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Preclinical report

In vitro cellular accumulation and cytotoxicity of liposomal and conventional formulations of daunorubicin and doxorubicin in resistant K562 cells

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Previous investigations have indicated the possibility to circumvent multidrug resistance (MDR) by incorporation of an anthracycline into liposomes. We examined the *in vitro* cytotoxicity and cellular drug accumulation of the anthracyclines daunorubicin and doxorubicin compared with the commercially available liposomal formulations DaunoXome® and Caelyx® in human myelogenous leukemia K562 cells. The drug-sensitive parental K562/K line was compared with the P-glykoprotein (P-gp)-expressing cell lines K562/Dnr and K562/Vcr. Two cell lines with reduced levels of topoisomerase II (K562/Nov and K562/Ida) were also included. The cytotoxicity was determined by fluorometric microculture cytotoxicity assay and the cellular drug levels were determined by high performance liquid chromatograghy. There was a strong inverse correlation between P-gp levels and celluar drug accumulation ($\rho = -0.83$, $p = 0.04$) and cytotoxicity ($\rho = -0.95$, $p = 0.01$) of daunorubicin. Also the cytotoxicity of DaunoXome and doxorubicin was related to P-gp levels ($\rho = -0.96$, $p = 0.01$ and $\rho = -0.90$, $p = 0.07$, respectively). Caelyx did not show any cytotoxic effect due to impaired cellular uptake of the pegylated liposome. Regardless of the P-gp levels of the treated cells, DaunoXome showed the same cytotoxic effect despite lower intracellular accumulation (range 22–47%), compared with conventional daunorubicin. [© 1999 Lippincott Williams & Wilkins.]

Key words: Cellular drug accumulation, cytotoxicity, daunorubicin, doxorubicin, liposome, multidrug resistance.

Introduction

Clinical drug resistance is an important reason for the failure to cure patients with solid tumors or hematologic malignancies. Among numerous potential mechanisms of resistance, multidrug resistance (MDR) has been most extensively studied and proven to be of clinical importance, in acute myelogenous leukemia[1,2] and its reversal by inhibition of the P-glycoprotein (P-gp) appears feasible.[3,4]

Liposomes are microscopic vesicles consisting of a lipid bilayer membrane enclosing an aqueous space. Drugs can be internalized in the membrane or solubilized in the aqueous space depending on their polarity.[5] The incorporation of anti-cancer drugs into liposomes to minimize side effects and deliver the drugs to the tumor efficiently has been extensively studied, and some formulations are already used in the clinic.[6–9] It has been shown that liposomal incorporation of doxorubicin can inhibit the active drug efflux caused by P-gp.[10,11] A hypothesis is that the liposomes may inhibit P-gp-mediated drug efflux by avoiding a step of dissolution within the cell membrane, which is believed essential for this efflux.[10]

DaunoXome® (liposomal daunobicin) is a formulation of small unilamellar vesicles containing distearoyl glycerophosphotidylcholine plus cholesterol which increases the stability and the small size prolongs the systemic half-life of the liposomes.[12,13]

Caelyx® (liposomal doxorubicin) is a third-generation liposome, which is a small sterically stabilized unilamellar vesicle, with extensive systemic half-life due to the addition of polyethyleneglycol (PEG) to the surface.[14]

Many studies assessing liposomal drug effects have been performed in animal models and patients. Some studies of *in vitro* cytotoxicity and intracellular drug

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uptake were reported.[15-17] Our aim was to determine the cytotoxicity and cellular drug accumulation *in vitro* of liposomal daunorubicin and doxorubicin compared with the conventional drug formulations. We also wanted to investigate the impact of P-gp expression of the treated cells, on drug accumulation and cytotoxicity. A fluorometric microculture cytotoxicity assay (FMCA) was used to determine the cytotoxicity using human myelogenous leukemia K562/K cells and drug-resistant sublines, expressing high levels of P-gp or reduced levels of topoisomerase II. The intracellular drug uptake of daunorubicin and doxorubicin was determined by HPLC. The P-gp protein levels of all investigated cell lines were determined by Western blot.

Materials and methods

Cells and incubation for high-performance liquid chromatography (HPLC) analysis

Human myeloid leukaemia K562/K cells and three sublines (K562/Vcr30 and K562/Vcr150 induced by vincristin exposure, and K562/Dnr induced by daunorubicin exposure) expressing P-gp were used for the study. Also three sublines showing decreased topoisomerase II levels, K562/Nov induced by mitoxantrone exposure, and K562/Ida20 and K562/Ida60 induced by idarubicin exposure, were investigated. They were continously grown in medium RPMI 1640 (Gibco, Life Technologies, Paisley, UK) containing 10% FCS, 1% glutamine and 2% penicillin in a standard incubator. All the drugs had been removed for 2 weeks before starting experiments.

The cells (1×10^6/ml) were incubated with daunorubicin (Rhône-Poulenc Rorer, Helsingborg, Sweden), DaunoXome (NeXstar Pharmaceuticals, Wilrijk, Belgium), doxorubicin (Pharmacia & Upjohn, Stockholm, Sweden) and Caelyx (Schering-Plough Europe, Brussels, Belgium) at 1 μM for 1 h. The incubation was stopped by washing the cells twice with ice-cold PBS. After sonication for 10 s the samples were frozen at −70 C until HPLC analysis. All incubations were performed in duplicates. An additional incubation with all the four drugs at 1 μM for a period of 1–20 h and following HPLC analysis were performed in K562/K cells, and the cell viability after 1, 3, 7 and 20 h incubation was determined with Trypan blue staining.

Analysis of intracellular drug accumulation

Prior to extraction all samples were buffered by the addition of phosphate buffer pH 7.0. Sep-PAK C18 extraction columns (Waters, Boston, MA) were preactivited with 5 ml methanol and 9 ml phosphate buffer (pH 7.0, μ=0.1) before 100 μl sample solution and 100 μl internal standard was applied onto the column. After washing with 5 ml phosphate buffer, the columns were dried before elution with 4 ml of methanol into a tapered base centrifuge tube containing 100 μl despramine hydrochloride (25 μg/ml) in methanol. The eluates were initially evaporated to about 1 ml under flowing nitrogen. Then 400 μl of phosphoric acid (0.1 M) was added and the evaporation proceeded to about 200 μl. After the addition of 50 μl phosphoric acid, the tubes were vortexed for 1 min and centrifuged for 15 min (1700 *g*).

Then 50 μl of the samples was injected onto the chromatographoic column (4 μm Nova-Pak Phenyl; 120 × 5 mm; mobile phase: acetonitrile 35% in phosphoric acid, 0.01 M; flow rate: 0.8 ml/min). The elute was monitored with a fluorescence detector (Shimadzu RF-511, 501/600 nm). Quantification was based on peak area measurements and internal standardization using daunorubicin as standard for doxorubicin and idarubicin for daunorubicin. Results were expressed as drug amount per 10^6 cells.

Determination of cytotoxicity by FMCA

The FMCA is based on the measurement of fluorescence generated from the hydrolysis of flurescein diacetate (FDA) to fluorescein by cells. The assay was performed according to Larsson *et al.*[18] Briefly, cells were suspended in complete medium to 0.11×10^6/ml. Then 180 μl of cell suspension was seeded into the wells of 96-well microtiter plates prepared with drugs. Cell density was 2000 cells/well. Each drug at each concentration was tested with six samples. Six wells with cells but without drugs served as control and six wells with only medium as blank.

The plates were incubated for 72 h at 37 C. The incubation was stopped by centrifugation at 200 *g* for 5 min and the medium was removed by aspiration. After washing once with PBS, 100 μl FDA (10 μg/ml in PBS) was added to each well and kept for 45 min. The general fluorescence (excitation 480 nm) from each well was measured at 538 nm in a 96-well scanning fluorometer (Fluoroscan II; Labsystems, Finland). The cell survival (CS) of treated wells was expressed as percentage of control wells. The chemosensitivity of the cells was determined by the calculated lethal concentration (LC_{50}) which was defined as the drug concentration that results in 50% CS and derived by calculating the point where the dose-response curve

crosses the 50% LCS. The resistance factor (RF) was calculated as follows: RF=LC_{50} of resistant cells/LC_{50} of parental cells.

Western blot analysis of P-gp

Western blot was performed according to standard methods. In brief, membrane protein was prepared from 50×10^6 cells from each cell line. The cells were suspended in 0.5 ml lysis buffer (10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4, 2 mM phenylmethylsulfonylfluoride, 5 μg/ml leupeptin, 5 μg/ml pepstatin, 100 μg/ml DNase and 2 μg/ml RNase) and sonicated for 10 s at 50 W. After centrifugation for 10 min at 800 *g* at 4 C, the supernatant was collected and centrifuged for 30 min at 800 *g* at 4 C. The precipitate was suspended in 30-50 μl sample buffer (40 mM boric acid, 5 mM EDTA, 700 mM 2-mercaptoethanol, 150 mM sucrose, 10% SDS and 40 mM Tris-HCl, pH 8.64) and kept at −70 C. Determination of protein concentration was performed by the method described by Lowry *et al.*[19]

A sample of 4 μg membrane protein from each cell line was separated simultaneously on 4-15% polyacrylamide gradient gels by Phast System (Pharmacia Biotech, Uppsala, Sweden) and then transferred to nitrocellulose membranes (Amersham, Little Chalfont, UK). The membranes were blocked with 5% dry milk in TBST (137 mM NaCl, 0.05% Tween 20 and 20 mM Tris-HCl, pH 7.6) and subsequently incubated for 2 h with rabbit anti-MDR-1 polyclonal antibody diluted 1:50 in TBST. After washing, the membranes were incubated with donkey anti-rabbit IgG conjugated with horseradish peroxidase (Amersham) at a dilution of 1:1250 for 2 h. Autoradiograms were obtained by exposing the membranes to (enhanced chemiluminescence) (ECL) reagents and Hyperfilm-ECL (Amersham). Quantifications were performed by using Image Analysis System (Molecular Analyst Alias; BioRad, Hercules, CA) and the results were expressed as optical density (OD) × area (mm^2).

Calculations

The statistical calculations were performed by using a Macintosh computer equipped with StatView software (Abacus Concepts, Berkeley, CA). Spearman rank regression was used to assess the association between all the investigated parameters. The comparison between two groups of parameters was tested by the Mann-Whitney *U*-test. p values <0.05 were considered significant.

Results

The drug cytotoxicity in K562/K, K562/Vcr150, K562/Dnr, K562/Nov and K562/Ida60 cells is summarized in Table 1, and the dose-response curves are shown in Figure 1. The maternal K562/K cells were more sensitive to daunorubicin (LC_{50}=0.08 μg/ml) compared with doxorubicin (LC_{50}=0.32 μg/ml). There was no difference between the resistance factors of the liposomal formulation of daunorubicin, DaunoX-

Table 1. Cytotoxicity of daunorubicin, DaunoXome and doxorubicin in K562 cells

Drug	Cell line	LC_{50} (μg/ml)	RF (resistance factors)
Daunorubicin	K562/K	0.08	
Daunorubicin	K562/Vcr150	5.00	62.5
Daunorubicin	K562/Dnr	>5.00	>62.5
Daunorubicin	K562/Nov	0.32	4.0
Daunorubicin	K562/Ida60	0.22	2.8
DaunoXome	K562/K	0.07	
DaunoXome	K562/Vcr150	5.00	71.4
DaunoXome	K562/Dnr	>5.00	> 71.4
DaunoXome	K562/Nov	0.32	4.6
DaunoXome	K562/Ida60	0.28	4.0
Doxorubicin	K562/K	0.31	
Doxorubicin	K562/Vcr150	5.00	15.6
Doxorubicin	K562/Dnr	>5.00	>15.6
Doxorubicin	K562/Nov	0.32	1.0
Doxorubicin	K562/Ida60	0.31	1.0

K562/K is the parental cell line, and resistant sublines were induced by exposure to vincristine (Vcr), idarubicin (Ida) and mitoxantrone (Nov).

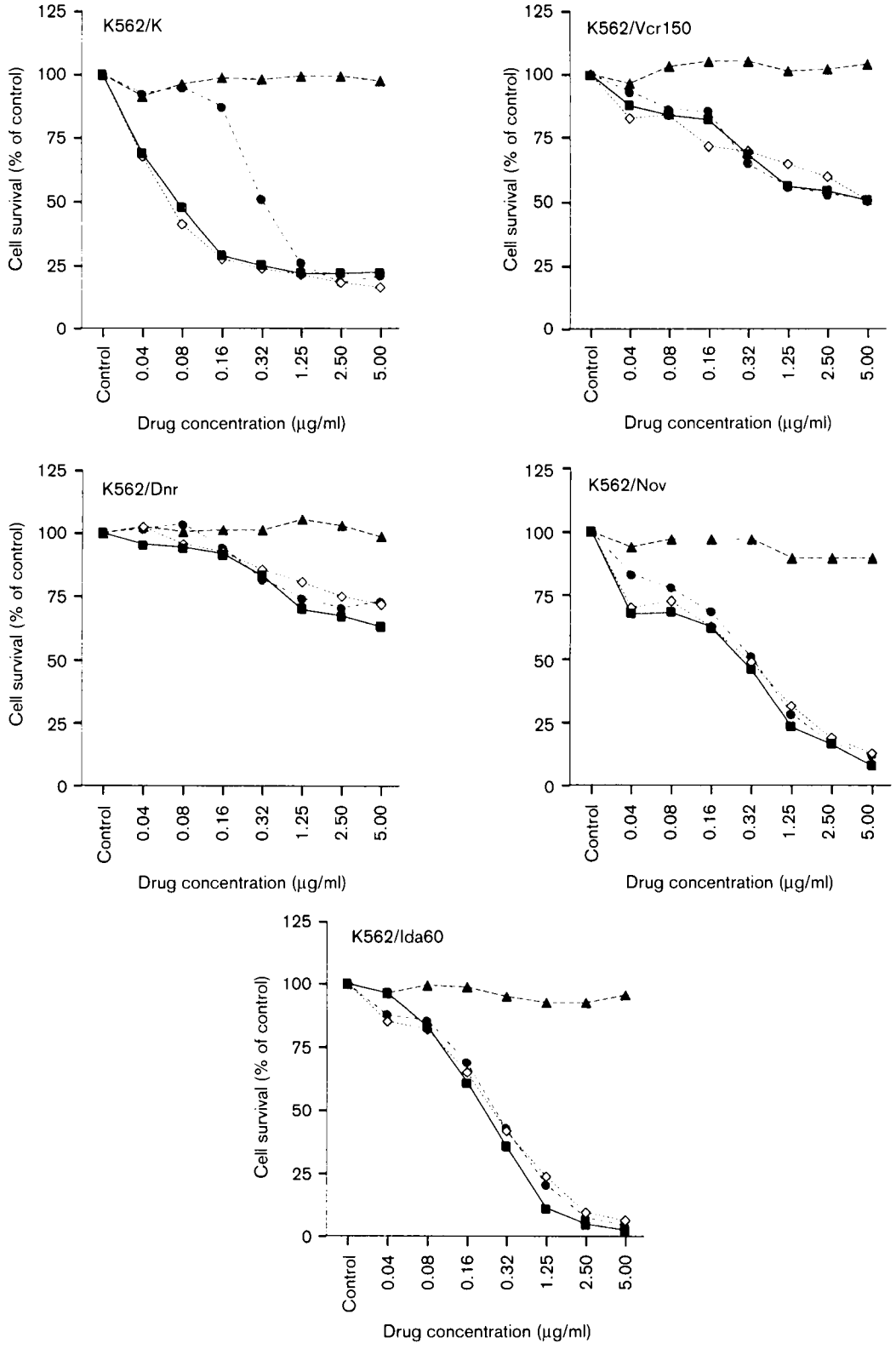

Figure 1. Cytotoxicity of daunorubicin (■), doxorubicin (●) and their liposomal formulations DaunoXome (◇) and Caelyx (▲) in K562 cells. Results are mean values of two or three independent experiments and expressed as the survival (%) compared with untreated control cells.

ome and the free drug. Caelyx was completely non-toxic in every cell line. In all resistant sublines there were no major differences between the cytotoxicity of daunorubicin, DaunoXome and doxorubicin, and the LC_{50} range was between 0.22 and greater than 5.00 μg/ml.

The intracellular drug accumulation after 1 h treatment with daunorubicin, DaunoXome, doxorubicin and Caelyx is shown in Figure 2. There was no intracellular accumulation of the pegylated liposome Caelyx. The non-pegylated liposome DaunoXome was accumulated to a considerably lesser extent than free daunorubicin (p=0.04, range 22-47%). Also, doxorubicin was taken up to a consideraby lesser extent than daunorubicin (p=0.01, range 12-81%) (Table 2).

Figure 3 illustrates the drug accumulation in K562/K cells following treatment with daunorubicin, DaunoXome, doxorubicin and Caelyx for a period of 1-7 h. K562/K cells demonstrated a time-dependent increase of drug accumulation of all drugs except Caelyx. At each time period, cells exposed to DaunoXome and doxorubicin showed lower accumulation than cells exposed to daunorubicin. The cell viability in all samples was greater than 90% at 3 h and greater than 85% at 7 h. At 12 and 20 h the viability of the cells was too low (55-78%) to determine cellular drug accumulation, except for cells incubated with Caelyx which were unaffected by the drug.

The P-gp levels determined in all the cell lines by Western blot are shown in Figure 4 and Table 2 shows the quantitive data. The resistant cells induced by daunorubicin exposure, K562/Dnr, had the highest P-

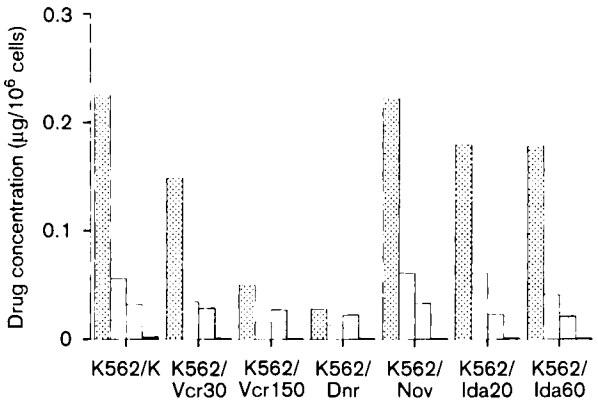

Figure 2. Intracellular drug accumulation after 1 h incubation with daunorubicin (▒), doxorubicin (□) and their liposomal formulations DaunoXome (■) and Caelyx (■) at 1 μM in K562 cells. Results are expressed as the amount of drug/10^6 cells, showing the mean of duplicate samples.

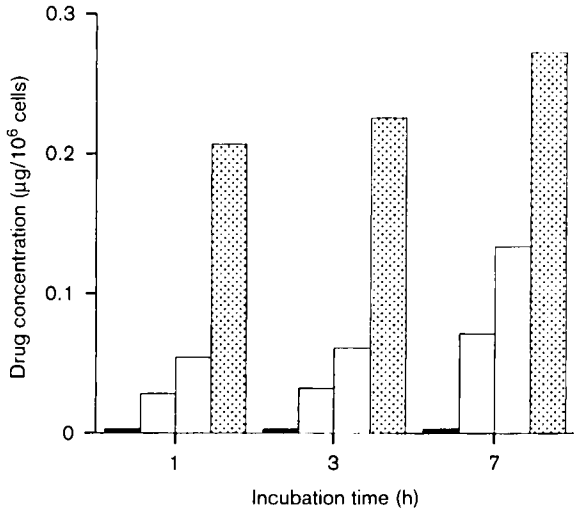

Figure 3. Intracellular drug accumulation in K562 cells after incubation with 1 μM daunorubicin (▒), doxorubicin (□) and their liposomal formulations DaunoXome (■) and Caelyx (■) for a period of 1–7 h. Results are expressed as the amount of drug/10^6 cells.

Table 2. P-gp levels and ratio of intracellular accumulation of doxorubicin (Dox) and DaunoXome compared with daunorubicin (Dnr) in K562 cells

Cell line	Pgp protein level (OD × mm^2)	Accumulation of Dox/accumulation of Dnr (%)	Accumulation of DaunoXome/accumulation of Dnr (%)
K562/K	0.00	14	22
K562/Vcr30	1.01	19	23
K562/Vcr150	13.32	54	30
K562/Dnr	20.78	81	47
K562/Ida20	0.13	15	27
K562/Ida60	0.63	13	34
K562/Nov	0.04	12	23

K562/K is the parental cell line, and resistant sublines were induced by vincristine (Vcr), idarubicin (Ida) and mitoxantrone (Nov).

gp level. The P-gp level in vincristine-selected K562/Vcr150 cells was also extensive. The cells growing in lower vincristine concentration, K562/Vcr30, expressed low Ppgp levels. There was no detectable P-gp in the mitoxantrone selected cell line K562/Nov, and a very faint band was visible in the idarubicin-selected cells K562/Ida20 and K562/Ida60.

Table 3 shows the relations between the determined parameters. There was a relationship between P-gp levels and intracelluar drug accumulation of daunorubicin ($\rho=-0.83$, $p=0.04$). The same trend was seen with DaunoXome, but not doxorubicin. The cytotoxicity of daunorubicin was correlated with both P-gp levels and drug accumulation ($\rho=-0.95$, $p=0.01$ and $\rho=-0.83$, $p=0.09$, respectively). The cytotoxicity of DaunoXome and doxorubicin was correlated only with P-gp levels ($\rho=-0.96$, $p=0.01$ and $\rho=-0.90$, $p=0.07$, respectively).

Discussion

The correlations between P-gp levels, drug accumulation and cytotoxicity of daunorubicin support that high expression of P-gp can induce decreased intracellular drug accumulation and result in decreased drug cytotoxicity. Doxorubicin was less accumulated than daunorubicin ($p=0.01$). No correlation was found between P-gp levels and doxorubicin accumulation, but the accumulation ratio of doxorubicin/daunorubicin is correlated with P-gp levels ($\rho=0.87$, $p=0.03$), suggesting that the accumulation of doxorubicin was less affected by the efflux function of P-gp compared with daunorubicin.

In vivo studies have shown that DaunoXome has activity against murine lymphoma[20,21] and Kaposi's sarcoma in AIDS patients,[22,23] and Caelyx was an active agent in advanced breast cancer[24] and AIDS-related Kaposi's sarcoma.[25] However, *in vitro* cytotoxicity studies indicated that both DaunoXome and Caelyx had reduced activity compared to free drugs in two myeloma cell lines (U266 and JJN3), one glioblastoma cell line (U251) and one lymphoma cell line (U937), and the reduction was greater for Caelyx.[15] In rat C6 glioblastoma cells, doxorubicin in different liposome formulations (all were unpegylated) had different cytotoxic effects; two formulations had similar cytotoxicity with the free drug, whereas another formulation was significantly less toxic and no reversal of resistance was found through liposomal encapsulation.[16] Other studies reported the reversal of MDR by liposomal doxorubicin. In HL-60/VCR leukemia cells that express P-gp, doxorubicin encapsulated with unpegylated liposomes had 2- to 3-fold higher accumulation and 5-fold increased cell sensitivity compared with the conventional drug.[17] In another resistant cell line HL-60R with P-gp expression, doxorubicin encapsulated with unpegylated liposomes had more cytotoxic effects than free doxorubicin.[26]

Figure 4. P-gp protein levels in K562 cells. Resistance was induced by vincristine (K562/Vcr30 and K562/Vcr150), daunorubicin (K562/Dnr), mitoxantrone (K562/Nov) idarubicin (K562/Ida20 and K562/Ida60).

Table 3. Correlations between P-gp protein level and drug cytoxicity and drug accumulation in K562 cells

Drug	P-gp and cytotoxicity	P-gp and accumulation	Cytotoxicity and accumulation
Daunorubicin	$\rho=-0.95$ $p=0.01$	$\rho=-0.83$ $p=0.04$	$\rho=-0.83$ $p=0.09$
DaunoXome	$\rho=-0.96$ $p=0.01$	$\rho=-0.66$ $p=0.11$	$\rho=-0.53$ $p=0.30$
Doxorubicin	$\rho=-0.90$ $p=0.07$	$\rho=-0.19$ $p=0.65$	$\rho=-0.15$ $p=0.76$

In the present study, daunorubicin and DaunoXome were equally potent in all five cell lines determined by the FMCA cytotoxicity assay (Figure 1). However, the intracellular drug accumulation of DaunoXome was 22-47% of the accumulation of free daunorubicin. How the liposomal daunorubicin could kill cells efficiently despite lower intracellular accumulation than the conventional daunorubincin could not be explained by our present results. It may suggest another mechanism adding to the intracellular cytotoxicity. Possibly, DaunoXome could interfere with the cell membrane and induce cell death. It has been suggested that anthracyclines have several parallel actions involving both the membrane and nuclear matrix, and both of them are responsible for the cytotoxic effects.[27] It was reported that the uptake of liposomal drugs was a result of release of free drug from liposomes and subsequent uptake.[28,29] The liposomal stability might cause only low amounts of drugs to be released into medium and taken up to cells during the short 1 h incubation for HPLC analysis. We prolonged drug incubation time to 20 h in K562/K cells. After treatment with doxorubicin, daunorubicin and DaunoXome for 12 h, cell viability was less than 80%. In this case the HPLC analysis of drug accumulation could not be trusted because of the increased drug binding to dead cells or cell fragments. Figure 3 illustrates drug accumulation in K562/K cells following treatment with all four drugs for a period of 1-7 h. At each time period, cells exposed to DaunoXome showed lower accumulation than cells exposed to daunorubicin, but the ratio of DaunoXome/daunorubicin increased from 22 to 49%.

Caelyx had neither intracellular accumulation nor cytotoxic effect in any cell line. It might be caused by its great stability due to its PEG moieties. It also suggests that *in vitro* incubation in regular cell medium is not feasible for determining the effects of the pegylated liposome. *In vivo* Caelyx treatment has resulted in increased accumulation of doxorubicin in animal tumors compared with free drug.[30]

Our results do not show circumvention of drug resistance caused by P-gp overexpression. However, the retained cytotoxicity of DaunoXome despite significantly decreased drug accumulation compared with free daunorubicin is an unexpected and interesting finding. The mechanism of this effect needs further investigation.

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