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Intracellular localization, vesicular accumulation and kinetics of daunorubicin in sensitive and multidrug-resistant gastric carcinoma EPG85-257 cells

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Abstract In the human gastric carcinoma cell line EPG85-257P (parent) induction of resistance to daunorubicin (DAU) was achieved by selection with stepwise increased concentrations of the drug. The new vairant was named EPG85-257DAU and was shown to overexpress the *mdr*1 gene product 170 kDa P-glycoprotein (P-Gp) as demonstrated by immunocytochemistry and *mdr*1-specific RT-PCR. To investigate the intracellular pathway of DAU the subcellular distribution of this autofluorescent drug was studied in the resistant cells and compared to its chemosensitive counterpart EPG85-257P. When sensitive cells were exposed to DAU the drug rapidly accumulated in the nucleus until cell death. No redistribution of DAU to the cytoplasm was observed. In resistant cells exposed to the drug DAU also accumulated in the nucleus but to a lesser extent than in parent cells. Following exposure, nuclear fluorescence was observed to decrease over a time period of up to 48 h. Six hours after DAU exposure formation of fluorescent vesicle formation started in the perinuclear region and increased continously. After 48 h nuclear fluorescence was no longer detectable and DAU was located exclusively in vesicles. During this period the vesicles moved from the region of origin to the cell periphery. A pulse chase experiment showed, that vesicles may contain DAU derived from the nucleus. Treatment of EPG85-257DAU cells with DAU in conjunction with the chemosensitizer cyclosporin A (CsA) increased nuclear fluorescence without impairing vesicle formation. Disruption of microtubules by nocodazole led to an accumulation of vesicles in the perinuclear region indicating that microtubules are involved in vesicular transport. Treatment of EPG85-257DAU cells with the actin disruptor cytochalasin B led to accumulation of vesicles in the cell periphery indicating that actin may be involved in exocytosis. Uptake and efflux of DAU and rhodamin (RH) were determined in sensitive and resistant cells using a fluorescence activated cell sorter. Uptake of both compounds was distinctly lower in resistant than in sensitive cells. When resistant cells preloaded for 2 h with RH subsequently were incubated in drug free medium the substance was rapidly released indicating transmembrane transport by P-Gp. In contrast, despite expression of P-Gp in resistant cells no considerable release of DAU was observed for up to 2 h under the same experimental protocol. This indicates that in resistant cells intracellular DAU at least in part may be inaccessible for P-Gp and that vesicular drug transport appears to contribute to DAU resistance by removing intracellular DAU via exocytosis.

Key words P-Glycoprotein · Multidrug resistance · Vesicle formation · Daunorubicin

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Introduction

Studies of chemoresistance in tumour cells have revealed the existence of several mechanisms for cell survival in the presence of cytostatic drugs (for review [4, 10, 16]). One of the most thoroughly investigated mechanisms is the expression of a transmembrane glycoprotein (P-glycoprotein; P-Gp) which serves as an efflux pump removing several types of cytostatic drugs from the cell. This has been named multidrug resistance (MDR) (for review see [3]). Other strategies of drug resistance involve alteration of enzyme activities, cytoskeletal proteins or intracellular distribution pattern (for review see [1, 2, 23]). In a mitoxantrone-resistant human gastric carcinoma cell line lacking P-Gp, vesicular transport of mitoxantrone has been identified as part of an excocytotic pathway to reduce intracellular drug concentration [11]. Vesicle formation also occurs in other drug-resistant cell lines [22, 29, 34] some of which express P-Gp. However, the importance of vesicular compartmentalization and transportation in drug resistane of P-Gp positive cells is still unclear.

The aim of the present study was to investigate the intracellular daunorubicin (DAU) distribution by means of fluorescence microscopy in a DAU-resistant *mdr*1-positive human gastric carcinoma cell line and its drug-sensitive counterpart. To elucidate a possible role of P-Gp and other mechanisms involved in vesicular transport we studied the effect of the chemosensitizer cyclosporin A (CsA), the tubulin disruptor nocodazole and the actin inhibitor cytochalasin B on vesicle formation.

To correlate differences in the intracellular distribution of DAU with the kinetics of the drug in sensitive and resistant cells we also determined uptake and efflux of DAU from both sensitive and resistant cells.

Materials and methods

Characterization of cell lines

EPG85-257 cells were grown in L-15 medium (Boehringer Mannheim) supplemented with 10% fetal calf serum (Gibco) as described previously [11]. Cells were made resistant by exposure to stepwise increased concentrations of daunorubicin (DAU) for ca. 1 year. This subclone was designated EPG85-257DAU. Resistant cells were maintained in medium containing 2.5 µg/ml DAU to assure the stability of resistance. The growth inhibition of daunorubicin (Farmitalia, Freiburg, FRG) was assessed by determination of the IC_{50} in the monolayer-proliferation assay as described previously [11]. Briefly, cells were seeded in multi-wellplates and were allowed to grow for three days at 37° C at a humidity of 95%. Following this pre-incubation period cells were exposed to varying concentrations of the drugs for 3 days. Cell proliferation was assessed by photometrical determination of DNA content. Cells removed before the addition of the drug were used as *pre-controls,* whereas cells grown without drugs for the entire period of time served as *post-controls*. IC_{50} was determined after plotting the net proliferation (growth with drug – pre-control) at each drug concentration as percent of growth of controls (post-controls – pre-controls) against the drug concentration on a semilogarithmic scale. The role of P-Gp was examined by incubating cells either with the drug alone or in conjunction with 1 µmM CsA to block P-Gp (CsA kindly donated by Dr. Ryffel, Sandoz AG, Basel, Switzerland). Immunocytochemistry of P-Gp was performed as described earlier [12].

RT-PCR for P-glycoprotein mRNA

For RNA extraction total RNA from EPG85-257P and EPG85-257DAU cells was prepared according to the protocol given by Chominski and Sacci [9]. The primers used were designed for cDNA resulting from human *mdr*1-mRNA [15]. For reverse transcription 1 µg of total RNA from EPG85-257DAU cells was incubated for 1 h at 37° C at a final volume of 10 µl in 1× reverse transcription buffer (50 mM TRIS-HCl, pH 8.9; 75 mM KCl; 3 mM $MgCl_2$; 10 mM dithiothreitol), 5 pmol of one of the specific primers, 2 nmol of each deoxynucleoside triphosphates (dATP, dTTP, dGTP, dCTP) and 10 U of AMV reverse transcriptase (Promega, Madison, Wis, USA [27]).

For PCR every 10 µl probe (reverse transcription mixture) was mixed with 10 nmol of each deoxynucleoside triphosphate, with 40 pmol of each primer and 1.5 U Taq-Polymerase (Gibco BRL, Eggelstein, FRG) in 1× amplification buffer (10 mM TRIS-HCl, pH 8.4; 50 mM KCl. 1.5 mM $MgCl_2$ and 20 µg/ml gelatine) with a final volume of 50 µl. The mixture was covered with 100 µl mineral oil to avoid evaporation during heating. The samples were placed in a thermal cycler (Perkin Elmer Cetus, Überlingen, FRG) and 40 cycles were performed, each consisting of 90 sec. at 91° C, 2 min at 66° C and 2 min at 72° C. The mineral oil was then removed with chloroformisoamyl alcohol (24:1) and the samples were stored at 5° C.

For restriction analysis and gel electrophoresis 12 µl of each sample was digested with 30 U of the restriction enzymes Pst I and Msp I and 1× restriction buffer (Gibco BRL, Eggenstein, FRG). The digestion products were precipitated and diluted in 5 ml water. A 2% agarose gel was prepared and the digested probes and 7 ml of the non-digested probes were separated. The pattern of the restriction analysis was in accordance with the pattern reported by Chen et al. [7].

Determination of drug distribution

Monitoring of drug distribution was achieved by first plating cells on glass slides and allowing them to grow for 3 days in drug-free medium. DAU was then added to the medium and slides were removed at the times indicated. A short rinse in phosphate-buffered saline (PBS) was followed by fixation in 4% paraformaldehyde in PBS. The rationale for using fixated cells was that autofluorescence of DAU was too low to be documented with an inverted photomicroscope. Control experiments using unfixed cells showed that the subcellular distribution of DAU was similar in fixed and fresh cells. Autofluorescence of DAU was observed under an Olympus BH2 microscope using an excitation wavelength of 535 nm. Micrographs were taken on Kodak TMAX 400 film at a magnification of 75× or 125×. For sake of clarity micrographs of sensitive cells were taken at an exposure time of 5 to 16 s, whereas in resistant cells exposure time was 2 min. In the pulse-chase experiment micrographs of cells after 48 h were recorded for 5 min to compensate for the low fluorescence. Micrographs recorded at the same exposure time in both resistant and sensitive cells proved to be not useful for showing the intracellular localization of the drug because of the high difference in drug accumulation between both cell lines.

The effect of different doses of DAU on subcellular distribution was investigated by incubating drug sensitive cells with 0.25 and 1 µg/ml DAU, while resistant cells were exposed to 0.25, 1 and 10 µg/ml DAU for 1, 6, 24 and 48 h. Because of complete and rapid destruction of sensitive cells by 10 µg/ml DAU this dose could not be applied (data not shown).

In the pulse-chase experiment the intracellular fate of DAU was monitored. Resistant cells were labelled with 10 µg/ml DAU for 2 h, then rinsed once with drug-free medium, and finally kept in this medium. The remaining concentration of DAU was not detectable by fluorescence microscopy. Cells were removed immediately before and 6, 24 and 48 h after medium exchange.

The effects of the resistance reversing compound CsA on DAU distribution was studied by adding 1 µM CsA to medium containing 10 µg/ml DAU. CsA was dissolved in Tween 80 and 10 mM ethanol and diluted in medium to a final concentration of 1 mM. To modify the function of the cytoskeleton 100 µg/ml nocodazole, or 5 µg/ml cytochalasin B both from Sigma, Deisenhofen, FRG) were added. Fresh stock solutions were prepared in dimethylsulphoximide (nocodazole) and ethanol (cytochalasin B). Vehicle concentration in the medium never exceeded 0.1%. Cells were removed after 24 h incubation and prepared for fluorescence microscopy as described above.

Fluorescence measurement

DAU uptake and efflux were measured in a fluorescence-activated cell sorter (FACS) using an excitation wavelength of 535 nm. Threshold sensitivity for fluorescence was 1 arbitrary unit (AU). Propidium iodide (40 µg/ml) was used as a marker for cell viability. For short-term uptake and efflux measurements resistant cells were incubated in suspension with 10 µg/ml DAU. Preliminary experiments using lower concentrations of DAU have shown, that in resistant cells the dose of 10 µg/ml led to an uptake of DAU, which was just sufficient to measure a decrease in fluorescence after removal of the drug. Fluorescence of 5,000 to 10,000 cells was

Table 1 IC_{50} of cytostatic drugs in sensitive EPG85-257P and daunorubicin-resistant EPG85-257DAU cells

Drug	IC_{50} in µg/ml culture medium[a]		Factor
	EPG85-257P	EPG85-257DAU	
Daunorubicin	0.0013+0.0002[b]	11.0+0.04	8460
+1 µM CsA		0.32+0.05	246
Adriamycin	0.016+0.001	20.0+0.3	1250
Epirubicin	0.0014+0.0003	8.0+0.7	5714

[a] Determined by the monolayer proliferation assay [11]
[b] Means+sd of three measurements

determined for a period up to 120 min. For determination of drug efflux, resistant EPG85-257DAU cells were incubated with 10 µg/ml DAU for 2 h, pelleted and resuspended in drug-free medium and subsequently measured for fluorescence for a period up to 120 min. In EPG85-257P cells the same protocol was applied except that 0.25 µg/ml DAU was used to avoid cell damage considering the higher uptake of sensitive cells. During the experiment viability decreased by 30 to 35% in both subclones.

As an alternative to fluorescent anthracyclines the fluorescent dye rhodamine 123 (RH) has previously been used as a marker for MDR cells [17]. However, this compound binds only to mitochondria in the cytoplasm [6], whereas anthracyclines bind to structures within the nucleus. In an attempt to investigate whether these different targets are associated with kinetic differences, we compared uptake and efflux of RH and DAU in both subclones. RH was used at a concentration of 10 µg/ml in both subclones with identical protocols as in the DAU experiment. Viability decreased by 35% during the experiment in both variants. Two experiments using cells from different passages gave identical results.

Results

Characterization of cell lines

EPG85-257DAU cells developed a 8450-fold resistance to DAU when compared with the parent strain EPG85-257P (Table 1). Incubation of EPG85-257DAU cells with DAU plus 1 µM CsA reduced drug resistance to 246-fold while 1 µM CsA alone did not inhibit proliferation of resistant and sensitive EPG85-257 cells (data not shown). By PCR it was demonstrated that EPG85-257DAU cells expressed the 4.5 kb mRNA coding for P-glycoprotein. This was confirmed by restriction analysis of the *mdr*1 mRNA in resistant cells (Fig. 1). Furthermore, resistant cells reacted with the monoclonal antibody C219 when stained for immunocytochemistry showing a strong reaction of the cell membrane, whereas sensitive cells were found to be completely negative (Fig. 2a, b). These data indicate presence of the MDR phenotype.

Kinetics of RH and DAU

Uptake of RH was 10-fold higher in sensitive cells when compared to the resistant subclone (Fig. 3a) as indicated by the increase in fluorescence measured by FACS. After incubation of RH-loaded sensitive cells in drug-free medium fluorescence decreased only slightly after 2 h probably due to diffusion of RH from the cells. In the resistant subclone a rapid efflux was observed, leading to a marked reduction of fluorescence to baseline levels within 2 h. This is consistent with the MDR phenotype.

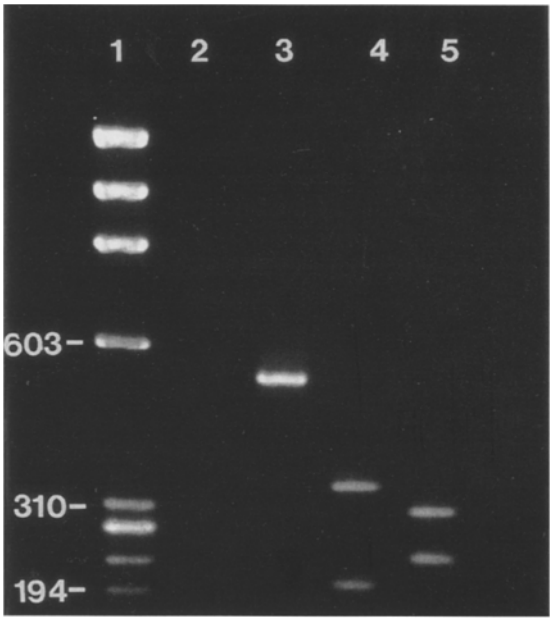

Fig. 1 Detection of the *mdr*1 mRNA in EPG85-257DAU cells with RT-PCR. Lane one: molecular weight marker. Lane two and three: PCR products of 542bp; lane two: product of EPG85-257P cells; lane three: product of EPG85-257DAU cells. Lanes four and five: digestions of the PCR products from EPG85-257DAU cells with Pst I (340 and 202 bp; lanes four) and Msp I (305 and 237 bp; lane five)

DAU uptake in sensitive cells was about 7-fold higher than in the resistant cells (Fig. 3b) although they received only 2.5% of the concentration applied to the resistant variant. Subsequent to a period of DAU loading both cell clones were incubated in drug-free medium (Fig. 3b). Due to outward diffusion of DAU, fluorescence of sensitive EPG85-257P cells decreased from 70 to 50 units within 120 min. Interestingly, within 120 min there was only a minor outward flux originating from the DAU load in resistant EPG85-257DAU cells. Fluorescence remained approxm. at 8 units (8-fold threshold of assay sensitivity) despite expression of P-Gp in these cells. This indicates that the DAU fluorescence at least in part may be confined to compartments (nucleus, vesicles) inaccessible to P-Gp during the time of measurement.

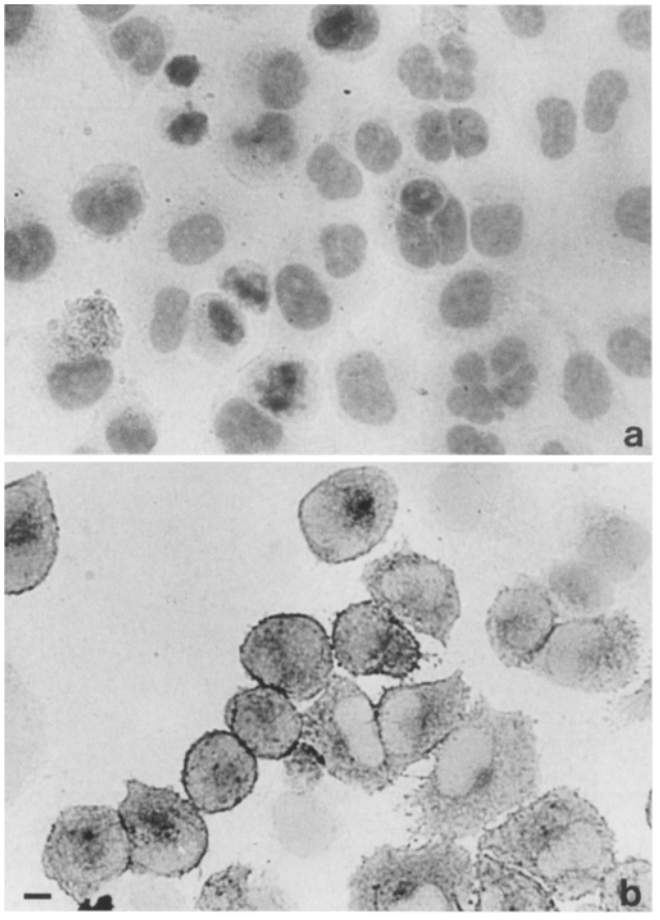

Fig. 2 Immunocytological detection of P-Gp in sensitive EPG85-257P (**a**) and resistant EPG85-257DAU cells (**b**) using the monoclonal antibody C219. Bar represents 5 µm

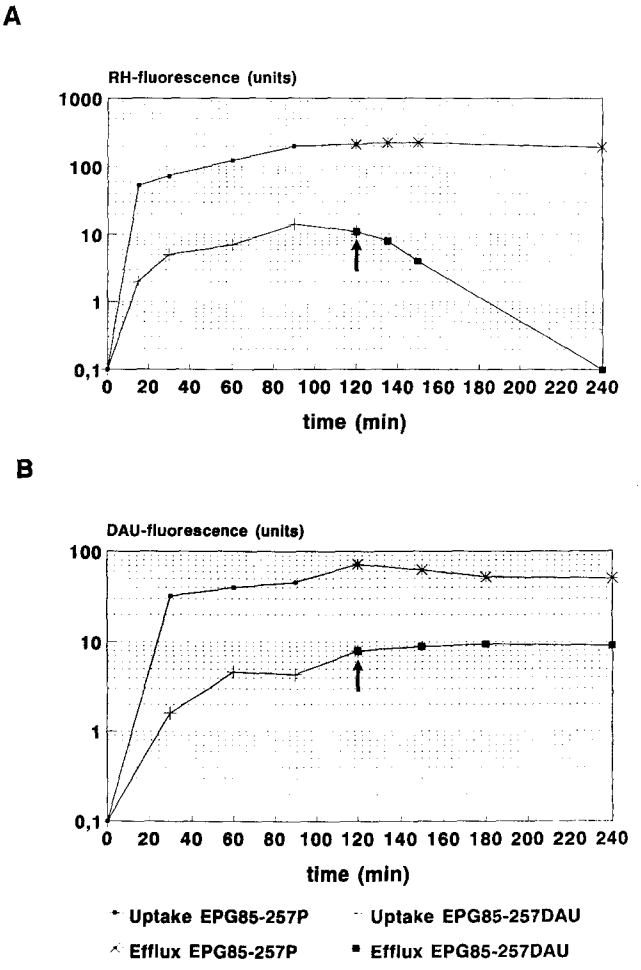

Fig. 3 Kinetics of RH (**a**) and DAU (**b**) in sensitive and resistant EPG85-257 cells. Cells were incubated with 10 µg/ml RH for the times indicated. DAU was used at a concentration of 0.25 µg/ml for sensitive and 10 µg/ml for resistant cells, respectively. *Arrow* indicates the time of drug removal from the medium. Mean fluorescence of 5,000 to 10,000 cells is given

Time course of intracellular distribution of DAU

In both sensitive and resistant cells removed from drug-free medium and fixed prior to drug exposure only a faint fluorescence of the nucleus was observed (data not shown). In the sensitive subclone 0.25 and 1.0 µg/ml DAU added to the medium bound rapidly to the nucleus leading to an intense fluorescence of nuclear structures. Fluorescence persisted and led to shrinkage of the cytoplasm and nuclear swelling after 24 h followed by condensation of the nuclei after 48 h (Fig. 4a–c).

In resistant cells studied 6 h after drug exposure DAU was also localized in the nucleus at all concentrations applied (0.25 to 10 µg/ml) thus showing a distribution similar to that in sensitive cells. However, even at higher doses of DAU the cellular accumulation was distinctly lower than in sensitive cells as shown by the reduced fluorescence (Fig. 4d, for details of exposure time see legend) and as measured by FACS. Nuclear fluorescence was observed in decreasing intensity during the first 24 h of the experiment (Fig. 4e). Formation of fluorescent vesicles started in the perinuclear region in a few cells approximately 6 h after drug exposure. The extent of vesicle formation appeared to be dose-dependent. At 0.25 µg/ml DAU vesicles were sparse and most of the cells showed a faint fluorescence of the cell membrane. At 1 µg/ml DAU the formation of vesicles was more prominent and 10 µg/ml DAU induced fluorescent vesicles in more than 90% of the cells (Fig. 4f). After 48 h the nuclei were free of fluorescence at all concentrations of DAU, whereas vesicular fluorescence was most intense (Fig. 4f). During this period the vesicles showed a movement from the perinuclear region towards the cell border.

The pulse-chase experiment (Fig. 5) showed that in EPG85-257DAU cells after 2 h of drug exposure the nuclei were uniformly labelled with DAU, whereas the cytoplasm was free of DAU (Fig. 5a). Vesicle formation started after 6 h and intensified up to 24 h (Fig. 5b). After 48 h in drug-free medium DAU-fluorescence in the nuclei was weak and almost exclusively located in vesicles (Fig. 5c). Thus, the pattern observed in this experiment was similar to that induced by the constant presence of DAU, except that after 24 h nuclear fluorescence

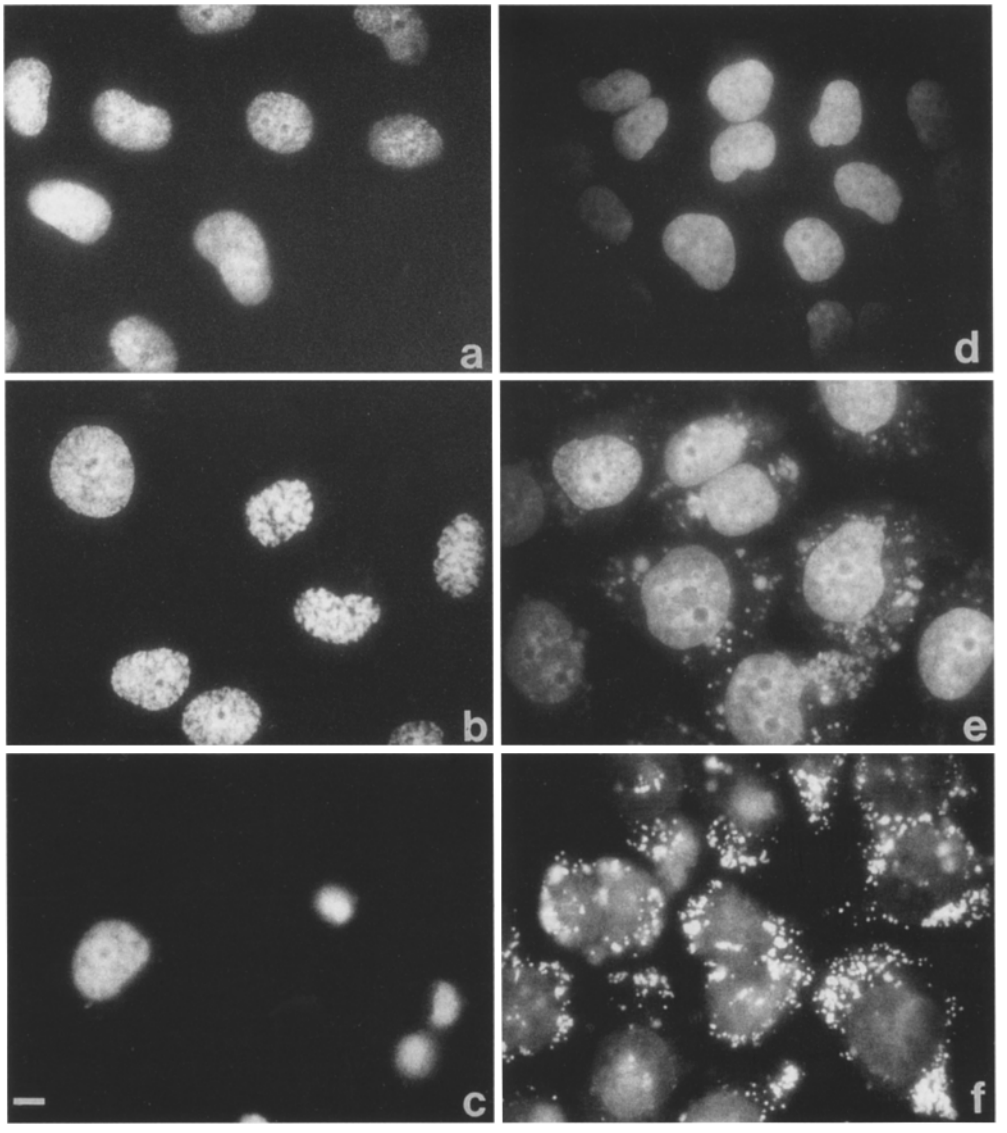

Fig. 4 a–c Distribution of daunomycin in drug-sensitive EGP85-257P cells exposed to 1 μg/ml of the drug for 1 (**a**), 24 (**b**) and 48 (**c**) h. **d–f** Distribution of daunomycin in drug-resistant EPG85-257DAU cells exposed to 10 μg/ml of the drug for 1 (**d**), 24 (**e**) and 48 (**f**) h. *Bar* represents 20 μm. Exposure time was reduced by the factor of 12 for EPG85-257P cells to compensate for the much higher fluorescence

was barely detectable and thus distinctly lower as compared to continuous exposure to the drug (Fig. 4e, 5b). These results indicate that the vesicles contained DAU derived probably from the nucleus.

Incubation of resistant cells with 1 μM CsA in conjunction with DAU led to resensitization and increased fluorescence of the nucleus, indicating enhanced uptake of DAU. Nuclear fluorescence was observed throughout the experiment, unlike in resistant cells exposed to DAU only. Vesicle formation was not impaired and occurred in almost every cell (Fig. 6a).

Disruption of microtubule formation by 24 h exposure to 100 μg/ml nocodazole did not inhibit vesicle formation. Instead we observed an accumulation of vesicles in the perinuclear space (Fig. 6b), whereas the peripheral area of the cell was virtually vesicle-free. This indicates that the vesicles are transported from the perinuclear region to the cell's border and that this mechanism is dependent on intact microtubule formation. Vesicle formation is independent of microtubule function.

The actin inhibitor cytochalasin B (at 5 μg/ml) induced accumulation of vesicles at the cell border, leaving the perinuclear area of most cells virtually free of vesicles (Fig. 6c). This indicates that exocytosis of drug-filled vesicles may occur and actin filaments are possibly involved in this process.

Discussion

In EPG85-257 cells development of daunorubicin resistance is accompanied by increased expression of the *mdr*1 gene product and P-Gp as shown by PCR and immunocytochemistry as well as by the reduced accumulation of DAU and RH in resistant cells and by the rapid export of RH. Expectedly, slow outward diffusion of DAU was observed from sensitive EPG85-257P cells. In contrast, DAU accumulated in EPG85-257DAU cells after a loading period of 120 min – while being clearly detectable in the fluorescence assay – had no access to dif-

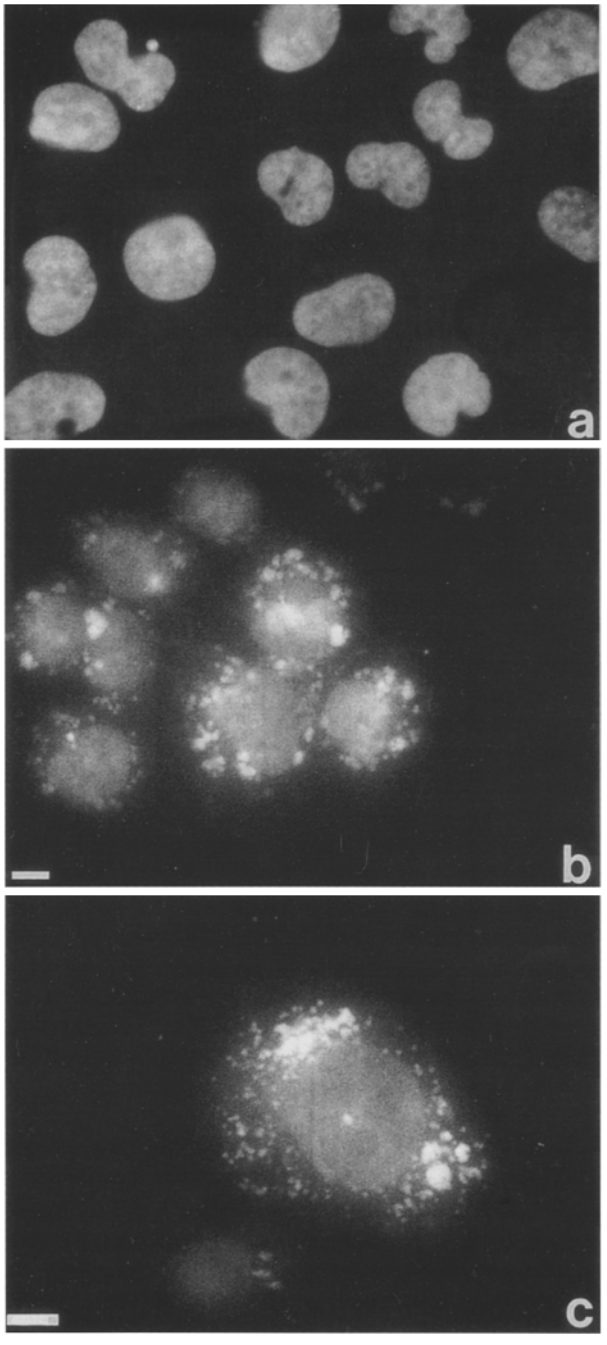

Fig. 5 Distribution of DAU in drug-resistant cells after 2 h incubation in presence of 10 μg/ml DAU (**a**) Following DAU exposure cells were kept in drug-free medium for 24 (**b**) and 48 (**c**) h. *Bar* represents 20 μm

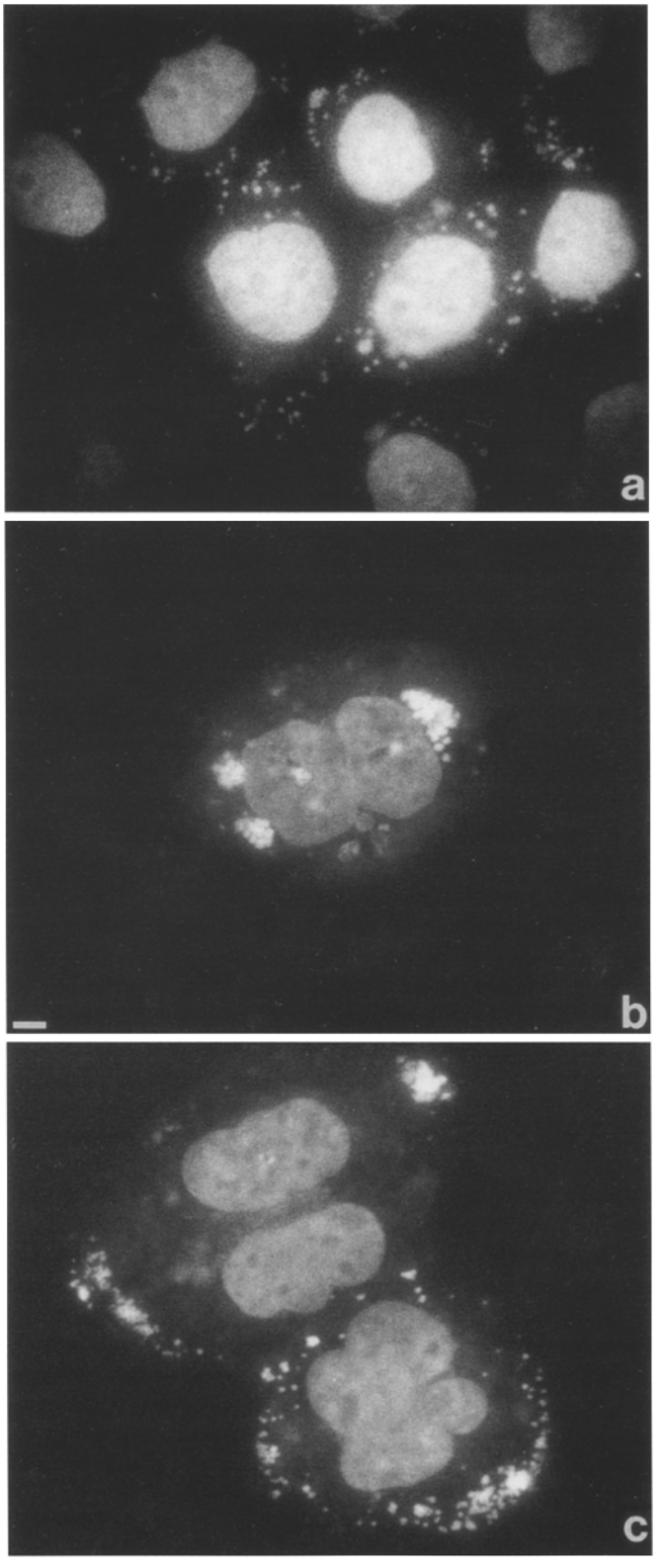

Fig. 6 Effect of 1 μM CsA (**a**), 100 μg/ml nocodazole (**b**) and 5 μg/ml cytochalasin B (**c**) on intracellular distribution of DAU in drug-resistant EPG85-257DAU cells. Cells were incubated with the drug plus 10 μg/ml DAU for 24 h. For further details see text. *Bar* represents 20 μm

fusional transmembrane exit into the drug-free medium. This indicates for the resistant variant confinement of DAU to compartments separate from the cytosol, as exemplified by tight bindung to nuclear structures or compartmentalization in vesicles.

The functional importance of P-Gp is inferred by the reduction of DAU resistance by concomitant incubation with CsA. However, complete reversal was not achieved by CsA, indicating that in highly resistant EPG85-

257DAU cells other mechanisms may contribute to DAU resistance. The existance of additional mechanisms is strongly supported by drug transport studies using labelled DAU suggesting different cellular pools for DAU in resistant versus sensitive cells [32]. Furthermore, it is in agreement with results obtained on ovarian cancer cells resistant to adriblastin. In a less resistant subclone of this cell line verapamil reversed resistance completely whereas in a highly resistant subclone only a partial reversion was achieved [28]. This indicate that MDR cells of high drug resistance may not rely solely on the expression of P-Gp.

Despite expression of P-Gp, DAU entered the cells rapidly and accumulated in the nucleus of resistant cells. Nuclear fluorescence was observed far below the DAU levels which inhibit cell growth (data not shown). This suggests that in the present study the nuclear accumulation of DAU was not due to an overload of the cells with the drug but rather reflects a general pathway of DAU in resistant cells. In other words, EPG85-257DAU cells can tolerate the presence of DAU in the nucleus for 24 to 48 h. With respect to the nuclear accumulation of DAU, the EPG85-257DAU cells differ from DAU-resistant KB cells in which DAU binds to lysosomes predominantly and only to a very limited extent to nuclei [34], while also differing from drug-resistant cells investigated by Gervasoni and co-workers [14] which accumulate the drug in the perinuclear region but not in the nucleus.

The results of our pulse-chase experiment show that the vesicles contain DAU derived from the nucleus, because after removal of the drug from the medium no fluorescent DAU could be detected outside the nucleus. Therefore a redistribution of DAU from the nucleus to transport vesicles occurs. The efficiency of this redistribution process is demonstrated by the fact that even at high DAU concentrations all nuclei were almost free of fluorescence after 48 h. Initial binding of adriamycin or daunorubicin to the nucleus and subsequential removal of the drug has been described in lung adenocarcinoma cells and in HL60 cells resistant to adriamycin [8, 21, 30] but in these cell lines no vesicular transport has been described.

The question of the significance of vesicular transport in DAU resistance in EPG85-257DAU cells is raised because the increase in nuclear fluorescence after CsA treatment indicates that intracellular DAU concentration is also reduced due to P-Gp. The fraction of DAU tightly bound to intracellular structures or confined to compartments other than the cytosol, obviously would be inaccessible for P-Gp mediated export functions. Therefore we presume that vesicular transport is responsible for the removal of nuclear-bound DAU in EPG85-257DAU cells. Complementing P-Gp function – clearing the cytosolic compartment from free DAU – the DAU – containing vesicles bypassed the cytosolic compartment after removal of DAU from the nucleus. The independent nature of the vesiculation process is illustrated by the fact, that CsA (known to inhibit P-Gp function) did not interfere with DAU-induced vesicle formation and exocytosis. This view is supported by the fact that the appearance of vesicles precedes the decreases in nuclear fluorescence. Vesicular transport has been described in MDR as well as in non-MDR cell lines [11, 14, 33, 34] and its importance in intracellular drug transport has been suggested.

CsA partially reverses DAU resistance in EPG85-257DAU cells [31] by inhibiting P-Gp-mediated drug efflux [5, 12]. In our experiments, this inhibition resulted in a higher binding of DAU to nuclear structures leading to a pronounced increase of nuclear fluorescence. This pattern is in good agreement with results found in MDR-NIH3T3 cells, in which marked nuclear fluorescence was observed after reversion of chemoresistance by verapamil [19]. However, in our experiments CsA did not inhibit DAU accumulation in vesicles and also vesicle formation was not reduced by CsA. Thus, it seems that P-Gp is not responsible for accumulation of DAU in vesicles. In accordance with this we could not detect P-Gp in vesicles by light and eletronmicroscopical immunocytochemistry (data not shown).

Nocodazole treatment inhibited the transport of vesicles, showing that tubulin fibres participate in this process. The role of the cytoskeleton in cytostatic drug resistance has been shown by Mujai'c & Mujai`c [23] who compared the tubulin fibres in cells resistant to colchicine with those of sensitive cells. They noted a more sparse network of tubulin fibres in resistant cells and differences in colchicine binding of purified tubulin. In EPG85-257DAU cells we observed in more dense network of tubulin fibres in comparison to the parent strain [25, 31]. This may be related to the increased vesicle transport in EPG85-257DAU cells which requires a well-developed cytoskeleton.

Unlike tubulin, actin fibres do not appear to be involved in the movement of vesicles from the perinuclear space to the cell border. However, disruption of actin filaments by treatment with cytochalasin B led to an accumulation of vesicles at the cell membrane, indicating that exocytosis at the cell periphery was impaired. Regulation of exocytosis by microfilaments in leukaemia cells has been demonstrated by Narasimhan et al. [24]. Involvement of microfilaments in the secretory process of mast cells [18] and in the release of chromaffin granules has been suggested by several studies (for review see [26]).

In summary our results support the importance of vesicular drug transport at least in some resistant cell lines. For the first time it could be shown that vesicles may be responsible for the removal of nuclear bound fraction of DAU suggesting an auxiliary mechanism of resistance.

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