Inhibitors of vacuolar H⁺-ATPase impair the preferential accumulation of daunomycin in lysosomes and reverse the resistance to anthracyclines in drug-resistant renal epithelial cells

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It has been suggested that the inappropriate sequestration of weak-base chemotherapeutic drugs in acidic vesicles by multidrug-resistance (MDR) cells contributes to the mechanisms of drug resistance. The function of the acidic lysosomes can be altered in MDR cells, and so we investigated the effects of lysosomotropic agents on the secretion of lysosomal enzymes and on the intracellular distribution of the weak-base anthracycline daunomycin in drug-resistant renal proximal tubule PKSV-PR_{col50} cells and their drug-sensitive PKSV-PR cell counterparts. Imaging studies using pH-dependent lysosomotropic dyes revealed that drug-sensitive and drug-resistant cells exhibited a similar acidic lysosomal pH (around 5.6–5.7), but that PKSV-PR_{col50} cells contained more acidic lysosomes and secreted more of the lysosomal enzymes *N*-acetyl- β -hexosaminidase and β -glucuronidase than their parent PKSV-PR cells. Concanamycin A (CCM A), a potent inhibitor of the vacuolar H⁺-ATPase, but not the P-glycoprotein modulator verapamil, stimulated the secretion of *N*-acetyl- β -hexosaminidase

in both drug-sensitive and drug-resistant cells. Fluorescent studies and Percoll density gradient fractionation studies revealed that daunomycin accumulated predominantly in the lysosomes of PKSV-PR_{col50} cells, whereas in PKSV-PR cells the drug was distributed evenly throughout the nucleo-cytoplasmic compartments. CCM A did not impair the cellular efflux of daunomycin, but induced the rapid nucleo-cytoplasmic redistribution of the drug in PKSV-PR_{col50} cells. In addition, CCM A and bafilomycin A1 almost completely restored the sensitivity of these drug-resistant cells to daunomycin, doxorubicin and epirubicin. These findings indicate that lysosomotropic agents that impair the acidic-pH-dependent accumulation of weak-base chemotherapeutic drugs may reverse anthracycline resistance in MDR cells with an expanded acidic lysosomal compartment.

Key words: anthracycline, lysosome, multidrug resistance, proton pump, renal cells.

INTRODUCTION

The multidrug-resistance (MDR) phenomenon that occurs in cancer cells is characterized by the development of resistance to a wide range of structurally and functionally diverse drugs, and it constitutes a major obstacle to chemotherapy. The mechanisms underlying MDR have been studied extensively in models of cultured drug-sensitive and -resistant tumour cells. The change most often observed in MDR cells is increased expression of the P-glycoprotein (P-gp) encoded by the *MDR* gene, belonging to the superfamily of ATP binding cassette transporters (ABC transporters) [1,2], which causes a decrease in the accumulation of a variety of cytotoxic drugs [1–3]. However, it has been suggested that mechanisms in addition to the extrusion of drugs via ATP-dependent membranous ABC transporters, which result from biophysical changes in either transmembrane pH gradients or electrical potentials, may also play a role in decreasing the accumulation of chemotherapeutic drugs in several cultured MDR cell models [3–5].

The MDR phenomenon can be associated with the over-expression of many other ABC transporters, such as the MDR-associated protein (MRP) and the lung resistant-related protein, and with many changes in the expression of a variety of

cytoplasmic and plasma membrane proteins, such as DNA topoisomerase II, vacuolar H⁺-ATPase, glutathione S-transferase π and catalase (for review, see [3]). As a consequence, the intracellular concentration and distribution of drugs may differ considerably in drug-sensitive and drug-resistant cells. Chemotherapeutic drugs such as the anthracyclines are distributed mainly in the cytoplasm and nucleus of drug-sensitive cells, but can accumulate preferentially in the acidic organelles of a variety of drug-resistant cancer cells [6–11]. Schindler et al. [12] have proposed a ‘protonation, sequestration and secretion (PSS)’ model that could account for the relative sensitivity of tumour cells to anthracyclines. These authors have reported that adriamycin is concentrated in acidified organelles (i.e. lysosomes and recycling endosomes) from drug-resistant MCF-7/ADR breast cancer cells, but not in those from their drug-sensitive MCF-7 counterparts exhibiting a vesicular acidification defect [11]. These authors and others have also shown that disruption of the pH gradient between the cytoplasm and the acidic organelles by a variety of lysosomotropic agents leads to the release of chemotherapeutic drugs accumulated in the acidic organelles from drug-resistant cells [10,11]. This intravesicular relocation of anthracyclines can obviously be expected to impair their cytotoxic action, which involves nuclear effects, including binding of DNA

Abbreviations used: ABC transporter, ATP binding cassette transporter; AcP, acid phosphatase; CCM A, concanamycin A; MDR, multidrug resistance; MRP, multidrug-resistance-associated protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAG, *N*-acetyl- β -hexosaminidase; P-gp, P-glycoprotein; pH_{lys}, lysosomal pH.

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and interference with topoisomerase II [13]. However, the contribution of the inappropriate sequestration of drugs in acidic organelles to the MDR phenomenon is still debated [14]. Disruption of the pH gradient between the cytoplasm and the acidic organelles by a variety of lysosomotropic agents may lead to the release of chemotherapeutic drugs accumulated in the acidic organelles from MDR cells; however, its impact on resistance to weak-base chemotherapeutic drugs has not been fully evaluated.

In the kidney, P-gp is expressed in the apical brush border of intact proximal tubule cells [15], which possess an active secretory lysosomal machinery [16]. Most forms of renal adenocarcinoma, which are mainly of proximal tubule origin, are highly resistant to chemotherapy. We established a line of drug-sensitive PKSV-PR cells derived from L-PK/Tag 1 transgenic mice harbouring the pyruvate kinase (PK)-Tag transgene [17], which have maintained the main features of the parent cells from which they were derived [18]. Drug-resistant MDR mouse proximal tubule cells (PKSV-PR_{col150}) were then obtained by continuous selection of parent PKSV-PR cells with colchicine [19]. These drug-sensitive and drug-resistant proximal tubule cells therefore appeared to be suitable cell models for an analysis of lysosomal functions and the intracellular distribution of daunomycin. We also used these cells to investigate the consequences of the rapid alkalization of lysosomes induced by inhibitors of vacuolar H⁺-ATPase for the cellular redistribution of anthracyclines and the restoration of drug sensitivity to drug-resistant proximal tubule cells.

MATERIALS AND METHODS

Cell culture

We used PKSV-PR_{col150} cells, a mouse kidney proximal tubule drug-resistant cell line, selected by colchicine from the parent drug-sensitive PKSV-PR cells [19]. PKSV-PR cells were cultured routinely in a modified culture medium [Dulbecco's modified Eagle's medium/Ham's F12, 1:1 (v/v), 60 nM sodium selenite, 5 µg/ml transferrin, 2 mM glutamine, 5 µg/ml insulin, 50 nM dexamethasone, 5 nM tri-iodothyronine, 10 ng/ml epidermal growth factor, 20 mM D-glucose, 2% (v/v) fetal calf serum, 20 mM Hepes, pH 7.4] at 37 °C in a 5% CO₂/95% air atmosphere. PKSV-PR_{col150} cells were grown in the same medium supplemented with 50 ng/ml colchicine. All experiments were performed between the 45th and 65th passages, and media were changed every 2 days.

Daunomycin efflux studies

The rate of efflux of daunomycin was determined as described previously [19] from confluent PKSV-PR and PKSV-PR_{col150} cells grown on collagen-coated six-well plates incubated with 100 nM [G-³H]daunomycin (specific radioactivity 5 Ci/mmol; NEN Life Science Products, Paris, France) for 60 min at 37 °C (loading period). The cell layers were then rinsed with ice-cold serum-free medium and incubated with 2.5 ml of fresh, serum-free, CO₂-free medium pre-warmed to 37 °C. Samples of 50 µl were collected at various intervals from 5 to 180 min, cells were lysed in 1 M NaOH and radioactivity was counted.

Enzyme assays

The activities of lysosomal enzymes were measured in the medium and in confluent cells grown on six-well plates. The culture medium was removed and cells were rinsed with PBS, scraped off, centrifuged (100 g, 5 min) at 4 °C, and stored at -70 °C.

Pelleted cells were resuspended in 1 ml of 10 mM phosphate buffer, pH 7, sonicated and then incubated with 0.1% (v/v) Triton X-100 for 2 h at 4 °C. The *N*-acetyl- β -hexosaminidase (NAG) activity of the cell lysates and culture medium was measured as described in [16]. Aliquots of cell lysate and medium were incubated in 0.2 ml of 50 mM citric acid/sodium citrate, pH 4.6, 2.5 mM 4-methylumbelliferyl β -*N*-acetylglucosaminide (Sigma) and 0.2 mg/ml BSA (Miles) for 10 min (lysate) or 30 min (medium) at 37 °C. The reaction was stopped by adding 2.85 ml of 400 mM glycine/NaOH, pH 10.8. The activities of β -glucuronidase and acid phosphatase (AcP) were determined as described above, using 2.5 mM 4-methylumbelliferyl β -glucuronide in 0.1 M sodium acetate/acetic acid buffer, pH 4.5, as the substrate for β -glucuronidase activity, and 1 mM 4-methylumbelliferyl phosphate in 0.1 M sodium acetate/acetic acid buffer, pH 5, as the substrate for AcP activity. The fluorescence intensity of the 4-methylumbelliferone released was measured by fluorimetry (excitation 368 nm, emission 448 nm), using 2.25 mM quinine sulphate solution to calibrate the fluorescence intensity. All measurements were performed in triplicate. The percentage of NAG, β -glucuronidase or AcP secreted into the culture medium was calculated from the ratio of enzymic activity in the culture medium over total activity (medium plus cell lysate).

Flow cytometry

The nuclear accumulation of daunomycin was assessed by cytofluorimetry. Briefly, cells were incubated sequentially with or without 20 nM concanamycin A (CCM A) for 24 h and with 50 µM daunomycin for 2 h at 37 °C. After rinsing, the cells were resuspended in a lysis buffer containing 0.3% Nonidet P40 and incubated for 5 min at 4 °C as described in [10]. Pelleted nuclei resuspended in 400 µl of PBS were fixed using IntraPrep™ fixation and permeabilization reagents (Immunotech, Marseilles, France). The fluorescence intensity of daunomycin accumulated in the nuclei was measured using a flow cytometer (Elite; Beckman-Coulter), assuming that the fluorescence emission recorded at 625 nm was proportional to the amount of daunomycin incorporated into DNA after its translation into the nuclei.

Cell imaging studies

Cells were seeded and grown to confluency on Lab-Tek coverglass chambers (Nalge Nunc International, Naperville, IL, U.S.A.). They were incubated in Phenol Red/fetal calf serum-free Dulbecco's modified Eagle's medium containing 20 mM Hepes, pH 7.4, at 37 °C under a flux of a humidified 5% CO₂/95% air atmosphere. Imaging was conducted on living cells using a confocal laser scanning inverted microscope (TCS 4D; Leica, Heidelberg, Germany) equipped with an argon/krypton laser. Cells were viewed with an $\times 63/1.4$ oil-immersion objective. The fluorescence of daunomycin incorporated into living cells incubated with 10 µM daunomycin for 1 h was visualized using the confocal microscope with an isothiocyanate (FITC) fluorescent set: $\lambda_{\text{ex}} = 488$ nm, a 515 nm beam splitter and an emission band-pass filter set for FITC. Cells were incubated with 10 µM LysoSensor Green DND-189 (Molecular Probes, Eugene, OR, U.S.A.) for 30 min using the FITC fluorescence set. PKSV-PR_{col150} cells were also incubated with 75 nM LysoTracker Red DND-99 (Molecular Probes) for 2 h, or with a conjugate of 50 µg/ml transferrin from human serum and Alexa fluor 568 (Molecular Probes) for 30 min as described [11], before being rinsed and incubated subsequently with daunomycin for 60 min. The red LysoTracker Red or Alexa fluor 568 emission was

viewed using the confocal microscope ($\lambda_{\text{ex}} = 568 \text{ nm}$). Emission light was passed through a 580 nm dichroic mirror and a 590 nm long-pass filter. The green daunomycin emission was viewed as described above, and the green and red emissions were collected using two photomultiplier tubes.

The grey-scale digital images were visualized with a 24-bit imaging system including Leica's Scanware software. The images generated passing through the middle plane of the nuclei were selected, imported into Adobe Photoshop 5.5 (Adobe, San Jose, CA, U.S.A.), pseudo-coloured, and in some cases overlapped to produce merged images. Quantitative analysis were performed using Metamorph 4.6 software (Universal Imaging Corp.). The relative mean fluorescence levels were obtained on a single-cell basis for each digital image. The lysosomal radius and number of lysosomes per cell were measured after segmentation, and regions of interest around these organelles were created. Segmentation required a series of consecutive image processing, including the application of a High Pass Fast Fourier Transform Filter, a morphometric classification to select individual round objects and the creation of a binary image mask.

Lysosomal pH (pH_{lys}) recordings

pH_{lys} in living cells was measured as described [11,19] using the pH-dependent fluorescent properties of FITC-dextran incorporated into the lysosomes of living cells by fluid-phase endocytosis [20,21]. Briefly, confluent PKSV-PR and PKSV-PR_{col50} cells grown on glass coverslips were incubated overnight at 37 °C with culture medium containing 1.5 mg/ml 10 kDa FITC-dextran (Sigma, St. Louis, MO, U.S.A.). Cells were placed in a PDMI-2 temperature-controlled microincubator (Medical Systems Corp., Greenvale, NY, U.S.A.) mounted on the stage of an inverted microscope (Nikon Diaphot 300; Nikon Corp., Tokyo, Japan). Before measuring pH_{lys} , extracellular dye was removed by prolonged superfusion of the cells with dye-free medium at 37 °C. pH_{lys} was measured after the FITC fluorescence signals had stabilized, during superfusion with a CO_2 -free solution containing (in mM): 140 NaCl, 5 KCl, 0.4 MgCl_2 , 1 CaCl_2 , 10 D-glucose, 20 Hepes/NaOH, pH 7.4. FITC-dextran fluorescence was monitored using video-enhanced fluorescence microscopy and a BCECF [2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein] filter set [19], at 37 °C. After correction for shading, images pairs were ratioed on a pixel-by-pixel basis, and ratio values were converted into pH units using the *in situ* high K-nigericin method [22], over the 5.5–6.5 pH range, as described previously [19]. Background noise was not detectable. pH_{lys} was measured using single cells (PKSV-PR_{col50}, $n = 62$; PKSV-PR, $n = 330$) from six to eight different passages.

Percoll density-gradient fractionation

Confluent PKSV-PR and PKSV-PR_{col50} cells were rinsed with ice-cold PBS containing 1 mM CaCl_2 and 1 mM MgCl_2 , and then loaded with 2 $\mu\text{Ci}/\text{ml}$ [G^3H]daunomycin (specific radioactivity 5 Ci/mmol; NEN) for 60 min at 37 °C. Unloaded or daunomycin-loaded cells were washed with PBS and then once in ice-cold fractionation buffer (250 mM sucrose, 20 mM Hepes, 1 mM EDTA, pH 7.2) supplemented with 1 mM PMSF and 100 $\mu\text{g}/\text{ml}$ protease inhibitor cocktail (Boehringer, Mannheim, Germany). Cells were scraped into 2 ml of fractionation buffer. Protein content was determined by the method of Lowry et al. [23] using BSA as standard. Cell homogenization was performed at 4 °C using a tight-fitting Dounce homogenizer (six passages). The resulting homogenate was centrifuged at 600 g for 10 min at 4 °C. The supernatant was adjusted to 5 ml with fractionation buffer and then 3 ml of an 80 % Percoll solution was added. The

gradient was performed by centrifuging for 35 min at 100 000 g at 4 °C [24]. The fractions were collected from the bottom of the tubes using a needle, and the radioactivity was counted in aliquots (50 μl) of each fraction collected.

The NADPH:cytochrome *c* reductase activity (used as a marker of the endoplasmic reticulum) and lysosomal mannosidase activity were determined for each fraction collected. NADPH:cytochrome *c* reductase activity was measured in 50 mM phosphate/0.1 mM EDTA buffer, pH 7.7, using 1 mg/ml NADPH and 25 mg/ml cytochrome *c* as substrates. Absorbance was measured at 550 nm. Lysosomal mannosidase activity was measured in 500 mM sodium acetate buffer, pH 4.5, using 10 mM *p*-nitrophenyl α -D-mannopyranoside as substrate. Absorbance was measured at 410 nm.

Drug resistance assay

Drug resistance was assayed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] dye assay [25]. Cells were seeded and grown in 96-well plates (8000 cells per well) for 2 days. The different agents to be tested were then added to the medium for 48 h. The medium was removed and replaced by 100 μl of MTT (400 $\mu\text{g}/\text{ml}$), and incubation was continued for 4 h at 37 °C. After centrifuging, the medium was removed, 100 μl of DMSO was added to each well and the plates were shaken for 10 min. Absorbance at 540 nm was determined for each well using a microplate autoreader (Titertek Multiskan MC). IC_{50} values, normalized against values obtained from cells incubated without drugs, were determined as described in [19].

Statistical analysis

Results are reported as means $\pm$ S.E.M. from (n) experiments. Statistical differences between groups were assessed using Student's *t* test. A *P* value of < 0.05 was considered significant.

RESULTS

Daunomycin efflux and lysosomal enzyme secretion by drug-sensitive and drug-resistant renal epithelial cells

We have shown previously that PKSV-PR_{col50} cells exhibit the typical features of MDR cells, including higher levels of *mdr1b* gene expression, than their drug-sensitive PKSV-PR counterparts, and high resistance to colchicine, vinblastine [19] and anthracyclines (the present study). The efflux of daunomycin was twice as great from PKSV-PR_{col50} cells than from PKSV-PR cells preloaded with 100 nM [^3H]daunomycin for 60 min (Figure 1, left panel). The amount of daunomycin remaining in the cells after efflux was also significantly lower in PKSV-PR_{col50} cells than in their drug-sensitive counterparts (Figure 1, right panel). The P-gp modulator verapamil (10 μM) did not affect the amount of daunomycin retained in the PKSV-PR cells, but significantly enhanced the cellular retention of the drug by PKSV-PR_{col50} cells (2.7-fold; Figure 1, right panel). These results indicated that these MDR PKSV-PR_{col50} cells retained less daunomycin and exhibited greater rates of drug efflux than their parent drug-sensitive PKSV-PR cells [19].

These MDR renal epithelial proximal tubule cells also exhibited changes in lysosomal structure. As reported by Beck [26], more abundant intracellular acidic vesicular structures that accumulated 9-aminoacridine and FITC-dextran were detected in PKSV-PR_{col50} cells than in PKSV-PR cells [19]. To analyse further the acidic compartments of these drug-sensitive and drug-resistant renal cells, imaging studies were performed using the lysosomotropic agent LysoSensor Green DND-189. The

Figure 1 Efflux and retention of daunomycin in drug-sensitive and drug-resistant renal proximal tubule cells

Confluent cells were loaded with 100 nM [G - 3H]daunomycin for 60 min at 37 °C and washed with ice-cold serum-free medium. Left panel: the efflux of daunomycin, expressed as a percentage of the total radioactivity incorporated, was measured over 3 h. Right panel: the bars represent the cellular content of [G - 3H]daunomycin (expressed as pmol/ 10^6 cells) remaining after the 3 h efflux measured in the absence (–) or presence (+) of 10 μ M verapamil (VRP) in PKSV-PR cells (□) and PKSV-PR_{col50} cells (■). Values are the means $\pm$ S.E.M. from five separate experiments; ** P < 0.01, *** P < 0.001.

fluorescence of LysoSensor Green is pH-dependent, and increases as the pH decreases. PKSV-PR cells exhibited a discrete punctuate LysoSensor Green fluorescence, distributed throughout the cytoplasm (Figure 2A), whereas PKSV-PR_{col50} cells exhibited numerous, densely packed fluorescence signals concentrated near the nucleus (Figure 2C). Quantitative analysis of these fluorescence signals revealed that the number of LysoSensor Green-labelled organelles (i.e. lysosomes) per cell was 1.8-fold greater in PKSV-PR_{col50} than in PKSV-PR cells (Figure 2E). The mean radius of the fluorescent vesicles (ranging between 235 and 364 nm) was also slightly greater in drug-resistant PKSV-PR_{col50} cells than in their drug-sensitive PKSV-PR counterparts (Figure 2E). Similar results were obtained by using the pH-independent LysoTracker Red DND-99 (results not shown). Although one cannot exclude the possibility that the incorporation of the fluorescent dyes induced swelling of the organelles, these results were in accordance with our previous observations using 9-aminoacridine and FITC-dextran [19]. The fact that there was no major difference in the intensity of the signals provided by the pH-dependent LysoSensor Green signals in PKSV-PR and PKSV-PR_{col50} cells (Figure 2F) also suggested that the lysosomes from drug-resistant and drug-sensitive renal cells exhibited a normal acidic pH. Vacuolar H^+ -ATPases contribute to the acidification of a variety of intracellular organelles in eukaryotic cells [27]. They are also highly expressed in endosomes and lysosomes of proximal tubule cells [28,29]. Cells were therefore incubated with CCM A, a potent vacuolar H^+ -ATPase inhibitor that does not affect ATP levels [30,31], to evaluate the vacuolar H^+ -ATPase-dependent acidification of lysosomes in PKSV-PR and PKSV-PR_{col50} cells. The addition of 50 nM CCM A to the medium bathing the cells loaded with the LysoSensor Green induced within 15 min a substantial decrease in the intensity of the fluorescence signals in both PKSV-PR (–75%) and PKSV-PR_{col50} (–65%) cells (Figure 2F), leading to almost complete disappearance of the green fluorescence from both cell types (Figures 2B and 2D). Overall, these results strongly suggest that PKSV-PR_{col50} cells have an expanded functional acidic lysosomal compartment.

We next performed pH measurements to estimate pH_{lys} . As reported for drug-sensitive MCF-7 breast cancer cells [11], we

Figure 2 LysoSensor Green labelling of acidic compartments of PKSV-PR and PKSV-PR_{col50} cells

Shown is the fluorescence of PKSV-PR (A, B) and PKSV-PR_{col50} (C, D) cells incubated with LysoSensor Green dye used to label the lysosomes. Untreated PKSV-PR_{col50} cells (C) exhibited more numerous punctate cytoplasmic organelles than PKSV-PR cells (A). CCM A (20 nM for 15 min) induced a rapid decrease in pH-dependent LysoSensor Green labelling in both PKSV-PR (B) and PKSV-PR_{col50} (D) cells. Scale bar = 10 μ M. The density and radius of lysosomes labelled with the LysoSensor Green fluorescent dye (E) and the mean LysoSensor Green fluorescence intensity per cell (F) were quantified from confocal laser scanning inverted microscopy images of passing through the middle plane of the nuclei of PKSV-PR and PKSV-PR_{col50} cells, as described in the Materials and methods section. Values are the means $\pm$ S.E.M. from individual cells analysed using images (11–20 cells per image) generated by confocal laser scanning inverted microscopy from three or four separate experiments for each condition tested. Significance of differences: * P < 0.05, *** P < 0.001.

Figure 3 Rates of secretion of lysosomal enzymes by drug-sensitive and drug-resistant renal proximal tubule cells

(A) The percentages of total NAG, β -glucuronidase (GUS) and AcP secreted by PKSV-PR cells ($\circ$) and PKSV-PR_{col50} cells ($\bullet$) were measured 1–4 days after confluency. Values are the means $\pm$ S.E.M. from six separate experiments. The percentages of secreted NAG and β -glucuronidase were significantly higher in PKSV-PR_{col50} cells than in PKSV-PR cells ($P < 0.01$). (B) The percentage of total NAG secreted was measured in confluent PKSV-PR cells ($\square$) and PKSV-PR_{col50} cells ($\blacksquare$) incubated without (Control) or with 10 μ M verapamil (VRP), 20 nM CCM A or verapamil plus CCM A for 24 h. Values are the means $\pm$ S.E.M. from five separate experiments; * $P < 0.05$, *** $P < 0.001$ compared with control values.

failed to obtain a correct measurement of pH_{lys} in the drug-sensitive PKSV-PR cells by using the pH-dependent LysoSensor Green DND-189. However, the results from the fluorescence studies showing that CCM A decreased the intensity of the pH-dependent LysoSensor Green DND-189 signal strongly suggested that the lysosomes from the parent, drug-sensitive PKSV-PR cells were acidic. Indeed, pH measurements using endocytosed 10 kDa FITC-dextran revealed that the drug-resistant PKSV-PR_{col50} cells had a slightly, but not significantly, lower resting pH_{lys} (5.66 ± 0.02) than drug-sensitive PKSV-PR cells (5.74 ± 0.02).

The activities of cellular and secreted lysosomal enzymes in PKSV-PR cells and PKSV-PR_{col50} cells were then measured to find out whether the rates of secretion of lysosomal enzymes were higher in drug-resistant renal proximal tubule cells, as has been reported for CEM/VLB₁₀₀ lymphoblastic cells [32]. The percentages of secreted NAG and β -glucuronidase were 1.3-fold and 1.8-fold greater respectively in PKSV-PR_{col50} cells than in PKSV-

Figure 4 Different effects of verapamil and CCM A on daunomycin efflux from drug-resistant PKSV-PR_{col50} cells

Left panel: the efflux and the cellular retention of [3 H]daunomycin were measured in PKSV-PR_{col50} cells incubated without ($\bullet$) or with verapamil (VRP; 10 μ M; $\circ$), CCM A (50 nM; $\triangle$) or verapamil plus CCM A ($\square$) for 24 h at 37 $^{\circ}$ C. Cells were then loaded with 100 nM [3 H]daunomycin as described in the legend of Figure 1, and daunomycin efflux was measured over 3 h. Right panel: the bars represent the cellular content of [3 H]daunomycin remaining after the 3 h efflux measured in the absence (None) and presence of verapamil, CCM A or verapamil + CCM A. Values are the means $\pm$ S.E.M. from five separate experiments; ** $P < 0.01$, *** $P < 0.001$ compared with untreated cells ('None').

PR cells, whereas the percentage of secreted AcP remained low ($< 2\%$) after 4 days, and was similar in PKSV-PR and PKSV-PR_{col50} cells (Figure 3A). CCM A (20 nM) stimulated the percentage of secreted NAG recovered from the culture medium after 24 h for both PKSV-PR (2.2-fold) and PKSV-PR_{col50} (1.8-fold) cells (Figure 3B). In contrast, verapamil (10 μ M) did not alter the level of secreted NAG in either untreated or CCM A-treated drug-sensitive or -resistant cells (Figure 3B). This raises the question of whether the rapid alkalization of the lysosomes induced by lysosomotropic agents, responsible for an increase in the secretion of lysosomal enzymes [16,33–35], could also affect the P-gp-dependent efflux of daunomycin from drug-resistant PKSV-PR_{col50} cells. The rate of [3 H]daunomycin efflux was almost identical in PKSV-PR_{col50} cells incubated without or with 20 nM CCM A (Figure 4, left panel). Moreover, CCM A did not significantly alter the rate of daunomycin efflux or the amount of drug remaining after efflux in either untreated or verapamil-treated PKSV-PR_{col50} cells (Figure 4, right panel).

Cellular distribution of daunomycin in drug-sensitive and -resistant renal epithelial cells

Cell imaging studies were performed to find out whether the subcellular distribution of daunomycin differed in drug-sensitive and drug-resistant proximal tubule cells. Daunomycin was widely distributed throughout the cytoplasm, in a few intracellular vesicular structures and in the nuclei of drug-sensitive PKSV-PR cells (Figure 5A), whereas in PKSV-PR_{col50} cells it was barely detectable in the cytosol and nuclei, but was concentrated in round structures around the nuclei (Figure 5G). Sequential labelling with the pH-insensitive LysoTracker Red DND-99 lysosomal marker and daunomycin showed that daunomycin was always co-localized with the lysosome-specific fluorescent dye (Figures 5C and 5D). In contrast, daunomycin did not co-localize with the transferrin–Alexa fluor 568 conjugate used to label the recycling endocytic compartment (Figures 5E and 5F).

Figure 5 Daunomycin distribution in drug-sensitive and drug-resistant renal proximal tubule cells

The pattern of daunomycin labelling (in green) was analysed by confocal laser scanning inverted microscopy in confluent living PKSV-PR cells (**A, B**) and PKSV-PR_{col50} cells (**G, H**) incubated without or with CCM A (50 nM for 15 min), and in PKSV-PR_{col50} cells incubated sequentially with LysoTracker Red DND-99 (**C, D**) or with the transferrin–Alexa fluor 568 conjugate (**E, F**) and daunomycin, as described in the Materials and Methods section. The fluorescence of daunomycin, widely distributed throughout the cytoplasm and nucleoplasm (**A**), was not altered by CCM A (**B**). The punctate fluorescence of LysoTracker Red DND-99 in PKSV-PR_{col50} cells (**C**) exactly matched the distribution of daunomycin (**D**, and inset), whereas the fluorescence of the transferrin–Alexa fluor 568 conjugate (**E**) did not co-localize with daunomycin (**F**, and inset). Daunomycin accumulated in the lysosomes from PKSV-PR_{col50} cells (**G**) was rapidly redistributed within the cytoplasm after addition of 20 nM CCM A for 15 min (**H**). Insets, *merge images. Scale bar = 10 μ m.

Effect of CCM A on the lysosomal accumulation of daunomycin in drug-resistant renal epithelial cells

Alkalinization of the lysosomal compartment by CCM A (20 nM for 15 min) did not significantly alter the nucleo-cytoplasmic distribution of daunomycin in PKSV-PR cells (Figure 5B), but did induce a marked decrease in the punctate fluorescence of the drug accumulated in lysosomes in PKSV-PR_{col50} cells (Figure 5H). Percoll density-gradient fractionation, which is able to

Figure 6 Lysosomal accumulation of daunomycin, and nuclear redistribution induced by CCM A

(**A**) Percoll density gradient fractionation was performed on homogenates of unloaded or [G - 3H]daunomycin-loaded (2μ Ci/ml for 60 min) cells, as described in the Materials and methods section. The profiles of lysosomal mannosidase and NADPH:cytochrome *c* reductase activities revealed two fractions: one highly enriched with lysosomes (fraction I) and the other with endoplasmic reticulum (fraction II). (**B**) A sharp peak of [G - 3H]daunomycin was detected in fraction I of the PKSV-PR_{col50} cells. (**C**) The relative percentage of [G - 3H]daunomycin accumulated in fraction I decreased significantly after addition of 20–50 nM CCM A during the loading period in PKSV-PR_{col50} cells, but remained unchanged in PKSV-PR cells; *** $P < 0.001$. (**D**) Three-dimensional galley plots of fluorescent daunomycin intensity analysed by flow cytometry in isolated nuclei from untreated PKSV-PR cells (1), untreated PKSV-PR_{col50} cells (2) and CCM A-treated PKSV-PR_{col50} cells (3). The main fluorescence peak corresponded to nuclei in G_0/G_1 phase.

resolve lysosomes from the endoplasmic reticulum, was then performed to measure drug accumulation in the lysosomal compartments of PKSV-PR and PKSV-PR_{col50} cells loaded with 2μ Ci/ml [G - 3H]daunomycin for 60 min at 37°C (Figure 6). The profiles of lysosomal mannosidase and NADPH:cytochrome *c*

Figure 7 Effects of CCM A and bafilomycin A1 on daunomycin sensitivity of drug-sensitive and drug-resistant renal proximal tubule cells

The sensitivity of the parent PKSV-PR cells and selected PKSV-PR_{col50} cells to CCM A, bafilomycin A1 (BFL) and daunomycin was determined by using the MTT assay after exposure to the drugs for 48 h. (A) IC₅₀ values for CCM A and bafilomycin A1. (B) The percentage of viable cells was measured in untreated PKSV-PR cells and in untreated or CCM A- or bafilomycin A1-treated PKSV-PR_{col50} cells incubated with increasing concentrations of daunomycin for 48 h. Values are the means $\pm$ S.E.M. from six to eight separate experiments; **P* < 0.05.

reductase activities revealed two main fractions, one highly enriched with lysosomes (fraction I) and the other with endoplasmic reticulum (fraction II) (Figure 6A). Consistent with the fluorescence studies, a sharp peak of [G-³H]daunomycin was recovered from the lysosome-enriched fraction I from PKSV-PR_{col50} cells (Figure 6B). Parent PKSV-PR cells exhibited a similar profile of daunomycin distribution (results not shown), but the percentage of radioactivity incorporated into cells and recovered from the lysosome-enriched fraction I was only about half as great for PKSV-PR cells as for PKSV-PR_{col50} cells (Figure 6C). Adding 20 nM or 50 nM CCM A to the culture medium during the loading period produced a significant and dose-dependent decrease in the percentage of [G-³H]daunomycin recovered from the lysosome-enriched fraction I of the PKSV-PR_{col50} cells, but not of the PKSV-PR cells (Figure 6C). The results from flow cytometry also showed that the fluorescence intensity of daunomycin accumulated in isolated nuclei from untreated PKSV-PR cells was about twice as great as that in nuclei from untreated PKSV-PR_{col50} cells (Figure 6D). Conversely, preincubating PKSV-PR_{col50} cells with CCM A (20 nM for 24 h), which significantly decreased the amount of daunomycin accumulated in the lysosomes (Figure 6C), caused a 2.2-fold increase in the nuclear fluorescent daunomycin intensity as compared with that in untreated PKSV-PR_{col50} cells (Figure 6D).

Inhibitors of the vacuolar proton pump restore the sensitivity of MDR renal epithelial cells to anthracyclines

The disruption of lysosomal acidification by CCM A caused a marked nuclear redistribution of daunomycin in the drug-resistant PKSV-PR_{col50} cells, without increasing the cellular efflux of the chemotherapeutic drug (see Figure 4). This raises the question of whether proton pump inhibitors also restore the sensitivity of PKSV-PR_{col50} cells to anthracyclines. Cells were first tested for their sensitivity to proton pump inhibitors. The results from the MTT assay showed that PKSV-PR_{col50} cells are about twice as sensitive to CCM A and bafilomycin A1 as PKSV-PR cells (Figure 7A). However, the concentrations of CCM A and bafilomycin A1 used (20–100 nM) did not alter cell viability

Table 1 Effects of CCM A and bafilomycin A1 on relative resistance to anthracyclines

PKSV-PR and PKSV-PR_{col50} cells seeded on 96-well trays were incubated with 10 μ M daunomycin, doxorubicin or epirubicin and without or with 20 nM CCM A or bafilomycin A1 (BFL) for 48 h. IC₅₀ values were determined using the MTT colorimetric assay, and were normalized against values obtained from cells incubated without drugs. Values are the means $\pm$ S.E.M. from (*n*) separate experiments. Fold resistance corresponds to the mean ratio of the IC₅₀ values of PKSV-PR_{col50} cells over those for PKSV-PR cells.

Treatment	IC ₅₀		Fold resistance
	PKSV-PR cells	PKSV-PR _{col50} cells	
Daunomycin	0.6 $\pm$ 0.2 (8)	57.7 $\pm$ 2.3 (6)	96.2
+ CCM A (20 nM)	1.4 $\pm$ 0.1 (6)	23.7 $\pm$ 1.1 (7)	16.9
+ BFL (20 nM)	1.0 $\pm$ 0.3 (5)	5.2 $\pm$ 1.2 (6)	5.2
Doxorubicin	12.3 $\pm$ 1.9 (9)	375.7 $\pm$ 38.7 (9)	30.5
+ CCM A (20 nM)	9.6 $\pm$ 2.1 (8)	14.2 $\pm$ 4.4 (6)	1.5
+ BFL (20 nM)	10.1 $\pm$ 1.4 (8)	17.9 $\pm$ 14.9 (6)	1.8
Epirubicin	21.0 $\pm$ 1.8 (8)	118.9 $\pm$ 14.9 (9)	5.7
+ CCM A (20 nM)	9.5 $\pm$ 1.0 (8)	7.3 $\pm$ 0.4 (8)	0.8
+ BFL (20 nM)	15.9 $\pm$ 3.2 (6)	8.2 $\pm$ 2.0 (6)	0.5

(> 90% viable cells) in either drug-sensitive or -resistant cells. Preincubating the cells with non-cytotoxic concentrations of CCM A (20–100 nM) or bafilomycin A1 (100 nM) restored the sensitivity of the PKSV-PR_{col50} cells to daunomycin, to levels close to that measured in their drug-sensitive PKSV-PR counterparts (Figure 7B). Adding 20 nM CCM A or bafilomycin A1 to the culture medium did not increase the cytotoxicity of anthracyclines in PKSV-PR cells (Table 1). In contrast, both vacuolar H⁺-ATPase inhibitors dramatically lowered the resistance of PKSV-PR_{col50} cells to daunomycin, and completely restored their sensitivity to doxorubicin and epirubicin to the same levels as in parent PKSV-PR cells (Table 1). These findings demonstrate that inhibitors of vacuolar H⁺-ATPase induced a nucleo-cytoplasmic redistribution of weak-base daunomycin within drug-

resistant PKSV-PR_{col50} cells, and restored the sensitivity of these drug-resistant renal epithelial proximal tubule cells to daunomycin, doxorubicin and epirubicin.

DISCUSSION

The findings of the present study show that daunomycin is sequestered in the acidic lysosomes of MDR renal proximal tubule cells, and that inhibitors of proton pumps cause a rapid redistribution of the drug in the cytoplasm and nucleus. In addition, both CCM A and bafilomycin A1 restore the sensitivity of the drug-resistant cells to anthracyclines. The contribution of the intracellular trapping of drugs to the MDR phenomenon is under debate [11,12,14]. The findings of the present study strongly suggest that the sequestration of weak-base therapeutic drugs in acidic organelles does indeed contribute to the acquired drug resistance phenotype of MDR epithelial renal cells. The results from fluorescence studies of living cells reveal that daunomycin is detected mainly, if not exclusively, in the lysosomes of drug-resistant PKSV-PR_{col50} cells. Although several authors have reported that anthracyclines can be detected in other acidic compartments, such as the *trans*-Golgi network or recycling endosomes [7,9,11], all of the fluorescent drugs tested have always been found solely in the more acidic lysosomal compartment of the various MDR cell lines analysed [7–11].

It has been suggested that the intracellular trapping of drugs in the expanded acidic organelle compartments of drug-resistant cells [10,26], plus the active drug export mediated by membranous ATP-dependent P-gps or MRPs, contributes to the reduced accumulation of drugs in MDR cells. Altan et al. [11] have reported that lysosomes and recycling endosomes are not acidified in drug-sensitive MCF-7 breast tumour cells, and that the cytosol of drug-sensitive MCF-7 cells is more acidic than the cytosol of their drug-resistant MCF-7/ADR counterparts [11]. Thus the increase in the pH gradient between the cytosol and acidic organelle compartments in MCF-7/ADR drug-resistant cells favours the protonation and sequestration of weak-base anthracyclines (with a pK_a of around 8) in acidic organelles. As in many MDR tumour cells [36], we also found that the intracellular pH of drug-resistant PKSV-PR_{col50} cells is slightly higher than that of the parent PKSV-PR cells [19]. However, in contrast with the situation reported for drug-sensitive and -resistant MCF-7 cells, the lysosomal compartments of drug-sensitive and -resistant renal proximal tubule cells exhibited quite similar acidic pH_{lys} values. The results from the pH_{lys} values measured using pH-sensitive FITC-dextran incorporated into the lysosomes by fluid-phase endocytosis [20,21] were in complete accordance with the relative fluorescence intensities provided by the pH-dependent LysoSensor Green DND-189 dye. Parent PKSV-PR cells and drug-resistant PKSV-PR_{col50} cells exhibited similar acidic pH_{lys} values, and were both sensitive to the action of CCM A (see Figure 2). These findings therefore indicate that the lysosomes of the parent drug-sensitive, but non-tumoral, PKSV-PR cells did not fail to acidify like the drug-sensitive MCF-7 cells. Weak-base anthracyclines are thought to enter P-gp-expressing cells by pinocytosis and then accumulate passively in the acidic compartments [37]. Thus the greater lysosomal accumulation of daunomycin in drug-resistant PKSV-PR_{col50} cells is consistent with an expanded lysosomal compartment. The fact that CCM A also inhibits the accumulation of daunomycin in lysosomes from PKSV-PR_{col50} cells is consistent with the view that this accumulation is passive and requires acidic lysosomes.

Consistent with their greater content of acidic lysosomal vesicles, the drug-resistant PKSV-PR_{col50} cells exhibited increased levels of secreted NAG and β -glucuronidase lysosomal enzymes.

Similar results have been reported in MDR human CEM/VLB₁₀₀ lymphoblastic cells [32]. These effects appear to be specific for lysosomal enzymes that require the mannose-6-phosphate receptor to be targeted in lysosomes [38,39], since the rate of secretion of AcP, which does not require the mannose-6-phosphate receptor [40], remained low and identical in PKSV-PR and PKSV-PR_{col50} cells. Interestingly, CCM A, which stimulates the secretion of NAG and β -glucuronidase in both untreated and verapamil-treated PKSV-PR_{col50} cells to the same extent, had no effect on the P-gp-dependent secretion of daunomycin. These findings indicate that the rapid alkaline shift of the lysosome induced by CCM A does not stimulate the apical P-gp-dependent secretion of daunomycin.

Thus the abundance of functional acidic lysosomes promotes the lysosomal trapping of protonated anthracyclines in MDR PKSV-PR_{col50} cells. Agents such as protonophores, the weak base chloroquine or inhibitors of the vacuolar H^+ -ATPase that block the acidification of organelles allow the release of anthracyclines from acidic organelles and lead to a substantial accumulation of the drugs in the cytosol and nucleus of MDR cells [10,11]. This enhanced accumulation of the drugs in the cytosol and nucleus should directly affect the cytotoxic nuclear effect of anthracyclines [13]. Our data now provide direct evidence that the inhibition of the vacuolar H^+ -ATPase by CCM A and bafilomycin A1 almost completely abolishes resistance to daunomycin, doxorubicin and epirubicin in the MDR PKSV-PR_{col50} cell line. CCM A does not affect the verapamil-dependent cellular efflux of daunomycin, and so the primary mode of action of vacuolar H^+ -ATPase inhibitors does not seem to require an intracellular P-gp. The fact that the IC_{50} values for CCM A and bafilomycin A1 are lower in PKSV-PR_{col50} cells than in PKSV-PR cells also may suggest that the drug-resistant cells overexpress vacuolar proton pumps. The acidic pH of vesicles is generated by vacuolar H^+ -ATPases that actively translocate protons across the organelle membranes. The differences between the steady-state pH of endocytic and secretory organelles (lysosomes being the most acidic compartment in the endocytic pathway) reflect differences in the regulation of organelle pumps and conductances [41]. Further studies will be needed to find out whether the loss of acidification observed in some drug-sensitive tumour cells is due to a defect in the density or assembly of the vacuolar H^+ -ATPase and/or whether, conversely, vacuolar proton pumps are overexpressed in MDR cells, as has been reported for MDR HL60 cells [42]. In conclusion, the findings of the present study provide direct evidence that the acidic lysosomal compartment of MDR cells offers a potential site for the reversal of anthracycline resistance by lysosomotropic agents.

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