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Report

Doxorubicin–peptide conjugates overcome multidrug resistance

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A well-known mechanism leading to the emergence of multidrug-resistant tumor cells is the overexpression of P-glycoprotein (P-gp), which is capable of lowering intracellular drug concentrations. To overcome this problem, we tested the capability of two peptide vectors that are able to cross cellular membranes to deliver doxorubicin in P-gp-expressing cells. The antitumor effect of peptide-conjugated doxorubicin was tested in human erythroleukemic (K562/ADR) resistant cells. The conjugate showed potent dose-dependent inhibition of cell growth against K562/ADR cells as compared with doxorubicin alone. Doxorubicin exhibited IC_{50} concentrations of 65 μM in the resistant cells, whereas vectorized doxorubicin was more effective with IC_{50} concentrations of 3 μM. After treatment of the resistant cells with verapamil, the intracellular levels of doxorubicin were markedly increased and consequent cytotoxicity was improved. In contrast, treatment of resistant cells with verapamil did not cause any further enhancement in the cell uptake nor in the cytotoxic effect of the conjugated doxorubicin, indicating that the conjugate bypasses the P-gp. Finally, we show by the *in situ* brain perfusion method in P-gp-deficient and competent mice that vectorized doxorubicin bypasses the P-gp present at the luminal site of the blood–brain barrier. These results indicate that vectorization of doxorubicin with peptide vectors is effective in overcoming multidrug resistance. [© 2001 Lippincott Williams & Wilkins.]

Key words: Blood–brain barrier, doxorubicin, multidrug resistance, peptide vector.

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Introduction

The resistance of some human tumors to multiple chemotherapeutic drugs is a major reason for the failure of cancer therapy. Limited uptake or increased drug excretion out of tumor cells is often noted when tumors acquire resistance to anticancer drugs. The mechanism underlying chemoresistance has been partly associated with overproduction of the drug-efflux pump, called gp 170 or P-glycoprotein (P-gp).[1,2] This protein functions as an ATP-driven pump capable of removing the drug from the cytoplasm and lowering intracellular drug concentrations.[2] Another related, but non-P-gp, multidrug resistance-associated protein (MRP) was also shown to be responsible for the reduced accumulation of drugs in tumor cells.[3] Doxorubicin has been shown to be transported out of tumor cells by either the P-gp[2,4] or the MRP.[3] The P-gp was also shown to be present at the luminal site of the endothelial cells of the blood–brain barrier (BBB).[5]

To circumvent multidrug resistance, most experimental and clinical efforts have attempted to block the efflux activity of P-gp.[6–9] Doxorubicin was given in combination with P-gp inhibitors: cyclosporine,[10] verapamil,[11] tamoxifen[12] and PSC 833.[13] However, if such drug combinations are effective *in vitro*, the high concentration of P-gp inhibitors necessary to overcome drug efflux limits their clinical application. Furthermore, co-administration of anticancer drugs and P-gp modulators may alter anticancer drug pharmacokinetics leading to an exacerbation of anticancer drug toxicity.[14] Some studies have focussed on new analogs of antitumor drugs whose uptake is not affected by P-gp.[4,15–17] An alternative strategy was developed to improve the action of various cancer drugs by linking them to various macromolecule carriers. The widely used doxorubicin has been

associated or conjugated to various molecules including transferrin, α-fetoprotein, dextran, liposomes, microspheres and polymers.[18–27] Although these molecules led to various beneficial effects including increased *in vitro* efficacy, the large size of some of them limits their clinical application.

In this study, we have linked doxorubicin to peptide vectors. The Pegelin and Penetratin peptides have been shown to translocate efficiently through biological membranes, thus providing the basis for the development of new peptide-conjugated drugs that cross cell membranes. The Penetratin peptides are derived from the transcription factor Antennapedia[28] and the region of the homeodomain of Antennapedia responsible for internalization has been mapped to its third helix.[29] A 16-amino-acid-long peptide corresponding to the third helix was found to translocate efficiently across biological membranes. Pegelin peptides (such as SynB1) are derived from natural peptides called Protegrins.[30,31] They are thought to form an antiparallel β-sheet, constrained by two disulfide bridges.[32] Replacement of the four cysteines with serines leads to linear peptides (Pegelin) that are able to cross efficiently cell membranes without any cytolytic effect.

In this report, we investigated the therapeutic efficacy of the peptide-conjugated doxorubicin on the growth of multidrug-resistant human K562 cells. We also examined the ability of the conjugated doxorubicin to circumvent the efflux activity of the P-gp present at the level of the BBB.

Materials and methods

Cell lines

K562 and K562/ADR cell lines were supplied by the ATCC (Rockville, MD) and cultivated as recommended by the supplier. The resistant K562/ADR subline was derived from K562 cells by a series of step selections for doxorubicin resistance. Culture medium (RPMI 1640, DMEM) purchased from Gibco (Cergy Pontoise, France) was supplemented with 10% heat-inactivated fetal calf serum (Gibco) and 1 mM HEPES. Incubations with free or vectorized doxorubicin were performed in culture medium exclusively composed of Optimem (Gibco).

Animals

The P-gp-deficient (mdr1a [−/−], 30–40 g, 6–8 weeks old) or competent (mdr1a [+/+], 30–40 g, 6–8 weeks old) CF-1 mice were bred from genotyped mice provided by Charles River (Wilmington, MA). Animals were maintained under standard conditions of temperature and lighting, and had free access to food and water. The research adhered to the ethical rules of the French Ministry of Agriculture for experimentation with laboratory animals (law no. 87-848).

Synthesis

Peptide synthesis. Peptides were assembled by conventional solid-phase chemistry using a 9-fluorenylmethoxycarbonyl/tertiobutyl (Fmoc/tBu) protection scheme.[33] The crude peptides were purified on preparative C_{18} reverse-phase HPLC after trifluoracetic acid cleavage/deprotection. The purity of peptides was assessed by C_{18} reverse-phase analytic HPLC (Beckman, Fullerton, CA; Gold) and MALDI-MS (Perseptive Biosystems, Framington, MA; Voyager Elite DE-RP). The peptide sequences were D-Penetratin (rqikiwfqnrrmkwkk, the amino acids are in D form, MW 2245 Da) and SynB1 (RGGRLSYSRRRFSTSTGR, MW 2099 Da).

Dox–D-Penetratin synthesis. Doxorubicin hydrochloride (1 molar equivalent; Fluka, Bushs, Switzerland) was suspended in dimethylformamide (DMF) containing diisopropylamine (DIEA) (2 molar equivalents; Fluka). *N*-Hydroxy-succinimidyl-maleimido-propionate (1 molar equivalent; Fluka) was added and incubated for 20 min. The thiol-containing peptide (as N-terminal 3-mercaptopropionic acid solubilized in DMF) was then added to this reaction mixture, followed by 20 min incubation. The reaction mixture was then injected on a C_{18} reverse-phase preparative HPLC column for purification. The acceptance criteria for the peptide and conjugates was HPLC purity of $>98\%$ at 215 and 480 nm in accordance with the molecular weight and fragmentation pattern for mass spectrometry. The molecular weight was found to be 3005 Da.

Dox–SynB1 synthesis. Doxorubicin hydrochloride (1 molar equivalent; Fluka) was suspended in DMF containing DIEA (2 molar equivalents; Fluka). Succinic anhydride (1 molar equivalent; Fluka) dissolved in DMF was added and incubated for 20 min. The resulting doxorubicin hemisuccinate was activated by addition of benzotriazol-1-yl-oxopyrrolidinephosphonium Hexafluorophosphate (1.1 molar equivalent; Novabiochem, Läufelfingen, Switzerland) dissolved in DMF. The peptide was then added to the reaction mixture after 5 min of activation and left for another 20 min for coupling. Further processing and purity check of the conjugate was performed as described above. The molecular weight was 2723 Da. The

radiolabeling of Dox–SynB1 was carried out as described in Rousselle *et al.*[34] The specific activity was (55 mCi/mmol, 2.04 Tbq/mol).

Drug transport kinetics

K562 and K562/ADR cells were diluted at 3×10^5 cells/ml 1 day before the experiment. Cell–drug association was measured by flow cytometry using a FACScan (Becton Dickinson, Mountain View, CA). Free and coupled doxorubicin were incubated with cells (5×10^5 cells/ml) in Optimem medium at 37°C for various periods of time (final volume was 0.5 ml). Cells were washed twice and resuspended in 0.5 ml of ice-cold PBS for FACS analysis. The samples were excited at 488 nm and fluorescence of doxorubicin was measured at 575 nm. A histogram of fluorescence intensity per cell (1×10^4) was obtained and the calculated mean of this distribution was considered as representative of the amount of cell-associated drug.

Confocal microscopy

K562/ADR cells were incubated with vectorized doxorubicin at a concentration of 25 μM. After 30 min incubation, the cells were washed and treated for analysis by confocal microscopy. We used a BioRad (Hercules, CA) 1024 CLSM system. This beam scanning system uses a Nikon Optiphot II upright microscope and an argon–krypton ion laser (15 mW) with an emission line at 488 nm. Either $\times 60$ (1.4NA) or $\times 20$ (0.75NA) objectives planapochromatiques (Nikon) were used. Images were collected sequentially to avoid cross-contamination between the fluorochromes. Series of optical sections were collected and projected onto a single image plane in the Laser Sharp 1024 software and processing system. Images were scanned at 512×512 or 1024×1024 pixel resolution.

Drug sensitivity assay

A colorimetric assay using MTT (3-(4.5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazorium bromide) was used to assess cytotoxicity of free and conjugated doxorubicin. The conditions of the assay allowed the measurement of cytotoxic effect on exponential growing cells. Briefly, cells were seeded at 10^4 cells/well in 96-well plates 24 h before incubation with the drug. After addition of increasing concentrations of either free or vectorized doxorubicin, the cells were grown for 48 h. After this time, 0.5 mg/ml MTT (Sigma, St Louis, MO) was added in each well and the plates were incubated for another 4 h at 37°C. Medium was removed and a DMF/SDS solution was added to each well. After dissolution of the formazan crystals, absorbance at 550 nm was determined using a microplate MR5000 auto-reader (Dynatech, Guyancourt, France). The IC_{50}, the drug concentration which decreases cell survival by 50% compared with control (drug-free) cultures, was determined by plotting control absorbance against log drug concentration. Each experiment was carried out six times.

The fold reversal factor was calculated using mean IC_{50} values as follows: fold-reversal=IC_{50} (doxorubicin − modulator)$_{ADR}$/IC_{50} (doxorubicin+modulator)$_{ADR}$.

In situ mouse brain perfusion study

The *in situ* brain perfusion in P-gp-deficient [mdr1a (−/−)] and competent [mdr1a (+/+)] mice was carried out as described in Dagenais *et al.*[35] Briefly, the right common carotid of anesthetized mice was exposed and ligated at the level of the bifurcation of the common carotid artery. Brains were perfused for 60 s at a calibrated flow rate of 2.5 ml/min. The perfusate consisted of a Krebs-bicarbonate buffer warmed at 37°C and gassed with 95% O_2 and 5% CO_2 for pH control (7.4) containing either [^{14}C]doxorubicin or [^{14}C]Dox–SynB1. At the end of the perfusion time, the mouse was decapitated and total radioactivity in the brain was determined. Doxorubicin uptake was expressed as a single-time-point, unidirectional transfer constant (K_{in}). Briefly, calculations were accomplished as described[36] from the relationship $K_{in}=(Q_{tot}-V_v \cdot C_{pf})/(T \cdot C_{pf})$, where Q_{tot} is the measured quantity of [^{14}C]Dox in brain (vascular and extravascular) at the end of the experiments, V_v is the cerebral vascular volume, C_{pf} is the perfusion fluid concentration of [^{14}C]Dox and T is the perfusion time (in s). V_v was evaluated by the sucrose space and calculated by the ratio between radioactivity of [^{3}H]sucrose (expressed in d.p.m. of sucrose per g of brain) and the perfusate sucrose concentration.

Results

DXR sensitivity of K562/ADR cells

As expected, continuous exposure to doxorubicin for 48 h was found to be much less cytotoxic to resistant cells (K562/ADR) than to sensitive cells (K562) (Figure 1). The 50% inhibitory concentration (IC_{50}) for doxorubicin in K562/ADR was 65 ± 7 μM, whereas the IC_{50} of the parental cell was 0.42 ± 0.06 μM (Table 1). Flow cytometry was then used to quantitate the cellular accumulation of

Table 1. Determination of cytotoxicity of free and vectorized doxorubicin

	IC_{50} values (μM) (mean $\pm$ SD)	
	K562	K562/ADR
Doxorubicin	0.42 ± 0.06	65 ± 7
Dox–D-penetratin	2.6 ± 0.94	3 ± 1.4
Dox + D-penetratin	0.53[a]	45 ± 5
Dox–SynB1	21.7 ± 2.7	21 ± 1.8
Dox + SynB1	0.6[a]	50 ± 4.2

Cells were cultured for 48 h with increasing drug concentrations. Cell viability was assessed as described in Materials and methods. IC_{50} values were calculated from complete dose–response curves. The values are means $\pm$ SD from three independent experiments.
[a]Values were obtained from a single experiment.

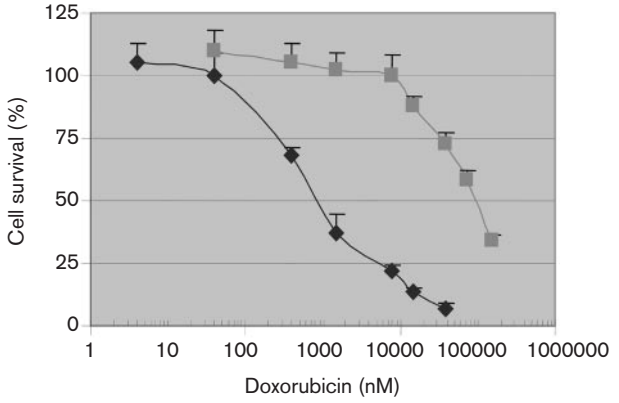

Figure 1. Dose–response curves of the K562 (■) and the resistant K562/ADR (◆) cell lines towards doxorubicin by continuous exposure for 48 h. Cytotoxicity was expressed as survival rate versus equivalent concentrations of doxorubicin. Results are means $\pm$ SD of sixplicate experiments. IC_{50} values are shown in Table 1.

doxorubicin in both sensitive and resistant cells. FACS measurements were carried out during 90 min incubation since the cell viability at this time point was greater than 90% in both cell types. At later times, the viability of the cells was too low to determine cellular drug accumulation. As shown in Figure 2, the uptake of doxorubicin at 5 μM in K562 cells was higher than in resistant cells.

Cytotoxic effect of doxorubicin conjugates

We assessed the cytotoxic activity of Dox–D-penetratin and Dox–SynB1 conjugates to K562/ADR cells. Both doxorubicin conjugates showed a potent and dose-dependent growth-inhibitory effect against K562/ADR cells (Figure 3). The IC_{50} concentrations for Dox–D-penetratin and Dox–SynB1 were 3 ± 1.4 and 21 ± 1.8 μM, respectively (Table 1).

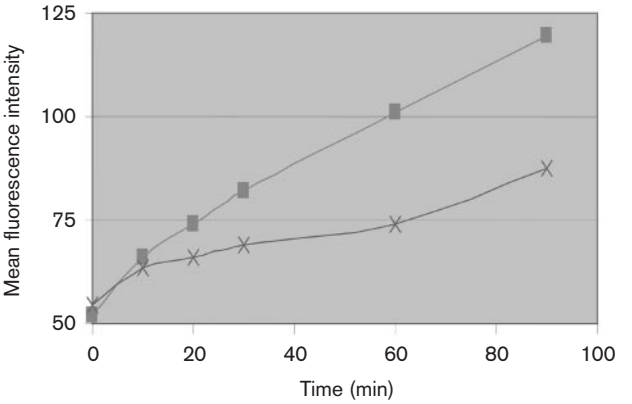

Figure 2. Time-dependent accumulation of doxorubicin in K562 (■) and K562/ADR (X) cells. After incubation of the cells with 5 μM doxorubicin, the internalized drug was measured by flow cytometry. Results are means $\pm$ SD of triplicate experiments.

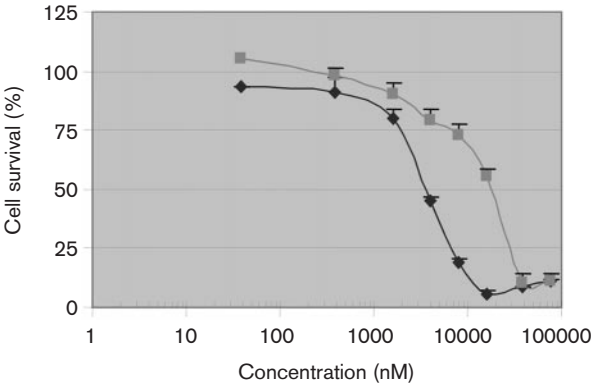

Figure 3. Cytotoxicity of vectorized doxorubicin against K562/ADR cells. Cytotoxicity was expressed as survival rate versus equivalent concentrations of vectorized doxorubicin. Results are means $\pm$ SD of sixplicate experiments. IC_{50} values are shown in Table 1. Dox–D-penetratin (◆); Dox–SynB1 (■).

The cytotoxic effect of doxorubicin conjugates in the K562 parent cell line was much less than the free doxorubicin. The IC_{50} concentrations for free doxorubicin, Dox–D-penetratin and Dox–SynB1 were 0.42 ± 0.06, 2.6 ± 0.94 and 21.7 ± 2.7 μM, respectively. Importantly, the IC_{50} values for the two conjugates were similar in both sensitive and resistant cells. Contrary to free doxorubicin, cell uptake of Dox–D-penetratin and Dox–SynB1 was similar in both sensitive and resistant cells (Figure 4). These data indicate a crucial difference between the mechanism of uptake of free and vectorized doxorubicin.

The conjugation of doxorubicin to the peptide vector was required to improve the cytotoxic effect of

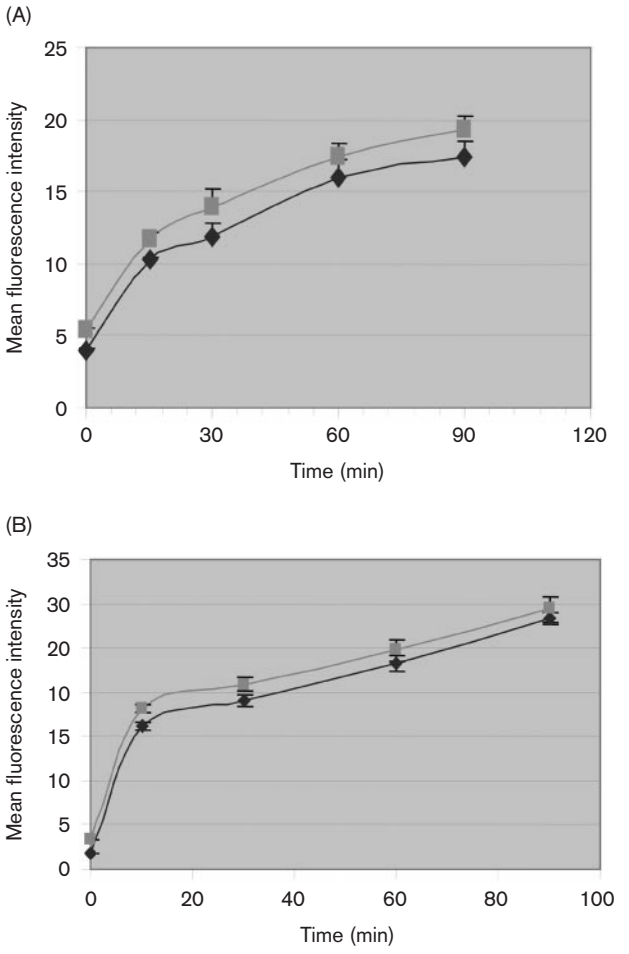

Figure 4. Time-dependent accumulation of vectorized doxorubicin in K562 (◆) and K562/ADR (■) cells. After incubation of the cells with 5 μM vectorized doxorubicin, the internalized drug was measured by flow cytometry. Results are means ± SD of triplicate experiments. (A) Dox–D-penetratin; (B) Dox–SynB1.

the drug since simultaneous addition of the vector and doxorubicin did not result in a significant enhancement of the cytotoxic activity of the drug (Table 1).

Intracellular distribution

The intracellular distribution of Dox–SynB1 and Dox–D-penetratin was investigated in K562/ADR cells using confocal microscopy. Resistant cells were incubated with coupled doxorubicin for 30 min, and the internalization and distribution was analyzed by confocal microscopy. This time point was chosen in order to have a cell viability of greater than 80%. At later times, the cell viability was too low. Figure 5 shows a significant distribution of Dox–D-penetratin through the cytoplasm and nucleus. By contrast, the

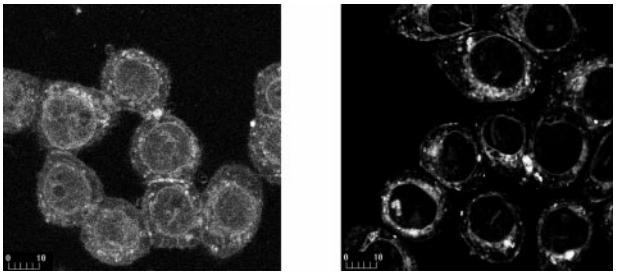

Figure 5. Analysis of drug distribution by confocal microscopy. K562/ADR cells were incubated with vectorized doxorubicin at a concentration of 25 μM. After 30 min incubation, the cells were washed and analyzed by confocal microscopy as described in Materials and methods.

distribution of Dox–SynB1 was detectable mostly in the cytoplasm and the perinuclear region. A very faint fluorescence in resistant cells was detected for free doxrubicin (data not shown). The slight enhanced cytotoxicity observed for Dox–D-penetratin compared to that of Dox–SynB1 could be due to this difference in intracellular biodistribution.

Effect of verapamil

Verapamil is able to block the export of molecules from the cell by the P-gp. Therefore, we determined the effect of verapamil on the accumulation and cytotoxicity of the free and coupled doxorubicin using K562/ADR cells. Simultaneous treatment of the cells with doxorubicin and 10 μM verapamil led to a marked increase in the accumulation and cytotoxicity of doxorubicin, as compared with that in the cells treated without verapamil (Figure 6A and Table 2). Verapamil lowered the IC_{50} values of doxorubicin in K562/ADR from 60 to 2.9 μM with a fold-reversal factor of 20 (Table 2). In contrast, the treatment of K562/ADR cells with verapamil did not induce any increase in accumulation nor in cytotoxicity of vectorized doxorubicin as compared with that in cells treated without verapamil (Table 2 and Figure 6). Treatment of K562/ADR cells with 10 μM verapamil alone did not show any cytotoxic activity (data not shown). These results indicate that the mechanism of uptake of vectorized doxorubicin does not depend on the presence of P-gp.

In situ brain perfusion in mice

We then investigated whether the vectorized doxorubicin is able to bypass the P-gp *in vivo*. Recently, we have developed an *in situ* mouse brain perfusion model[35] and shown that the brain uptake of vectorized

Table 2. Effect of verapamil (VPL) on IC_{50} values in K562/ADR cells

	IC_{50} values (μM) (mean $\pm$ SD)	
	K562/ADR	
	−VPL	+VPL
Doxorubicin	60 $\pm$ 3.6	2.9 $\pm$ 0.14
Dox–D-penetratin	2.8 $\pm$ 0.28	2.6 $\pm$ 0.56
Dox–SynB1	18 $\pm$ 1.5	19 $\pm$ 1.2

Cells were cultured for 48 h with increasing drug concentrations either in the absence or presence of 5 μM VPL. Cell viability was assessed as described in Materials and methods. IC_{50} values were calculated from complete dose–response curves. The values are means $\pm$ SD from three independent experiments.

doxorubicin was significantly higher than that of free doxorubicin. We applied this *in situ* mouse brain perfusion method to evaluate the BBB penetration of free and vectorized doxorubicin in both P-gp-deficient (mdr1a [−/−]) and competent (mdr1a [+/+]) mice.[35] P-gp has been identified as an efflux system on the luminal surface of the BBB.[5] Mice were perfused with either [^{14}C]doxorubicin or [^{14}C]Dox–SynB1. The distribution of doxorubicin to the brain of mdr1a [+/+] was quite limited, whereas a significant increase was observed in the mdr1a [−/−] CF-1 mice (65% after 120 s of perfusion) (Figure 7). On the other hand, no differences in the brain uptake of vectorized doxorubicin were observed between the wild-type mice and mdr1a [−/−] CF-1 mice. When doxorubicin was coupled to SynB1, an average of 30-fold increase in brain uptake was observed. This implies that contrary to free doxorubicin, vectorized doxorubicin is not recognized by the P-gp.

Discussion

Multidrug resistance is often the result of amplification of the MDR-1 gene leading to an overexpression of the P-gp, which is capable of pumping out cytotoxic drugs from cellular cytoplasm.[37,38]

The discovery that synthetic peptides derived from natural peptides can be used successfully to deliver biologically active substances inside live cells[28] has provided the basis for developing new effective strategies for drug delivery across cellular membranes. For this reason we have coupled the anticancer drug doxorubicin to two different peptides: D-penetratin and SynB1, which were expected to increase the uptake of doxorubicin to resistant cells.

In the present study, we have demonstrated that coupling doxorubicin with peptide vectors efficiently increased its cytotoxicity activity against doxorubicin-resistant K562/ADR cells. Coupling of doxorubicin

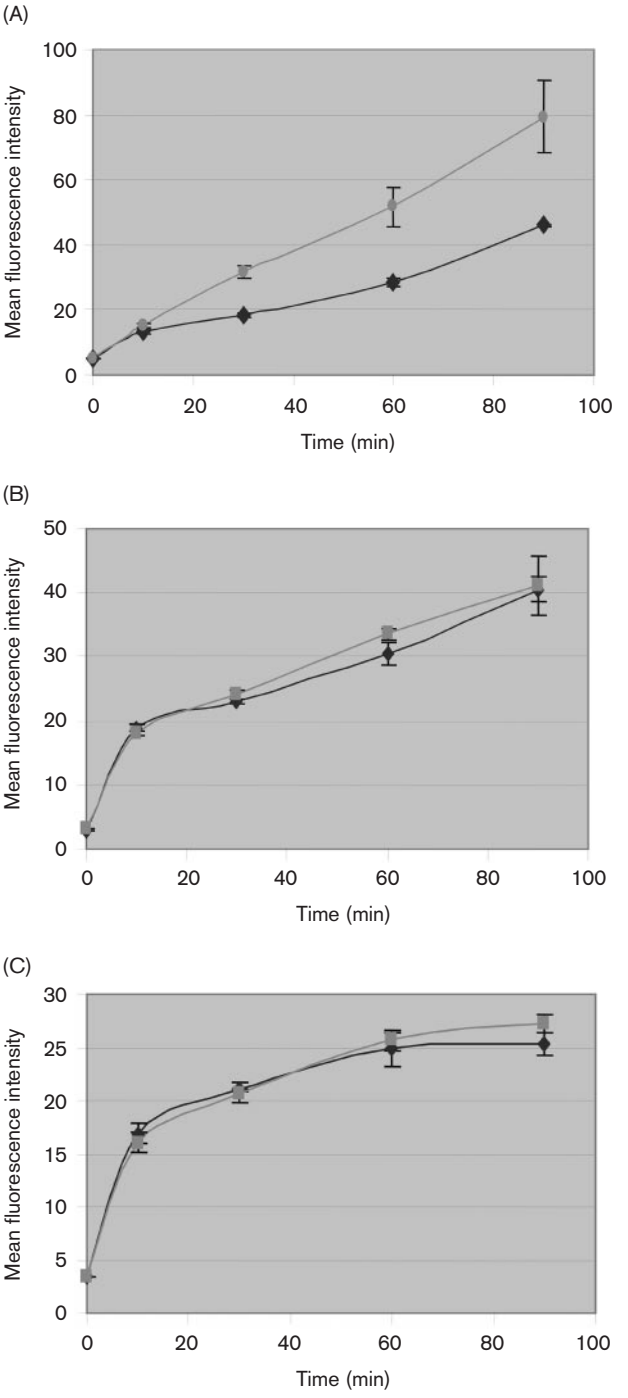

Figure 6. Effect of verapamil on drug accumulation in K562/ADR cells. Incubation was carried out in the presence (■) or absence (◆) of 10 μM verapamil. The internalized drug was measured by flow cytometry. Results are means $\pm$ SD of triplicate experiments. (A) Doxorubicin; (B) Dox-D-penetratin; (C) Dox–SynB1.

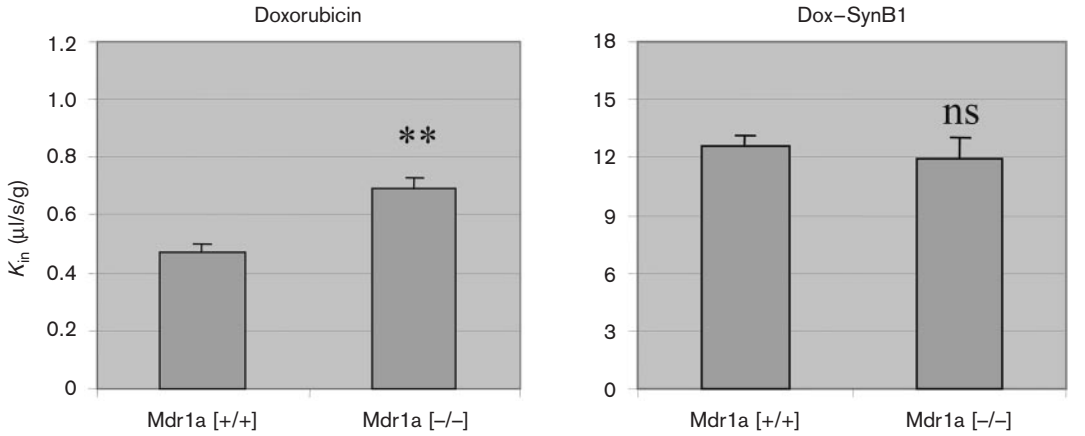

Figure 7. Unidirectional transfer constants (K_{in}) for [^{14}C]doxorubicin and [^{14}C]Dox–SynB1 uptake in the right hemisphere of wild-type and mdr1a [−/−] mice. The mice were perfused for 60 s with 3 µM of doxorubicin or 0.5 µM of Dox–SynB1. Each bar represents a mean ± SEM for n=5 animals. $^{**}p<0.01$; NS: not significant.

with D-penetratin led to an IC_{50} that was about 20-fold less than that of free doxorubicin (65 versus 3 µM). This increase in cytotoxic effect in K562/ADR cells might be explained by the translocation properties of these vectors as well as by the fact that vectorized doxorubicin is not recognized by the P-gp.

Quantitation of cellular accumulation by flow cytometry indicated that free doxorubicin at a concentration of 5 µM or higher had a lower uptake in resistant cells compared to that in sensitive cells. Nevertheless, if cells are incubated at low concentrations of doxorubicin (e.g. 1 µM), the drug uptake was similar in both K562 and K562/ADR cells, suggesting that the P-gp-mediated drug efflux would not occur unless a relatively high cellular drug concentration was reached. A similar observation has been previously reported.[39] Importantly, vectorized doxorubicin had a similar uptake in both sensitive and resistant cells, implying that the conjugate is transported across the cell by a mechanism that does not involve the P-gp.

Although the accumulation of doxorubicin in resistant cells was at a somewhat lower level than in sensitive cells, the cytotoxicity was about 160-fold higher in sensitive cells. This may be due to the fact that the total fluorescence measured by flow cytometry does not reflect the actual cellular drug content because anthracycline fluorescence is known to be quenched after intercalation between the base pairs of DNA.[40,41] Consequently, flow cytometry does not detect the fraction of drug mainly implicated in cell death (i.e. the fraction interacting with DNA). We noticed during this study that the doxorubicin fluorescence is also slightly quenched by the peptide vector. Therefore, we could not assess the comparison of the cellular uptake of free and vectorized doxorubicin. However, we have shown by the *in situ* brain perfusion method that by coupling doxorubicin with SynB1, a significant enhancement in brain uptake was observed.

In the parent sensitive cell line, cytotoxic effects of free doxorubicin were more potent than that of coupled doxorubicin. This somewhat reduced toxicity may be due to the covalent coupling between the drug and the vector. Actually, some loss of drug activity, as a consequence of the covalent linkage between drug and carrier, has been reported.[25,42] However, since vectorized doxorubicin is efficient in blocking the growth of resistant cells, this implies that conjugated doxorubicin is still able to exert its action. Actually, DNA binding studies demonstrated that the vectorized doxorubicin still intercalates with DNA base pairs (Drin *et al.*, unpublished results). The use of chemical linkages of different types and stability may critically influence the activity. In this study, the two different linkers used, succinate for SynB1 and thioether for D-penetratin, are not susceptible to hydrolysis. To potentiate cell killing of the conjugate it would be desirable to develop another linker strategy that will release the drug once it reaches its site of action. For that purpose, we have coupled doxorubicin to the SynB1 vector by a disulfide linker and preliminary experiments indicate that this conjugate is more potent than the one containing a succinate linker (data not shown).

Although, we found that the conjugate still intercalates with DNA, we cannot exclude that it may induce cell death by other mechanisms. It has been suggested that doxorubicin and anthracyclines might have several parallel effects involving both the membrane and nuclear matrix which are responsible

for the cytotoxic activity.[43–46] One possibility is that vectorized doxorubicin induces cell death by interfering with cell membrane.

To investigate whether or not P-gp caused efflux of the conjugate, the effect of verapamil on the accumulation and cytotoxicity of the vectorized drug was determined using K562/ADR cells. Verapamil is a calcium channel blocker that has been known for a long time to inhibit anthracycline efflux by direct interference with P-gp, thus increasing the intracellular accumulation of the drugs.[6,47] Co-treatment of the resistant cells with doxorubicin and verapamil caused a marked increase in intracellular drug level, leading to potent cytotoxicity against the cells. Consequently, verapamil lowered the IC_{50} values from 60 to 2.9 μM with a fold-reversal factor of 20. By contrast, the addition of verapamil to K562/ADR cells failed to cause any further increase neither in accumulation nor in the cytotoxicity of Dox–D-penetratin and Dox–SynB1 as compared with that in the cells cultured without verapamil. Our *in vivo* experiments also clearly show that the vectorized doxorubicin bypasses the efflux mechanisms of P-gp. First, we have observed in the present study that [^{14}C]Dox–SynB1 brain uptake in mdr1a [−/−] mice was identical to that observed in wild-type mice. Secondly, we recently reported[34] that pretreatment of rats with (±)-verapamil slightly increased the brain uptake of free doxorubicin but had no effect on the brain uptake of vectorized doxorubicin. All these results highlight a crucial difference between the mechanism of uptake of free and vectorized doxorubicin.

From the results of the present study, we conclude that vectorized doxorubicin accumulates rapidly in resistant cells with minimal efflux by P-gp, thereby resulting in potent cytotoxicity. The mechanism by which the vectorized doxorubicin is not recognized by the P-gp is still unknown. Priebe *et al.*[15] have shown that the amino group of doxorubicin plays an important role in the binding of the drug to P-gp. Since the coupling of the peptide vector occurred at this amino group, it is possible that the reduction of the basicity of the drug is responsible for the accumulation of the drug in resistant cells and, consequently, of its increased in cytotoxicity.

Doxorubicin has a broad spectrum of antitumor activity including a variety of human and solid tumors and leukemias. Its administration in humans and animals is known to cause an irreversible cardiotoxic effect with a major clinical handicap limiting its clinical use.[48,49] Therefore, the development of a carrier system that is able to decrease doxorubicin uptake in heart while increasing its antitumor activity will improve the therapeutic index of this drug. We have demonstrated recently that vectorization of doxorubicin with peptide vectors reduces significantly its accumulation in heart.[34] Furthermore, we observed that vectorization enhances significantly brain uptake of doxorubicin. This should be of great clinical interest because therapeutic use of doxorubicin and other cytotoxic agents for the treatment of glioblastomas and other tumors has been hampered by the presence of the P-gp in tumor cells. Our studies indicate that vectorized doxorubicin is active against multidrug-resistant cells *in vitro*, thus suggesting the possibility of utilizing this compound to overcome multidrug resistance. Towards this goal, future studies are planned to explore the antitumor potential of the conjugate *in vivo* using animals implanted with resistant tumors.

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