fluorescent signal when cells were incubated with BP1 only. As can be seen in Fig. 9, the amount of pirarubicin incorporated in the nucleus at steady state increased (V_a decreased) as the concentration

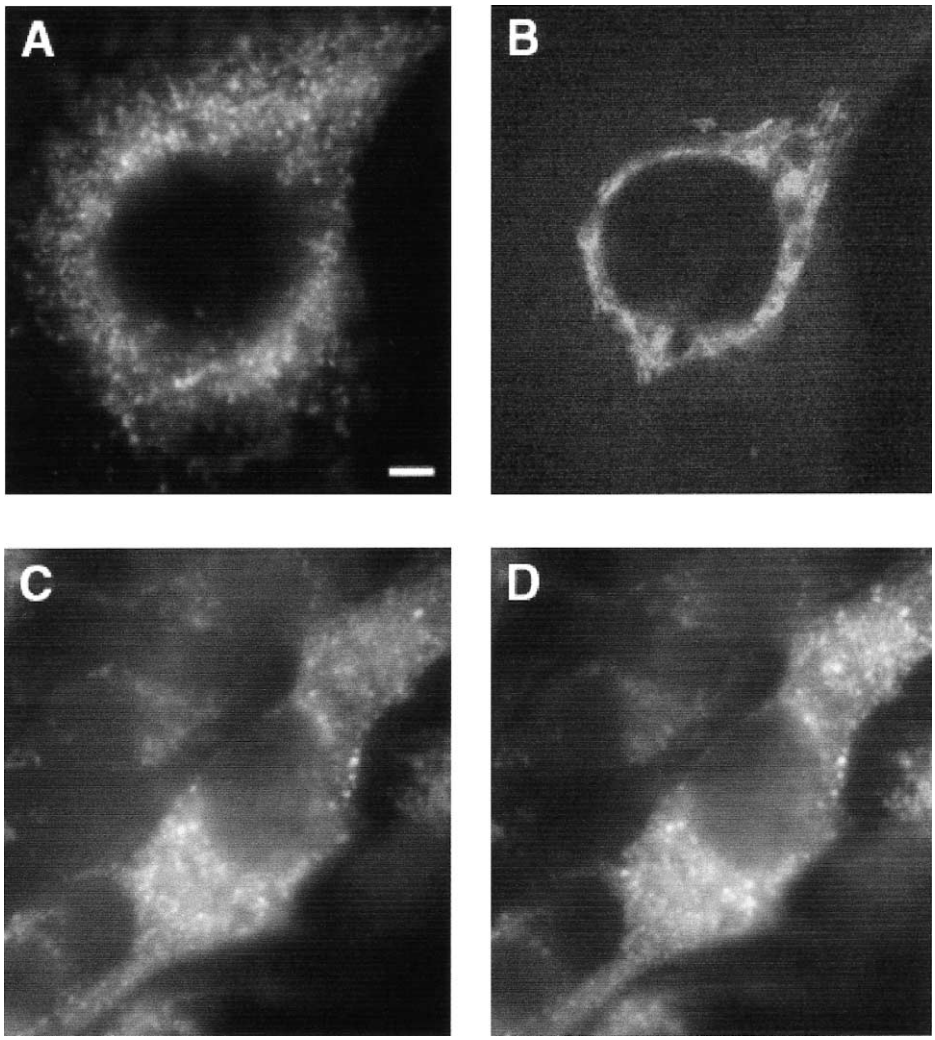


Fig. 7. Colocalization of BP1 with trans-Golgi and acidic organelles probes in NIH/3T3 cells. Cells were labelled according to Section 2 with 1 μM BP1 (A and C), C_6-NBD-ceramide (B) and 50 nM LysoTracker™ Green DND-26 (D).

of BP1 increased, indicating that BP1 inhibited the pumping of pirarubicin by both P-glycoprotein and MRP1. To get more quantitative information, $1/V_a$ was plotted as a function of the BP1 concentration added. The intersect of

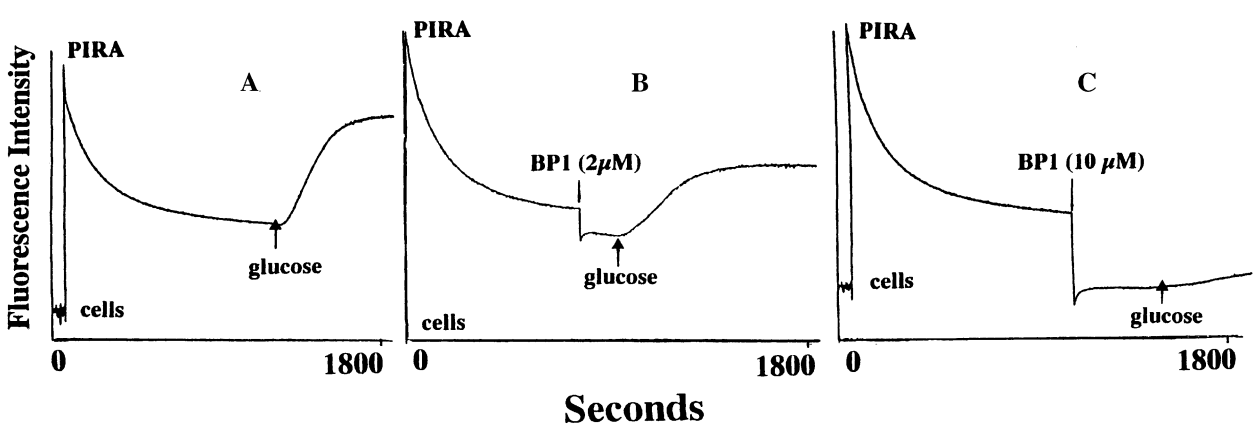


Fig. 8. Competition between pirarubicin and BP1 for efflux by the MRP1 pump. Fluorescence intensity at 590 nm was recorded as a function of the time of incubation of energy-deprived cells (10^6/ml) with 1 μM pirarubicin. At steady state 0 (A), 2 μM (B) or 10 μM (C), BP1 was added and glucose 5 min later. The kinetics for the MRP1-mediated efflux of pirarubicin were determined from the slope of the tangent to the curve after the addition of glucose. Each experiment was repeated three times and this figure is a representative example.

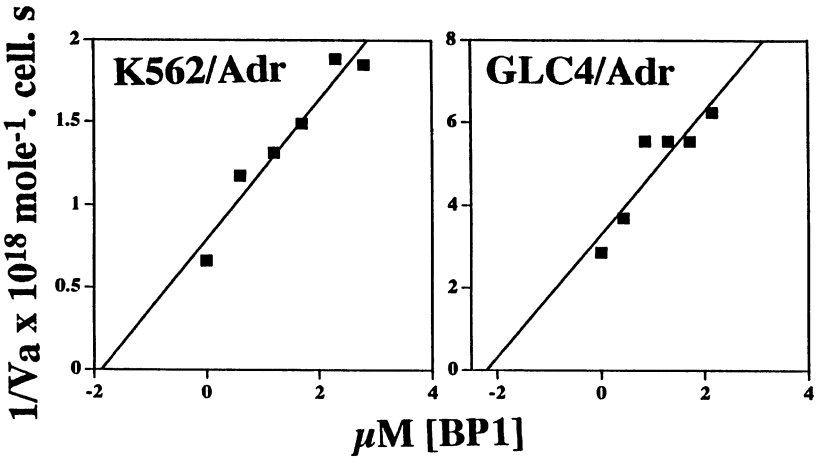


Fig. 9. Competition between pirarubicin and BP1 for efflux by the MRP1 and P-glycoprotein pumps. $1/V_a$ was plotted as a function of the concentration of BP1 free in the cytosol for K562/Adr and GLC4/Adr. V_a is the kinetics of the pump-mediated efflux of pirarubicin.

the straight line fitted with x-axis yielded $K_i = 1.5 \pm 0.5$ and 2.1 ± 0.8 μM for K562/Adr and GLC4/Adr cells, respectively.

4. Discussion

To date, three members of the superfamily of ATP-Binding Cassette (ABC) transporters, the 170-kDa P-glycoprotein, the 190-kDa MRP1 protein and the MRP2 protein, have been clearly shown to cause multidrug resistance, i.e. resistance to a spectrum of drugs with different structures and cellular targets (Cole et al., 1994; Leier et al., 1994; Borst et al., 1998; Zaman et al., 1994). Because of the importance of inherent and acquired multidrug resistance as obstacles to the success of anticancer chemotherapy for the treatment of disseminated cancers, great effort has been made to understand the mechanism of action and the physiological function(s) of these proteins, as well as to discover clinically effective inhibitors. Thus, one pharmacological approach to overcome drug resistance is based on the use of chemosensitizers to enhance the cytotoxicity of known antineoplastic agents (Wigler and Patterson, 1993; Lan et al., 1996; Sharom, 1997; Sumizawa et al., 1997; Wigler, 1999; Berger et al., 1999; Teodori et al., 1999). P-glycoprotein activity can be blocked by a wide variety of drugs (Sikic, 1993). Some of them have entered clinical trials. (Sikic, 1993; Sikic et al., 1997). In contrast to P-glycoprotein-mediated transport, MRP1 has been demonstrated to be inhibited by only a few agents (Manzano et al., 1996). The other approach to overcome cell resistance is to design and synthesize new non-cross-resistant drugs with physicochemical properties that enhance the uptake of the drug by resistant cells (Marbeuf-Gueye et al., 1999; Bassan et al., 1997; Kubota et al., 1998).

In this study, we show that benzoperimidine antitumor compounds, a new class of antitumor compounds, are able to almost completely reverse resistance in K562/Adr cells expressing P-glycoprotein, and in GLC4/Adr cells overexpressing MRP1. The cytotoxicity of BP1 and BP3 in sensitive cells was comparable to that of doxorubicin and pirarubicin (Table 1), while BP2 being a little less effective. However, the cytotoxicity of the three benzoperimidine compounds in resistant cells was also very high (RF ~ 1), whereas the cytotoxicity of doxorubicin in GLC4/Adr and K562/Adr was very low. The data were analysed by an ANOVA test and showed that the amount of drug incorporated inside both sensitive and resistant cells was comparable whatever the compound considered ($P > 0.05$), corroborating that cytotoxicity is closely related to the intracellular drug concentration (Pereira and Garnier-Suillerot, 1994).

In this study, one of our aims was to determine the reason for the high accumulation of drug inside resistant cells. Our data show that the three benzoperimidine derivatives interacted with DNA, either naked or in the nucleus. The interaction probably occurred through intercalation of the flat conjugated rings between the base pairs. Their affinity for DNA was comparable to that of anthracycline (doxorubicin, daunorubicin) for DNA. Our data also showed that these compounds are weak bases ($pK_a = 7.4$) which accumulate in acidic organelles, mainly lysosomes. Thus, the accumulation of drugs in cells, either sensitive or resistant, when the pH gradient was eliminated, i.e. in energy deprived cells or in cells incubated with FCCP, was significantly less than in ATP-rich cells ($P < 0.05$). Now, the very important point is that the uptake kinetics of these compounds were very rapid, similar to those of cells in which the plasma membrane had been previously permeabilized with Triton X-100—i.e. higher than $\sim 20 \times 10^{-18}$ mole cell^{-1} s^{-1}. The observation that the drug accumulation was the same in both sensitive and resistant cells could suggest that the drugs were not recognized by the transporter proteins and therefore, not pumped out. This has been already claimed several times in the case of some

anthracycline derivatives (Gros et al., 1992). However, we have recently shown that this is not true and that when the rate of transmembrane transport of anthracycline is high enough, the efflux mediated by the protein transporter is not able to keep pace with it (Marbeuf-Gueye et al., 1999). The protein transporter essentially operates in a futile cycle and the resistance factor approaches 1. This does not mean, however, that when the resistance factor is close to 1, the anthracycline is not transported by the pump. A strictly analogous phenomenon occurs in the case of benzoperimidines: the observation that the accumulation of the drugs was the same in both sensitive and resistant cells does not mean that the molecules were not pumped out. This was shown by the indirect observation that the P-glycoprotein- and MRP1-mediated efflux of pirarubicin, a well-known substrate of these transporters, was inhibited by BP1, the inhibitory constants being 1.5 and 2.1 μM for P-glycoprotein and MRP1, respectively. This strongly suggests that BP1 is transported by the two transporters with $K_m \sim 2$ μM. This value compares with that observed for most of anthracyclines ($K_m = 0.5–2$ μM).

In MDR cell lines, resistance is mainly due to the presence of a pumping system and, therefore, the resistance factor should theoretically be given as the ratio of internal drug concentrations at the IC_{50} measured when the pump is active (Stein, 1997). Taking into account that at steady state the kinetics of influx are equal to the kinetics of efflux and that the passive influx and efflux coefficients are equal, the resistance factor can be expressed as $RF = 1 + k_a/k_+$, where k_+ and k_a are the kinetics constants for passive and active efflux, respectively (Marbeuf-Gueye et al., 1999). In the case of the three new compounds studied here, RF tended towards 1, not because k_a tended towards 0, but because k_+ was very high. Of course, in the clinic, resistance is due to more factors than increased efflux alone.

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