Single Cell Analysis of Daunomycin Uptake and Efflux in Multidrug-resistant and -sensitive KB Cells: Effects of Verapamil and Other Drugs

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ABSTRACT

The accumulation of daunomycin in drug-sensitive and multidrug-resistant human KB cells was examined using light microscopy to detect the inherent fluorescence of daunomycin. Intracellular accumulation of fluorescent drug occurred rapidly in parental KB cells and was markedly reduced in several multidrug-resistant mutants. The addition of verapamil, which reverses multidrug resistance, resulted in increased accumulation of daunomycin in resistant cells. In living cells, most of the daunomycin was found in the nucleus, but significant amounts were detected associated with the plasma membrane, in the cytoplasm, in organelles of the Golgi region, and in lysosomes. The nuclear fluorescence was measured using a photometer system, and the loss of daunomycin from the cells was determined under various conditions. When sensitive cells were allowed to accumulate daunomycin for 5 min at 37°C and then placed in medium without the drug, daunomycin remained inside the nuclei for longer than 1 day. When resistant cells were loaded in the presence of verapamil and the verapamil was removed, the resistant cells lost daunomycin with a half-time of about 1 min. The continuous presence of verapamil markedly inhibited the loss of daunomycin from the cells. Similar results were obtained in separate experiments using [3H]daunomycin. Vinblastine, vincristine, and quinine were also effective in stimulating daunomycin accumulation in multidrug-resistant cells and in preventing the loss of daunomycin from these resistant cells. This effect required half-maximal concentrations of 1-2 μM for verapamil, vinblastine, and quinine. Ouabain, lanthanum, colchicine, amiloride, probenecid, and 1-β-D-arabinofuranosylcytosine had no effect on this process. Quinine was effective at 10μM and nifedipine was effective at 50 μM. Depletion of cellular adenosine triphosphate levels by preincubation of cells with azide and 2-deoxyglucose partially inhibited daunomycin loss from resistant cells. These single-cell measurements indicate that diminished daunomycin accumulation in multidrug-resistant cells results from accelerated energy-dependent efflux across the plasma membrane, and this efflux is inhibited by verapamil, quinine, vincristine, and vinblastine.

INTRODUCTION

Multidrug resistance of human cancer cells is a major problem in chemotherapy. To study this problem, various lines of cultured cells have been isolated which exhibit the multidrug-resistant phenotype (1-6). Cultured cells selected for multidrug resistance usually accumulate less drug (7-11). Many studies on intact cells have used radioactively labeled drugs; these methods do not allow examination of the intracellular compartmentalization of drugs in living cells.

The resistance to drugs in MDR1 cells has been previously shown to be largely reversed by treatment with the Ca2+ channel blocker verapamil and related compounds (11-16) as well as by quinidine (17). This reversal of resistance by verapamil is accompanied by increased accumulation of drugs by the resistant cells (11-17). Our laboratory has been studying multidrug resistance in the human KB carcinoma cell line and we have developed MDR KB cells by selecting with either colchicine, vinblastine, or Adriamycin. In this paper, we have examined the accumulation and loss of daunomycin by these resistant and sensitive KB cells using quantitative morphological methods which allow us to examine single cells. We have also investigated the effects of verapamil, vinblastine, vincristine, quinidine, and inhibitors of ATP synthesis on daunomycin accumulation and release.

MATERIALS AND METHODS

Cells. KB-3-1, KB-C3, KB-C4, KB-V1, and KB-A1 cells were isolated and propagated in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (Gibco), penicillin (50 units/ml), streptomycin (50 μg/ml), and their selecting agent (C4, colchicine, 4 μg/ml; V1, vinblastine, 1 μg/ml; A1, Adriamycin, 1 μg/ml) as described previously (4-6, 18). Cells were plated for uptake studies in 35-mm plastic tissue culture dishes 1 day prior to experiments in media without their selective agent. Relative resistance of cells to various drugs was calculated by comparing 50% lethal doses as described previously (5).

Drugs and Chemicals. Drugs and chemicals were obtained from Sigma. Concentrations used (unless otherwise indicated) were: daunomycin (20 μM); verapamil (20 μM); vinblastine (100 μM); vincristine (100 μM); amiloride (100 μM); probenecid (100 μM); quinidine (10 μM); quinine (20 μM); nifedipine (50 μM); colchicine (100 μM); ouabain (100 μM); lanthanum chloride (10 mm); and 1-β-D-arabinofuranosylcytosine (100 μM). For depletion of ATP, cells were preincubated in 15 mM sodium azide and 50 mM 2-deoxyglucose for 15 min at 37°C in Dulbecco's PBS (8, 9). Experiments with ouabain and lanthanum were performed in 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered saline.

Incubations with Daunomycin. Cells in serum-containing medium were washed and incubated in normal culture medium containing 20 μM daunomycin with or without 20 μM verapamil for 5 min at 37°C in a humidified air-CO2 incubator. The cells were then washed free of daunomycin. In some experiments, incubations were also carried out using buffered saline solutions, and some uptake experiments were performed at 23°C (e.g., Fig. 2). For observations of living cells, the dishes were then washed in PBS containing Ca2+ and Mg2+ at 37°C, overlaid with a 25-mm-diameter No. 1 coverslip, and observed directly under an oil immersion objective on an upright microscope (Zeiss RA; rhodamine epifluorescence optics). Photographs were made using Kodak Tri-X film with Diafine development. For observations of fixed cells, dishes were washed after daunomycin incubations, placed in a second incubation medium when required, and then fixed in 3.7% formaldehyde in PBS for 5 min at 23°C. Fixed cells were mounted in buffered glycerol under a coverslip prior to microscopy.

Epifluorescence Photometry. Dishes of fixed cells were placed on the stage of a Zeiss ICM-405 epifluorescence inverted microscope equipped with rhodamine epifluorescence optics, a ×63 Neofluor N.A. 1.25 objective, an EMI (Model 9658R) photomultiplier (EMI Gencom, Plainview, NY) operated at 1 kV, and a Keithley Model 480 picoammeter. The illumination field was restricted to a 30-μm-diameter circle using an iris diaphragm in the illumination light path. This circle was usually twice the diameter of the nuclei of the cells. Measurements of the fluorescent intensity of 10-20 nuclei in each sample were routinely made, and standard errors of these measurements for each dish are presented in the figures. Variation in intensity measurements from dish to dish of the same type of incubation on the same day was less than 10%. Not
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Fig. 1. Fluorescence visualization of daunomycin accumulation in living and fixed KB-3-1 and KB-C4 cells. KB-3-1 parental, drug-sensitive cells were incubated with daunomycin for 5 min at 37°C and then visualized live (A) or after formaldehyde fixation (C, E). KB-C4 drug-resistant cells were incubated in a similar fashion and viewed live (B) or after fixation (D, F). Daunomycin incubations were performed in the absence (A–D) or presence (E, F) of verapamil. Daunomycin is present in lysosomes (small arrows, ly), as well as in a diffuse accumulation in the perinuclear Golgi region (G) (A, B). After fixation, only the nuclear label is preserved (C–F), but labeling of mitotic chromosomes is also easily seen (C, inset). n, nucleus × 1400; bar, 10 μm.

RESULTS

Accumulation of Daunomycin in KB Cells. Living KB cells were incubated with daunomycin at 37°C and observed using epifluorescence microscopy without fixation. The fluorescence pattern showed a very bright signal in the nucleus (Fig. 1A), a bright signal in the Golgi region of the cell and in lysosomes (lysosomes were identified as dense organelles using phase contrast optics), and a small amount of fluorescence on the plasma membrane and in the cytoplasm. Mitotic chromosomes were also strongly labeled (Fig. 1C, inset), indicating that the accumulation of daunomycin in nuclei was not dependent on the presence of a nuclear envelope. The appearance of fluorescence in lysosomes and the Golgi region, but not the nucleus, was partially inhibited by coincubation with 200 μM chloroquine (results not shown), suggesting that the pH gradient across these intracellular membranes might favor accumulation of daunomycin from the cytosol. The nuclear and chromosomal fluorescence due to daunomycin was retained after fixing these cells in formaldehyde, but fluorescence in the Golgi region, lysosomes, plasma membrane, and the cytoplasm was not well preserved (Fig. 1C). This result suggested that daunomycin bound relatively well to nuclear and chromosomal structures, but not to other structures.
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Fig. 2. Accumulation of daunomycin in nuclei of KB cells. A, KB-3-1 cells were incubated in 20 μM daunomycin for various times at 23°C in either serum-containing culture medium (10% CS Med) or PBS. Each point represents means of 20–30 nuclei measured on the photometer. Bars, SE. In B, KB-3-1 and KB-C4 cells were incubated at different concentrations of daunomycin for 5 min at 37°C. One group of KB-C4 cells were incubated at the same time with 20 μM verapamil (V). Nuclear fluorescence in fixed cells was measured using a ×63 N.A. 1.4 objective to increase sensitivity. Bars, SE.

Fig. 3. Effect of verapamil on accumulation of daunomycin in nuclei of various cell types. KB-3-1, C3, C4, and A1 cells were incubated in daunomycin (20 μM) with or without verapamil (10 μM) for 5 min at 37°C in serum-containing culture medium. After fixation in formaldehyde, the nuclear fluorescence was measured. Bars, SE.

The amount of daunomycin fluorescence in each nucleus was quantitated using a microscope photometer system. Fig. 2A shows the rate of accumulation of daunomycin fluorescence in KB cell nuclei; this experiment was performed at 23°C to slow the accumulation rate so that early time points could be examined. Accumulation occurs more rapidly in complete culture medium than in a buffered saline solution which does not contain glucose, amino acids, or other nutrients. Accumulation was approximately twice as fast at 37°C than at 23°C (results not shown). The uptake appeared to be linear for the first 10 min of incubation at 23°C. Fig. 2B demonstrates the effect of varying the concentration of daunomycin during a 5-min incubation at 37°C. The significance of the results using KB-C4

Fig. 4. Loss of daunomycin from nuclei of KB and KB-C3 cells incubated in the presence or absence of verapamil. After incubation with 20 μM daunomycin in the presence or absence of 10 μM verapamil, KB-C3 or KB-3-1 cells were washed in media and incubated in a daunomycin-free medium in the presence (○) or absence (■) of verapamil at 37°C. Bars, SE.

Fig. 5. [3H]Daunomycin accumulation and efflux in intact cells. KB-3-1 and KB-C4 cells (10⁶ cells/assay) were incubated in Dulbecco's modified Eagle's medium, 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.3, and 12 nM [3H]daunomycin for 30 min at 37°C in the absence (−) or presence (+) of 10 μM verapamil, as described in "Materials and Methods." At 30 min the assay medium was replaced with fresh medium with (+) or without (−) 10 μM verapamil for 10 min, 37°C. After washing and trypsinization, accumulated [3H]daunomycin was measured by liquid scintillation counting. The association of [3H]daunomycin with dishes containing no cells was subtracted from the total measured in the presence of cells. The data are expressed as the means of triplicate determinations and normalized to the level found in KB-3-1 cells at 30 min in the absence of verapamil. Bars, SE.

Fig. 6. Daunomycin accumulation in nuclei of KB-C4 cells incubated with various concentrations of verapamil, vinblastine, and quinidine. Various concentrations (0.1–5.0 μM) of verapamil, vinblastine, or quinidine were mixed with daunomycin (20 μM) and incubated for 5 min at 37°C prior to fixation in formaldehyde and measurement of nuclear fluorescence. Bars, SE.

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Fig. 7. Daunomycin remaining in nuclei after incubation of KB-C3 cells. KB-C3 cells were incubated at 37°C with daunomycin (20 μM) in the presence of verapamil (10 μM) for 5 min and then incubated for 3 min in medium [NaCl-4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] alone at 37°C or with various drugs added to this medium: verapamil (verap.) (10 μM); vinblastine (vinbl.) (100 μM); vincristine (vincr.) (100 μM); 1-β-D-arabinofuranosylcytosine (ara C) (100 μM); ouabain (Ouab.) (100 μM). C, control. Bars, SE.

Fig. 8. Daunomycin remaining in nuclei after incubation of KB-C4 cells with various drugs. KB-C4 cells were incubated with daunomycin in the presence of verapamil (control (C)) and then incubated in complete culture medium containing 10% calf serum. As for Fig. 7, various drugs were added to this medium with no additions or with verapamil (verap.) (10 μM), quinidine (10 μM), vinblastine (vinbl.) (100 μM), or colchicine (colch.) (100 μM). Bars, SE.

Table 1 Relative resistance of MDR cell lines

<table>
<thead>
<tr>
<th>Drug</th>
<th>KB-3-1</th>
<th>KB-C3</th>
<th>KB-C4</th>
<th>KB-A1</th>
<th>KB-V1</th>
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<td>Colchicine</td>
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<td>262</td>
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<td>422</td>
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<tr>
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<td>141</td>
<td>254</td>
<td>97</td>
<td>422</td>
</tr>
<tr>
<td>Daunomycin</td>
<td>1</td>
<td>75</td>
<td>76</td>
<td>76</td>
<td>76</td>
</tr>
<tr>
<td>Daunomycin + verapamil</td>
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<td>0.76</td>
<td>0.76</td>
<td>0.76</td>
<td>0.76</td>
</tr>
<tr>
<td>1-β-D-Arabinofuranosylcytosine</td>
<td>1</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Ouabain</td>
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<td>0.68</td>
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<sup>a</sup> Fold resistance with respect to KB-3-1 (sensitive) cells.

Accumulation of Daunomycin in Multidrug-resistant KB Cells. Living KB-C4 cells incubated with daunomycin showed a significantly reduced amount of daunomycin fluorescence in their nuclei (Fig. 1B) compared to wild type sensitive cells (Fig. 1A). In contrast, fluorescence in Golgi and lysosomes of resistant cells (Fig. 1B) was at least as prominent as in sensitive cells (Fig. 1A). The poor accumulation in nuclei of resistant cells was also very evident after formaldehyde fixation (Fig. 1D), and, as for sensitive cells, fluorescence in Golgi and lysosomes disappeared after fixation. If these drug-resistant KB-C4 cells were incubated with daunomycin in the presence of verapamil, however, the accumulation in nuclei was substantially improved (Fig. 1F). The Ca<sup>2+</sup> channel blocker verapamil is one of several drugs which reverse the MDR phenotype of KB-C4 cells (Table 1). Quantitation of this accumulation of daunomycin fluorescence demonstrated that while verapamil had little effect on accumulation of daunomycin fluorescence in wild type cells, incubation of resistant cells with verapamil and daunomycin significantly increased the fluorescence in nuclei (Figs. 2B and 3). This effect was seen with the MDR mutants KB-C3, KB-C4, KB-V1 (not shown), and KB-A1 which were initially selected for resistance to colchicine, vinblastine, and Adriamycin, respectively (Fig. 3). Similar results were seen in KB-C4 cells with and without verapamil as described below. Using a more sensitive (N.A. 1.4) objective, this experiment showed that reproducible comparisons of daunomycin accumulation in 5-min incubations could be made at concentrations as low as 0.2 μM.

EXTRACELLULAR SPACE

PLASMA MEMBRANE

NUCLEUS

EFFLUX PUMP

Energy

CYTOSOL

GOLGI - LYSOSOME

Fig. 10. Scheme of daunomycin accumulation and efflux in multidrug-resistant cells.
at concentrations of daunomycin as low as 0.2 μM.
Pulse-Wash Experiments with Daunomycin and [3H]Daunomycin in Resistant Cells. A direct way to examine the presence of accelerated efflux in the resistant cells was to measure the loss of daunomycin from cells. The problem was that, in these cells, daunomycin alone did not accumulate and label nuclei well. However, when cells were coincubated with daunomycin and verapamil, enough daunomycin accumulated so that the nuclei labeled well enough to examine the loss of this label.

Wild type cells lost daunomycin from their nuclei at a very slow rate (half-time, >12 h; results not shown and Fig. 4; the decline in fluorescence shown in the single experiment in Fig. 4 is not statistically significant). Verapamil had no effect on this process. In KB-C3 cells loaded with daunomycin in the presence of verapamil, the rate of efflux of daunomycin in medium lacking daunomycin was found to be very rapid with a half-time of loss of approximately 1 min at 37°C (Fig. 4). When daunomycin release was studied in the presence of verapamil, the rate of daunomycin release slowed dramatically. This experiment suggests that these resistant cells have a very active efflux mechanism compared to wild type cells and that this efflux can be partially inhibited by verapamil. Inhibition of efflux by verapamil allows accumulation of daunomycin in nuclei and prevents loss of previously accumulated daunomycin from the cells.

To confirm that measurements of daunomycin fluorescence were accurate means of measuring daunomycin association with cells, we repeated these efflux experiments using [3H]daunomycin. As shown in Fig. 5, accumulation of [3H]daunomycin was reduced in the MDR cell line KB-C4 compared to parental KB-3-1 cells. Verapamil addition restored this accumulation closer to wild type levels. In washout experiments, removal of verapamil resulted in a marked increase in loss of [3H]daunomycin from resistant cells; this loss was prevented when efflux was examined in the presence of verapamil.

Effects of Various Drugs on Daunomycin Accumulation and Efflux. An experiment to determine the amount of verapamil, quinidine, and vinblastine required to enhance daunomycin accumulation in KB-C4 cells is shown in Fig. 6. Verapamil, vinblastine, and quinidine showed very similar effects on daunomycin accumulation with half-maximal enhancement of daunomycin accumulation at approximately 1–2 μM. Quinine showed a similar effect at a half-maximal concentration of 10 μM, while nifedipine enhanced accumulation half-maximally at 50 μM (results not shown).

We also examined the effects of various drugs on efflux in both KB-C3 and KB-C4 cells (Figs. 7 and 8). Vinblastine and vincristine, to which these cells are cross-resistant (see Table 1), and quinidine significantly slowed efflux, whereas 1-β-D-arabinofuranosylcytosine (100 μM) and ouabain (100 μM), to which these cells are sensitive (Table 1), as well as lanthanaum (10 mM) had no biologically significant effects at the concentrations tested. Although these cells were selected in colchicine and are cross-resistant to colchicine, colchicine had no measurable effect on daunomycin efflux.

Energy-Dependence of Efflux. After a 15-min preincubation in 15 mM sodium azide and 50 mM 2-deoxyglucose to deplete ATP pools (8, 9), KB-C3 cells were incubated with daunomycin in the presence of verapamil. These treated cells accumulated slightly more daunomycin than KB-C3 cells daunomycin loaded with verapamil alone (compare Columns A and B in Fig. 9), suggesting that the uptake of daunomycin is not strongly energy dependent. When KB-C3 cells preincubated with azide-2-deoxyglucose were incubated with daunomycin and verapamil and then placed in daunomycin-free medium in the absence of verapamil or azide-2-deoxyglucose, but in the presence of 20 mM glucose to regenerate ATP, the rate of efflux was rapid (Fig. 9C). However, if efflux was measured in the continued presence of azide-2-deoxyglucose without glucose, the rate of efflux was slow (Fig. 9D). These results suggest that the uptake of daunomycin is not energy dependent, but the accelerated efflux of daunomycin in these resistant cells is energy dependent.

DISCUSSION

Previous studies using radioactively labeled drugs have shown that multidrug-resistant cells have a decreased accumulation of drugs and that this decrease is due to some extent to an accelerated efflux of drugs across the plasma membrane (8, 16, 19). Measurements of total cellular accumulation of labeled drugs may not always be indicative of cytoplasmic concentrations if cells have other mechanisms of sequestration of these drugs in membrane-limited intracellular compartments. For this reason, direct morphological detection of drugs inside intact cells can yield important information on the intracellular distribution of drugs in living cells. Other investigators have previously shown that daunomycin accumulates in nuclei using fluorescence techniques similar to those shown in this paper (20). Chauffert et al. (21) demonstrated qualitative effects of verapamil and energy inhibitors on accumulation and efflux of Adriamycin in a drug-resistant rat colon carcinoma cell line. We have extended these observations in comparing resistant and sensitive human cell lines to show morphologically and quantitatively that daunomycin accumulation is defective in resistant cells and that the most obvious decrease occurs in the cell nucleus. Verapamil, quinidine, vincristine, and vinblastine have been shown to inhibit daunomycin efflux, thus enhancing the accumulation of daunomycin in these resistant cells. We also have shown that efflux is inhibited by azide-2-deoxyglucose, suggesting that efflux is energy dependent.

The most likely explanation of these findings is that daunomycin enters cells passively in an energy-independent process; this entry is similar in both resistant and sensitive cells. However, while the sensitive cells continue to accumulate drug in their cytoplasm, the resistant cells have an ATP-dependent efflux system that rapidly removes the drug from the cytoplasm, preventing a high cytoplasmic concentration. The binding of a drug such as daunomycin to nuclei reflects an equilibrium with daunomycin in the cytoplasm. The nuclear membrane apparently has no role in this process, because daunomycin was concentrated in mitotic chromosomes which are not surrounded by a nuclear envelope. Resistant cells can apparently remove daunomycin from the cell so that a high cytoplasmic concentration is never achieved and nuclear fluorescence cannot occur. An alternate explanation of these results, that loss of nuclear fluorescence represents quenching of fluorescence and not efflux of drug, has been shown to be incorrect by demonstration of loss of [3H]daunomycin from cells in a manner that parallels loss of fluorescence (Fig. 5). However, the fluorescence of daunomycin in living cells seems to be slightly less than that seen in these same cells after formaldehyde fixation (results not shown); this suggests that formaldehyde may allow daunomycin to be quenched less by its environment. It is also possible that the binding of daunomycin to nuclei in living cells is decreased in resistant cells and that nuclei of normal cells bind the drug more tightly. This possibility seems unlikely because of the broad cross-resistance of MDR cells to other drugs with no
affinity for the nucleus and the finding that prefixation of cells with formaldehyde or permeabilization with saponin completely eliminates the nuclear fluorescence differences between sensitive and resistant cells (data not shown). We favor the idea that the major alteration in resistant cells is in the plasma membrane efflux system, since the process is energy dependent and inhibited by membrane-interactive drugs such as verapamil. One possible scheme describing these relationships is shown in Fig. 10.

The apparent increase in accumulation of drug observed in the lysosomes and Golgi elements of drug-resistant cells (Fig. 1B) may reflect more than the concentricative effect of the pH gradient in these organelles. Since these cells appear to have a plasma membrane efflux pump that is very active, it is also possible that components of this same pump may occur in the membranes of lysosomes and Golgi elements which functionally mix in some ways with the plasma membrane. The orientation of such a pump would be such that the efflux that normally occurs to the outside of the cell would occur towards the inside of the lysosomal lumen. A low level of pump activity could result in a significant accumulation of drug in the lysosomal lumen or in the lumen of any other membranous organelles that contain this pump activity. Since a fixation-stable binding sink does not exist in these organelles, destruction of membrane barriers after fixation would release this accumulated drug into the medium. This is likely to be the reason we detect daunomycin only in nuclei after fixation.

The results shown in this paper indicate that verapamil, vinblastine, and vincristine can inhibit efflux of daunomycin across the plasma membrane. One explanation for their action would be inhibition of the binding of daunomycin to an efflux channel or a binding site on the inside of the plasma membrane. Cornell et al. (22) have recently reported that vinblastine binds to membranes from MDR cells and this binding is inhibited by vincristine, verapamil, and daunomycin, but not by colchicine. The inability of 100 µM colchicine to inhibit daunomycin efflux (Fig. 8) and vinblastine binding (21) is unexpected, since KB-C3 and KB-C4 cells are resistant to colchicine (Table 1) and do not accumulate it. Perhaps colchicine has a very low affinity for the efflux channel binding site shared by daunomycin and the Vinca alkaloids vinblastine and vincristine.

The protein(s) responsible for accelerated efflux has not been identified, but one candidate for this activity is a M, 150,000–170,000 membrane protein that is labeled with a photoaffinity label of vinblastine (23). This protein may be related to the M, 170,000 glycoprotein described by Juliano et al. (24) which is increased in amount in membrane vesicle preparations from MDR KB mutants (6). The localization of such a protein or proteins to the plasma membrane would be consistent with a role in drug efflux. The data in this paper raise the possibility that this protein may also be found in other intracellular membranes such as Golgi elements or lysosomes. The localization of such putative efflux pumps should be of help in understanding their role in mediating the multidrug resistance phenotype.

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