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A Comparison of Rhodamine 123 Accumulation and Efflux in Cells with P-Glycoprotein-mediated and MRP-associated Multidrug Resistance Phenotypes

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Rhodamine 123 (Rh123) is a fluorescent dye which locates in the mitochondria of cells. It is a substrate for P-glycoprotein (Pgp) and can, therefore, be used as a molecular probe in studies of the multidrug resistance (MDR) phenotype. However, not all MDR cells overexpress Pgp. In some, the MDR phenotype is associated with expression of an alternative transporter molecule, the multidrug resistance-associated protein (MRP). We have studied the accumulation and efflux of Rh123 in MDR cells having both Pgp-mediated and MRP-associated phenotypes. In the mouse tumour parental cell line, EMT6/P, Rh123 accumulates rapidly to reach plateau levels by 90 min. Confocal microscopy confirms a localisation to the mitochondria. In the MDR subline, EMT6/AR1.0, which overexpresses Pgp and which is 10-fold resistant to Rh123 cytotoxicity, accumulation is dramatically reduced. Efflux of Rh123 from both resistant and parental lines is rapid but can be inhibited by reduced temperature or by the presence of cyclosporin A (5 μg/ml). Efflux from the parental line is probably due to the presence of very low, but detectable, levels of Pgp but the existence of other mechanisms cannot be ruled out. In contrast, the human lung cancer parental cell line COR-L23/P, and its MRP-associated (but Pgp-negative) MDR subline, COR-L23/R (which is 23-fold resistant to Rh123 cytotoxicity), accumulate Rh123 at similar rates for the first 30 min. The curves then diverge so that, at 180 min, the resistant cells contain only 70% of the Rh123 of parental cells. Confocal microscopy demonstrates a similar distribution of fluorescence in resistant and parental cells. Essentially no efflux of Rh123 occurs from parental cells, whereas 70% of the content is lost from resistant cells over a period of 150 min. Such efflux may again be inhibited by reduced temperature but cyclosporin A (5 μg/ml) has little effect. These observations should be borne in mind when interpreting Rh123 efflux data in terms of MDR mechanisms.

Key words: cellular pharmacokinetics, rhodamine 123, P-glycoprotein, MRP, multidrug resistance
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INTRODUCTION

CANCER CELL lines treated *in vitro* with one of a group of amphipathic cytotoxic drugs frequently assume a multidrug resistant (MDR) phenotype [1]. Such cells are cross-resistant to other drugs within the group, which include doxorubicin, daunorubicin, vincristine, vinblastine, actinomycin D, etoposide and paclitaxel. In many MDR cells, resistance is mediated by hyperexpression of P-glycoprotein (Pgp), a putative energy-dependent efflux "pump" located at the plasma membrane. The result of this hyperexpression is that lower intracellular drug levels are achieved and hence cytotoxicity is reduced. A number of MDR cell lines have, however, been reported in which there is no hyperexpression of Pgp [2–5]. In some of these, high levels of a 190-k protein are seen [2, 5]. Recently, a gene has been isolated from a non-Pgp-mediated MDR cell line and sequenced [6]. The gene codes for a protein with a high degree of homology to Pgp, having two nucleotide-binding folds and 12 membrane-spanning regions. This protein has been termed the "multidrug resistance-associated protein" (MRP) by the group which carried out the sequencing [6]. We believe that the MRP is a component of the 190-k protein band detected by rabbit polyclonal anti-sera in several non-Pgp MDR cell lines [2, 5].

In addition to the cytotoxic drugs listed above, a variety of other chemicals act as substrates for Pgp. Amongst these are a number of fluorescent dyes which can be used as molecular probes for the study of MDR. One such probe, rhodamine 123 (Rh123), which selectively locates in mitochondria [7], has been previously shown to be effluxed more efficiently by MDR cells and to be relatively non-toxic [8, 9]. Such efflux could be inhibited by verapamil and other established modulators of MDR [9, 10]. Recently, it has been suggested that use of Rh123 together with flow cytometry can provide a useful approach to the determination of Pgp activity in human haemopoietic cells

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[11–13]. Although Pgp expression undoubtedly occurs in haemopoietic cells, it has also been reported that mononuclear blood cells contain high levels of mRNA from the MRP gene [6]. The possibility thus arises that the cellular pharmacokinetics of Rh123 in such cells may be influenced as much by MRP gene expression as by Pgp.

In this paper, we describe experiments carried out to examine the kinetics of Rh123 accumulation and efflux in a cell line which hyperexpresses MRP. The results are compared with those obtained in cells having a classical Pgp-mediated MDR phenotype.

MATERIALS AND METHODS

Cell lines and culture conditions

The present study used the murine mammary tumour cell line EMT6/P and the human large cell lung cancer cell line COR-L23/P, alongside their MDR sublines EMT6/AR1.0 and COR-L23/R, respectively [5, 14]. Both MDR sublines were derived by growth of parental cells in increasing concentrations of doxorubicin over a period of 8 weeks (EMT6/AR1.0) or 7 months (COR-L23/R). The EMT6/AR1.0 subline has a classical MDR phenotype, overexpressing P-glycoprotein and the mouse mdr1a gene [14, 15]. By contrast, the COR-L23/R subline does not overexpress P-gp but has high levels of a 190-k protein and overexpresses the MRP gene [5, 16, 17].

The EMT6 cell lines were maintained as monolayers in Eagles' minimal essential medium with Earles' salts and with 20% new born calf serum (Gibco Biocult, Paisley, U.K.) in 75-cm^2 flasks with penicillin and streptomycin (at concentrations of 100 U/ml and 100 μg/ml respectively). Stock cultures were grown in an atmosphere of 92% air, 8% CO_2 at 37°C. EMT6/AR1.0 was maintained in 1.0 μg/ml doxorubicin, with drug removal at least 2 days before use in experiments. The COR-L23 cell lines were maintained as monolayer cultures in RPMI medium (Gibco Biocult) with 10% fetal calf serum (Sigma, Poole, Dorset, U.K.). COR-L23/R was maintained in 0.2 μg/ml doxorubicin, but this was again removed at least 2 days before experiments. Other culture conditions were as for the EMT6 cell lines.

In order to obtain single cell suspensions, EMT6 cell line monolayers were subjected to two rinses with 0.1% trypsin in phosphate buffered saline (PBS) followed by a 15-min incubation at 37°C. Cells were then resuspended in complete Eagle's medium by repeated pipetting and subsequently counted using a haemocytometer. The COR-L23 cell lines were subjected to two rinses with trypsin (0.4%) and versene (0.02%) in PBS and incubated for 15 min. The cells were then reduced to a single cell suspension and counted as before.

Chemicals

Rh123 was obtained from Sigma. Cyclosporin A (CsA) was kindly supplied by Sandoz (Basle, Switzerland). Verapamil was from Abbot Laboratories (Queenborough, Kent, U.K.).

Chemosensitivity assay

Sensitivity of cell lines to Rh123 was determined using the MTT colorimetric assay as described previously [14, 18]. For the EMT6 cell lines, a 3-day assay was used, whereas a 6-day assay was used for the COR-L23 cell lines. The assay durations allow an approximately 10-fold increase in numbers of control cells. Rh123 was added 2 h after cell inoculation and the medium was unchanged throughout the assay period. Where the resistance modifying effect of CsA (5 μg/ml) was to be determined, this agent was added 1 h before Rh123. The IC_{50} (dose of Rh123 to reduce final absorbance to 50% of control) was read from dose–response curves as described previously [14].

Flow cytometry

Cells in exponential growth were reduced to single cell suspensions as above. Suspensions were diluted in their respective growth media (pH 7.2) to a concentration of 2×10^5 cells/ml and stored on ice. Thirty minutes before the start of experiments (except in one experiment where a longer time of 150 min was used), cell suspensions in plastic tubes were warmed to 37°C and maintained at this temperature on a roller platform in the warm room. Rh123 dissolved in sterile distilled water was added at time zero to produce a final concentration of 0.1 or 1.0 μg/ml. At various times afterwards, aliquots of 0.5 ml were removed, placed in Eppendorf tubes, and the cells rapidly pelleted on a microcentrifuge (2 min at 3000 *g*) at room temperature. After removal of the supernatant, cells were resuspended in 0.5 ml of ice-cold medium and the tubes were then kept on ice until cells were analysed by flow cytometry. Each time the tubes were opened, they were re-gassed with a mixture of 5% CO_2, 95% air to maintain the pH of the medium at 7.2.

At the 30-min time point, a larger volume of cell suspension was centrifuged (2 min at 200 *g*) and the cells resuspended in an equal volume of Rh123-free medium at 37°C. Portions of this suspension were removed and either placed on ice or supplemented with CsA (5 μg/ml). The remaining suspension, together with the portion containing CsA, were then returned to the roller platform at 37°C. Aliquots of these suspensions were also removed at various time intervals, centrifuged, resuspended in fresh medium and stored on ice as previously. In two experiments with COR-L23/R cells, the effect of verapamil (5 μg/ml) on Rh123 efflux was compared with that of CsA.

Cells were analysed (10 k cells per sample) on the Cambridge flow cytometer [19]. Excitation was by an argon laser operating at 488 nm and analysis of fluorescence was at 515–560 nm. Cells of the various lines which had not been exposed to Rh123 were used to determine the background of autofluorescence under these conditions.

Confocal microscopy

Single-cell suspensions resulting from trypsin/versene treatment and containing 5×10^4 cells/ml in 2-ml aliquots were allowed to attach overnight at 37°C on to glass coverslips in six-well multiplates. The growth medium was then removed and cells incubated at 37°C for 1 h with 2-ml aliquots of medium containing Rh123 at concentrations of 0.1 or 1.0 μg/ml. For the COR-L23 cell lines, an experiment using a 3-h incubation period was also carried out. Following a quick rinse in PBS, the coverslips with cells attached were inverted and mounted in PBS on glass slides, the edges being sealed to prevent drying out. The slides were then kept on ice until viewed under the confocal microscope, generally within 10 min of preparation. Fluorescence was observed with the Biorad MRC-600 laserscan confocal microscope (Biorad Ltd, Hemel Hempstead, U.K.) incorporating a Nikon Optiphot 2 microscope with a 60 × objective. The 488-nm line from the argon laser was used for excitation in the Biorad BHS filter block which allows detection of emitted light at all wavelengths above 515 nm. Pictures obtained by averaging the images from five separate scans were stored on optical disc and photographed from a high intensity screen.

RESULTS

Cellular sensitivity to Rh123

Data from a series of experiments to determine the sensitivity of the four cell lines to continuous incubation with Rh123 are shown in Table 1. It may be seen that the parental COR-L23/P cells were approximately 30-fold more sensitive than the parental EMT6/P cells. Both of the MDR sublines showed resistance to Rh123 compared to their respective parental lines, the mean resistance factors (i.e. ratio of IC_{50} of resistant versus parental cells) for COR-L23/R and EMT6/AR1.0 being 23 and 10, respectively.

Both parental EMT6/P and its MDR subline EMT6/AR1.0 showed considerable sensitisation to Rh123 by the addition of 5 μg/ml CsA. Mean sensitisation ratios (i.e. ratio of IC_{50} in the absence versus presence of CsA) were 30 and 8, respectively. Hence, in the presence of CsA, the mean resistance factor for EMT6/AR1.0 was 51 compared with 10 in the absence of the modifier. By contrast, relatively small effects of CsA were seen in the COR-L23 cell lines. Mean sensitisation ratios were 1.1 and 1.8 in the parent and resistant lines, respectively.

Rh123 accumulation and efflux

Accumulation and efflux of Rh123 from COR-L23/P and COR-L23/R cells as determined by flow cytometry are shown in Figure 1a. The accumulation curves for the two cell lines were similar for the first 30 min, but then separated with the parental cells showing more accumulation, the ratio of cellular contents being around 1.5 at 180 min. There was little or no efflux of Rh123 from parental cells over a period of 150 min, whereas the resistant cell line lost 70% of its Rh123 content over this time. The shapes of these curves were unchanged when the pre-incubation time at 37°C before Rh123 addition was increased from 30 to 150 min.

A quite different pattern was seen in the EMT6 cell lines (Figure 1b). The parental cells accumulated Rh123 rapidly, reaching a plateau by 90 min. The resistant cells, however, showed little or no increase in accumulation between 1 and 180 min. At 180 min, the relative Rh123 content was 5.2-fold lower in resistant than in parental cells. If the loading concentration of Rh123 was increased to 1.0 μg/ml for EMT6/AR1.0, the initial accumulation was greater but again did not increase with time.

Independent repeat experiments produced very similar accumulation and efflux curves to those shown for the four cell lines. Ratios of Rh123 content of resistant versus parental cells in the two independent experiments are shown in Table 2. The fluorescence of the different cell lines as shown in Figures 1a and 1b is expressed in arbitrary units and cannot, therefore, be directly compared as different amplifier settings were used in experiments with different cell lines. However, in a direct comparison using identical amplifiers settings, we found the Rh123 content of COR-L23/P cells at 1 h to be approximately 7-fold greater than that of EMT6/P cells.

The effects of reduced temperature or of CsA (5 μg/ml) on efflux of Rh123 from the cell lines are shown in Table 3. In COR-L23/P, there was minimal efflux even at 37°C. There did appear to be a somewhat greater efflux at 37°C by 150 min in the presence of CsA. In the MDR subline COR-L23/R, efflux reduced the Rh123 content at 37°C to 43 and 22% of the starting value at 60 and 150 min, respectively. The efflux was greatly reduced at 0°C, but CsA had little if any effect at 37°C. However, 5 μg/ml verapamil had a somewhat greater inhibitory effect on Rh123 efflux from COR-L23/R than 5 μg/ml CsA (data not shown).

In parental EMT6/P cells, there was extensive efflux. This was considerably reduced at 0°C or in the presence of CsA. The data for the EMT6/AR1.0 cell line are rather variable, probably because of the very low starting level (only twice the autofluorescence background). Clearly, however, there was again extensive efflux which was reduced at 0°C or in the presence of CsA. As the rate of efflux of Rh123 from both parental and resistant EMT6 lines was very rapid, we examined the time course over the first 30 min in more detail. Data from one such experiment are shown in Figure 1c. In this experiment, EMT6/P cells were loaded at 0.1 μg/ml Rh123 and EMT6/AR1.0 cells at 1.0 μg/ml Rh123 in order that approximately equal fluorescence was achieved following 30 min accumulation. During the initial spin down, resuspension in Rh123-free medium and sampling of the resuspended cells, there was a loss of 58% of the fluorescence from the resistant cells but only 14% from the parental cells. After 10 further min of efflux, 61% of the initial fluorescence remained in the parental cells but only 13% in the resistant cells. In a repeat experiment the corresponding figures for the 10-min time point were 75 and 25%, respectively. It is clear, therefore, that the initial rate of loss of fluorescence is considerably greater in the resistant than in the parental cells.

Table 1. Sensitivity of cells to rhodamine 123 in the absence or presence of cyclosporin A (5 μg/ml)

Cell lines	Parental line IC_{50}* (μg/ml) −CsA	Parental line IC_{50}* (μg/ml) +CsA	Parental line Sensitisation ratio†	Resistant line IC_{50} (μg/ml) −CsA	Resistant line IC_{50} (μg/ml) +CsA	Resistant line Sensitisation ratio	Resistance factor‡ −CsA	Resistance factor‡ +CsA
COR-L23	0.36§	0.20	1.8	6.4	3.6	1.8	17.8	18.0
	0.16	0.28	0.6	6.1	2.9	2.1	38.1	10.4
	0.29	0.29	1.0	4.1	2.8	1.5	14.1	9.7
EMT6	7.9	0.35	22.6	>50.0	7.5	>6.7	>6.3	21.4
	8.3	0.35	23.7	80.0	26.0	3.1	9.6	74.3
	7.6	0.28	27.1	93.0	6.3	14.8	12.2	22.5
	9.4	0.20	47.0	110.0	17.4	6.3	11.7	87.0

* IC_{50} = concentration of Rh123 to reduce final optical density to 50% of control in the MTT assay (see Materials and Methods). †Sensitisation ratio $= \frac{IC_{50}(-CsA)}{IC_{50}(+CsA)}$. ‡ Resistance factor $= \frac{IC_{50}(\text{resistant line})}{IC_{50}(\text{parental line})}$. §Each line of data is from an independent experiment with quadruplicate wells at each dose point.

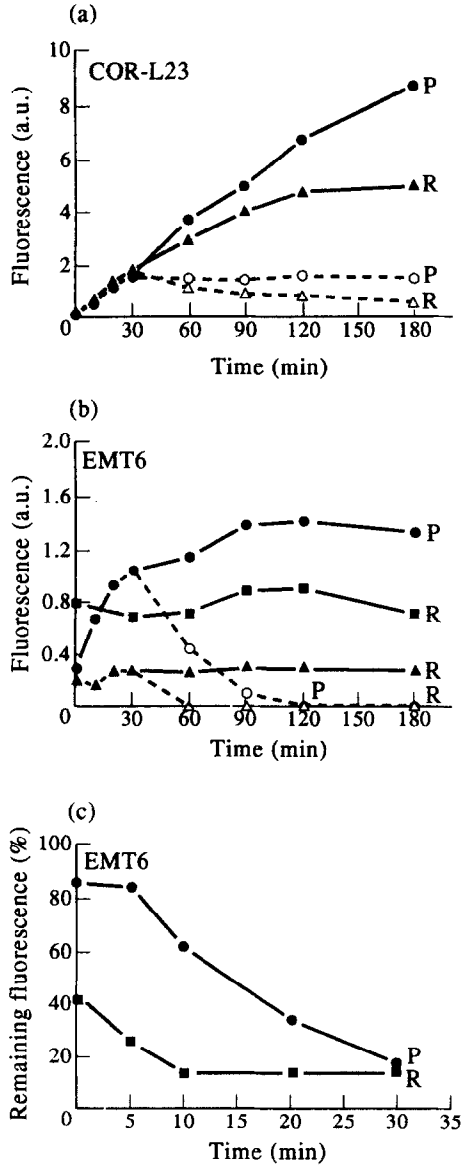

Figure 1. Accumulation (solid symbols, solid lines) and efflux (open symbols, broken lines) of Rh123 in (a) COR-L23/P (circles) and COR-L23/R (triangles) and (b) EMT6/P (circles) and EMT6/AR1.0 (triangles and squares). P, parental cell line; R, MDR-resistance sublines. The exposure concentration of Rh123 was 0.1 μg/ml, except for the squares in (b) where a concentration of 1.0 μg/ml was used. Data are from single experiments for each pair of cell lines with 10 k cells analysed at each point. Repeat experiments gave similar results (see Table 2). (c) Efflux from EMT6/P and EMT6/AR1.0 over the first 30 min. The fluorescence at zero time is expressed as a percentage of that measured at 30 min under accumulation conditions, before centrifugation and resuspension in warm, drug-free medium.

Subcellular distribution

Confocal microscope images of the four cell lines exposed to Rh123 (0.1 μg/ml) for 1 h are shown in Figures 2–4. Figure 2a and b shows Nomarski phase contrast and fluorescence image, respectively, of a single cell of the COR-L23 parental cell line. It may be seen the fluorescence was confined to bodies within the cytoplasm with a distribution consistent with the expected localisation in mitochondria. Figure 3a and b shows lower magnification images, taken at equal instrument settings, of COR-L23/P and COR-L23/R cells. The images are essentially identical, the subcellular distribution of fluorescence being the same and the overall intensity of fluorescence similar. We also examined COR-L23 parental and MDR cells after a loading period of 3 h. Qualitatively, the distribution of fluorescence was similar to that shown in Figure 3. A greater intensity of fluorescence in parental compared with resistant cells was, however, apparent at this time.

Table 2. Rhodamine 123 accumulation ratios in parental versus resistant cells at different times

Cell lines	Accumulation ratio* 30 min	90 min	180 min
COR-L23/P / COR-L23/R	0.88, 0.93†	1.27, 1.43	1.43, 1.54
EMT6/P / EMT6/AR1.0	3.8, 5.6	4.7, 5.8	5.2, 9.5

* Accumulation ratio = $\frac{\text{median fluorescence in parental cells}}{\text{median fluorescence in resistant cells}}$. † Results from two independent experiments are shown.

By contrast, images of EMT6/P and EMT6/AR1.0, again taken at equal instrument settings, are shown in Figure 4a and b. The resistant cells showed dramatically-reduced fluorescence compared with the parental cells. If equivalent cells were examined using reduced neutral density filtration in the microscope (Figure 4c), there was still a little discrete mitochondrial fluorescence, but a more diffuse cytoplasm fluorescence was seen. If, however, EMT6/AR1.0 was exposed to a 10-fold higher dose of Rh123 (i.e. 1.0 μg/ml) but images obtained at the original instrument settings (i.e. as used in Figure 4a and b), the distribution shown in Figure 4d was obtained. The fluorescence distribution was more similar to that seen in the parental cells, being located in discrete cycloplasmic bodies without the higher level of diffuse cytoplasmic fluorescence.

DISCUSSION

In previous studies, we have shown that the mouse tumour cell line EMT6/AR1.0 has a classic MDR phenotype [14]. It is 40–70-fold resistant to doxorubicin, vincristine, colchicine and etoposide [14], hyperexpresses P-glycoprotein as demonstrated by western blotting and immunocytochemistry [14, 15], hyperexpresses the mouse *mdr*1a gene [15] and shows approximately 10-fold reduced accumulation of tritium-labelled daunomycin compared with the parental cells [14]. The parental cell line

Table 3. Efflux of rhodamine 123 from parental and resistant cells under various conditions

Cell lines	% of Rh123 remaining* 60 min 37°C	0°C	37°C+CsA	150 min 37°C	0°C	37°C+CsA
COR-L23/P	90, 72†	98, 79	99, 78	89, 70	83, 77	71, 56
COR-L23/R	46, 41	88, 85	60, 57	25, 19	76, 83	21, 28
EMT6/P	19 (4)‡	66 (20)	63 (19)	12 (4)	52 (11)	58 (8)
EMT6/AR1.0	13 (6)	50 (11)	68 (20)	22 (14)	26 (4)	50 (19)

* The 100% value is that after 30 min of accumulation and before resuspension in Rh123-free medium (see Materials and Methods). †For the COR-L23 cell lines, values from two independent experiments are shown. ‡For the EMT6 cell lines, means and S.E. from five independent experiments are shown.

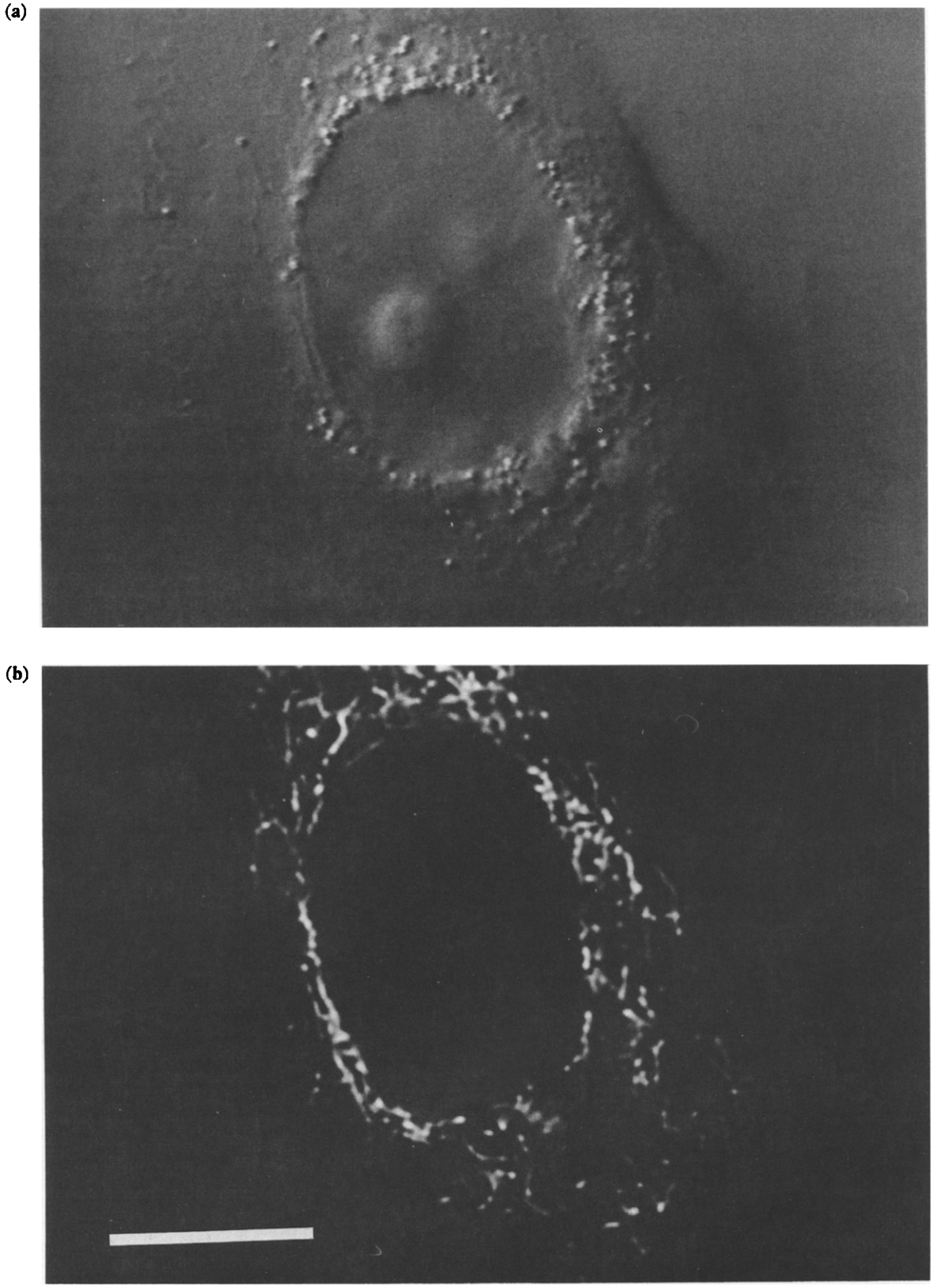

Figure 2. Nomarski phase contrast (a) and fluorescence (b) confocal microscope images of a single cell of the COR-L23/P line exposed for 1 h to Rh123 (0.1 μg/ml). Bar = 20 μm.

EMT6/P, although Pgp-negative by standard western blotting and immunocytochemistry techniques, does show low level expression of the protein (as demonstrated by overexposure of western blots) [14] and of *mdr*1a mRNA (as detected by reverse transcriptase PCR) [15]. The line can be sensitised by verapamil or by CsA, and this sensitisation is associated with increased drug accumulation [14].

By contrast, the human lung cancer cell subline COR-L23/R has an MDR phenotype which does not involve Pgp or overexpression of the human MDR1 gene [5, 16]. It is also

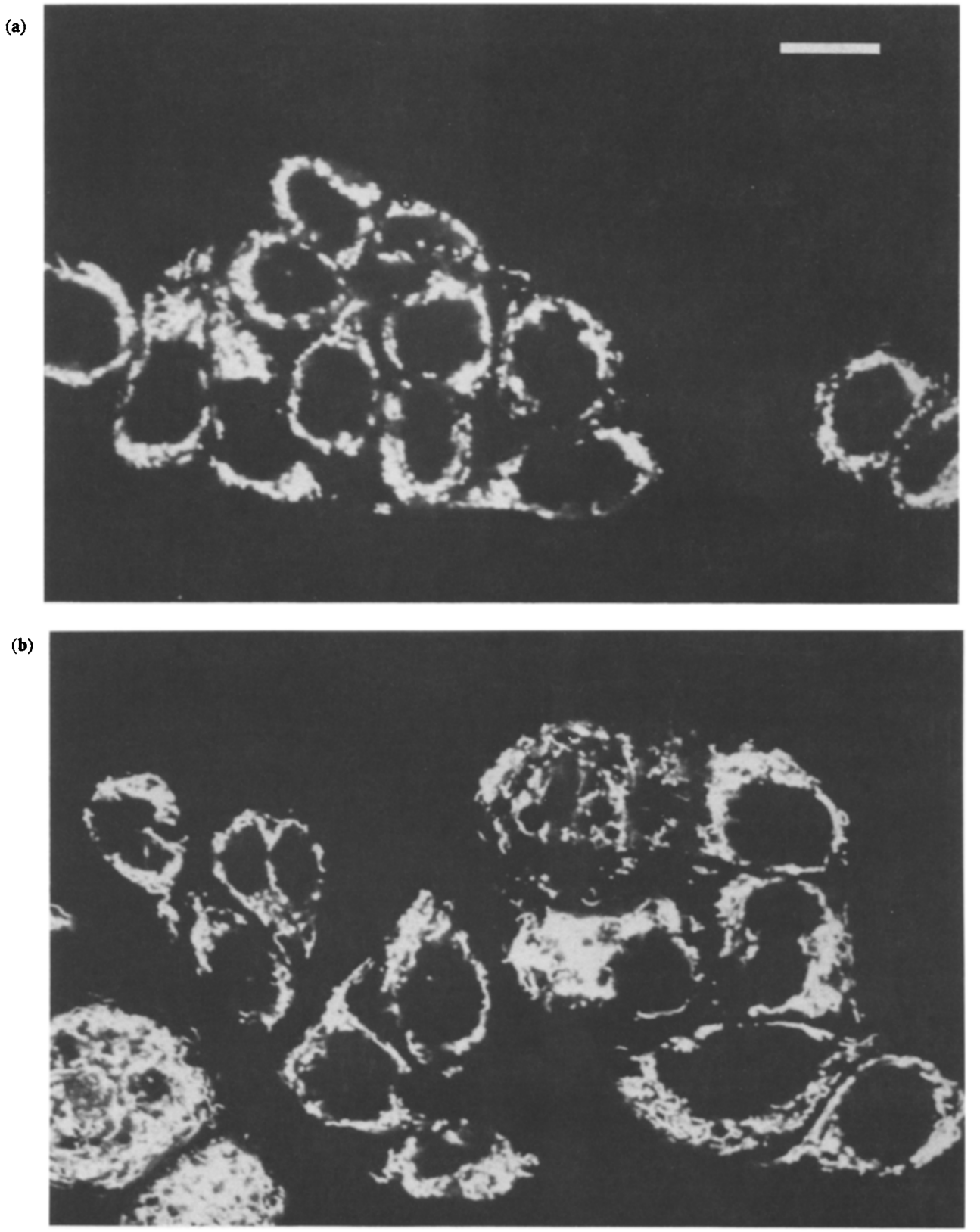

Figure 3. Confocal microscope fluorescence images of (a) COR-L23/P and (b) COR-L23/R cells exposed for 1 h to Rh123 (0.1 μg/ml). Images obtained at equal instrument settings. Bar = 20 μm.

resistant to doxorubicin, vincristine, colchicine and etoposide [18] and shows a drug accumulation deficit but only modest chemosensitisation by verapamil or CsA [16]. The intracellular distribution of both doxorubicin and daunorubicin is dramatically different between parent and resistant cells, the former showing mainly nuclear localisation, whereas the latter accumulate drug principally in cytoplasmic vesicles [16, 20]. The resistant line overexpresses a 190-k protein detected by polyclonal antisera ASP-14 [21] or CRA-1 [17, 22] raised to a 15 amino acid synthetic peptide corresponding to a highly conserved region of

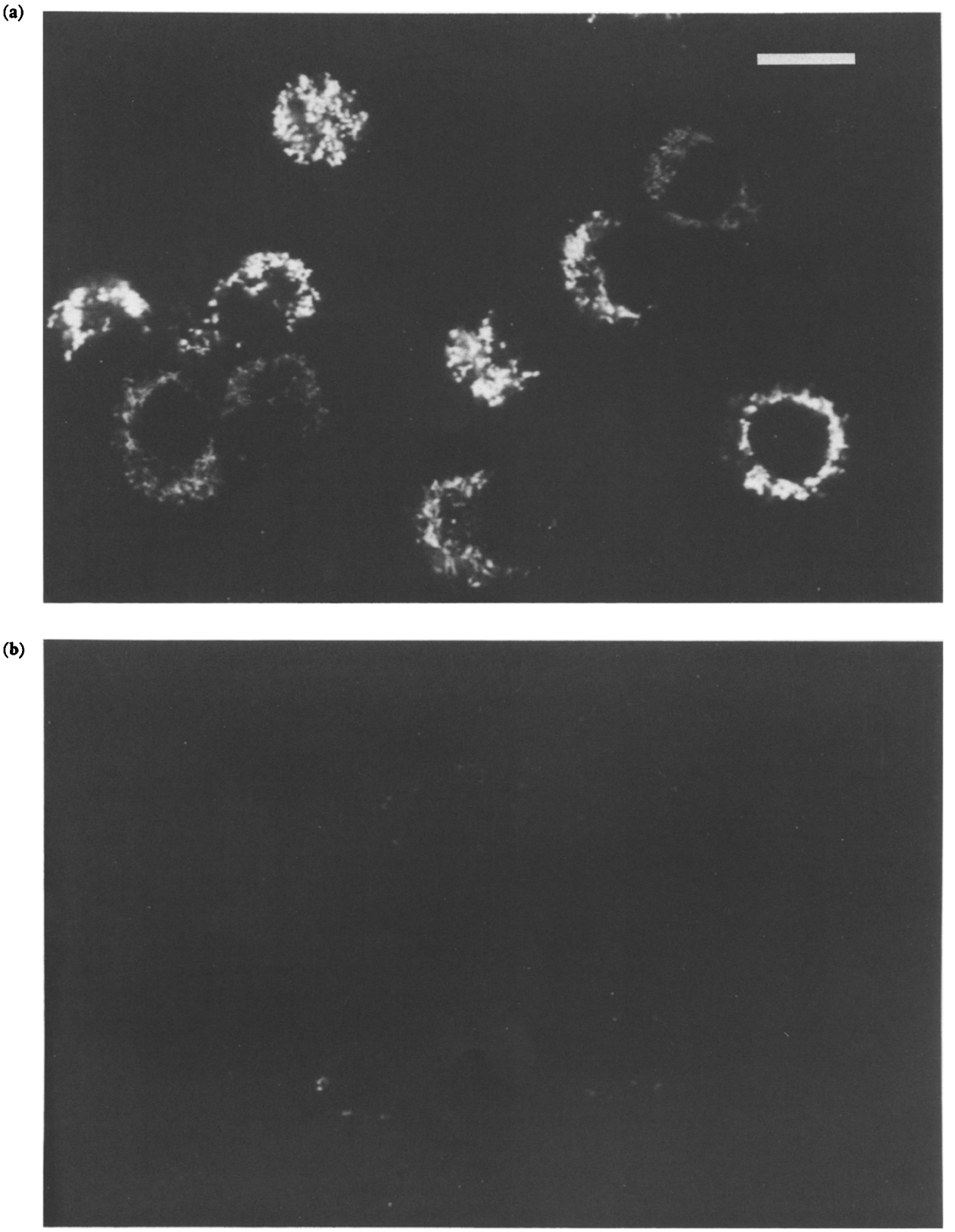

Figure 4. Confocal microscope fluorescence images of (a) EMT6/P and (b–d) EMT6/AR1.0 cells exposed for 1 h to Rh123 (a–c = 0.1 μg/ml; d = 1.0 μg/ml). Images obtained at equal instrument settings except (c) where the neutral density filtration was reduced 3-fold. Bar = 20 μm.

Pgp [21]. This protein is lost in revertant cells [17]. The resistant line also overexpresses the MRP gene, and we believe that the 190-k protein band includes the product of this gene [17].

Our results for accumulation and efflux of Rh123 in the EMT6 cell lines are generally in accordance with the known phenotype of these cells. The resistance factor for Rh123, as determined in the MTT assay, is lower than that seen for most cytotoxic drugs involved in the MDR phenotype [14]. The approximately 5–10-fold lower accumulation of Rh123 in resistant compared with parental cells is, however, similar to that which we have seen

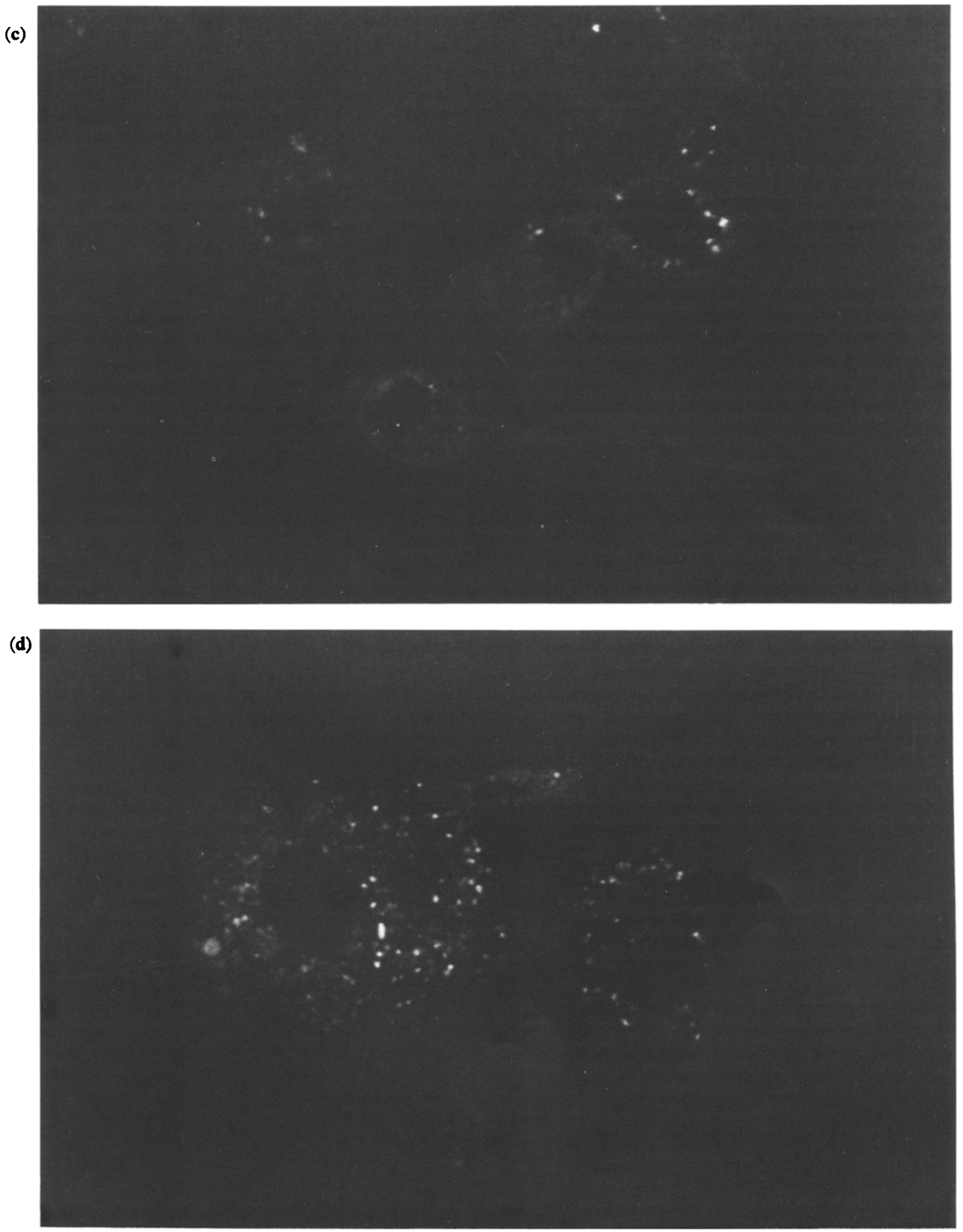

Figure 4. Continued

previously for tritium-labelled daunorubicin [14]. Although both parental and resistant sublines are sensitised by CsA in the MTT assay, the effect is somewhat greater in parental cells. This is the opposite to that which we have previously reported for high dose CsA in combination with cytotoxic drugs in these cells lines [14], and presumably reflects differences between Rh123 and the other drugs in their interaction with Pgp. However, clear sensitisation of parental EMT6 cells to various MDR drugs by both CsA and verapamil has been well established in our previous studies. We found that relative sensitisation of parental

and resistant cells was dependent upon the particular cytotoxic drug and the dose of the resistance modifier, presumably reflecting competition for the pumping activity of Pgp [14]. It is, therefore, perhaps not too surprising that drug/modifier combinations may exist where sensitisation is greater in low Pgp (parental) than in high Pgp (MDR) cells. Efflux of Rh123 occurs rapidly from parental and even more rapidly from resistant cells. The rate of efflux can be reduced by lowering the temperature or by adding CsA and is likely, therefore, to result from an active transport process, probably involving Pgp. If this is so, however, the very low levels of Pgp in the parent cells must be highly efficient in removing Rh123. Alternatively, a different temperature and CsA-dependent mechanism may be involved.

The low level of fluorescence seen on confocal microscope images of EMT6/AR1.0 cells after exposure to Rh123 at 0.1 μg/ml make it difficult to compare the intracellular distribution with that seen in the parent cells. At a higher concentration of Rh123, however, mitochondrial localisation similar to that in parental cells is seen, and it would therefore appear that high levels of Pgp do not result in an altered intracellular distribution of Rh123.

In the COR-L23/R cell line, the resistance factor for Rh123 in the MTT assay is greater than that seen for EMT6/AR1.0 cells. This is the opposite of that seen for cytotoxic drugs including doxorubicin, vincristine and colchicine [14, 18]. The modest sensitisation brought about by CsA in both parent and resistant cells is, however, similar to that seen for this agent in combination with doxorubicin or vincristine [16]. It is clear from the confocal microscope images that Rh123 content and distribution after 1 h of exposure are very similar in COR-L23/P and COR-L23/R cells. Intracellular distribution patterns were also similar after a longer (3-h) exposure although, at this time, the intensity of fluorescence was greater in the parental cells. This is quite different for the situation for doxorubicin or daunorubicin, where very different intracellular distributions are seen between parental and resistant cells [16, 20]. However, the observation is in agreement with the accumulation data at the 1- and 3-h time point obtained by flow cytometry. The accumulation curves are essentially identical for the first 30 min and then slowly diverge over the next 150 min such that the parental cells contain 50% more Rh123 than the resistant cells. These curves are remarkably similar to those which we have previously obtained for doxorubicin and daunorubicin in these cell lines [18]. The initial period of similar accumulation is quite different to the situation in the EMT6 cell lines, where the curves diverge at early times. It is noteworthy that accumulation curves with an initial lag before divergence have also been reported for a human leukaemic cell line which is Pgp negative, but which overexpresses a 190-k protein [21, 23].

Based on the accumulation of doxorubicin and daunorubicin into cytoplasmic vesicles in the COR-L23/R line, we have suggested previously [16] that the mechanism of resistance in this line may involve exocytosis following transport of acidic vesicles to the cell membrane as originally proposed by Sehested and colleagues [24]. The initial lag could represent an interval during which such transport occurs. However, the existence of such similar accumulation curves for a fluorescent dye which distributes in a similar manner in resistant and parent cells undermines the argument for such a mechanism.

Efflux of Rh123 is minimal in the COR-L23/P parental cells. This gives some support to the notion that the rapid efflux seen in the EMT6/P cell line results from an active process and not from simple diffusion out of the cell. However, in the resistant COR-L23/R efflux clearly occurs. Again, the inhibitory effect of low temperature supports the notion of an active transport process. Furthermore, the lack of effect of CsA is in agreement with the minimal chemosensitisation seen with this agent in this cell line as is the somewhat greater efficacy of verapamil [16]. It appears likely, therefore, that Rh123 acts as a substrate for an alternative transporter which is overexpressed in the COR-L23/R line.

The comparative accumulation and efflux of Rh123 in parental and resistant cells has previously been reported, as has the effect of the resistance modifier verapamil, on such kinetics [8, 10]. Separate resistant cell lines had been derived by growth in either doxorubicin or daunorubicin. Unfortunately, the P-glycoprotein status of the resistant Friend leukaemic cell used in these studies was not stated. However, in a more recent publication [25], the same authors demonstrated that MDR Friend leukaemic cell line derived in doxorubicin showed increased expression of a mouse *mdr* gene. Both doxorubicin- and daunorubicin-resistant sublines showed cross-resistance to Rh123, although there was not a direct relationship between the level of anthracycline resistance and the level of Rh123 resistance. It was also found that accumulation of Rh123 (determined by HPLC) was 20-fold lower in highly resistant versus parental cells, and that Rh123 was effluxed more efficiently by the resistant cells. Interestingly, the authors state that efflux was not inhibited by 5×10^{-2} M sodium azide, although no data are shown. As the experiments were not carried out in glucose-free medium, the significance of this observation is hard to assess. Subsequent studies showed that the doxorubicin-resistant Friend leukaemia cells could be sensitised by verapamil to both doxorubicin and to Rh123, whereas no effects were seen in the parental cells [10].

Rh123 was one of a large range of fluorescent compounds investigated as molecular probes for the study of MDR by Neyfakh [9]. They found that accumulation of Rh123 was much reduced in a range of hamster and mouse MDR cell lines compared with their respective parental lines. The resistant cells effluxed Rh123 much more rapidly than parental cells, and the efflux could be inhibited by weak detergents, metabolic inhibitors, calcium transport blockers, cadmodulin inhibitors and reserpine. Based on these results, these authors went on to show that using a 15-min Rh123 loading, followed by a 1-h efflux into drug-free medium, the amount of residual fluorescence was inversely proportional to the level of MDR in a series of T-cell lymphomas. This method has subsequently been used to demonstrate the expression of Pgp by human haemopoietic stem cells and the induction of MDR in human cells by transient exposure to various cytotoxic drugs [11–13].

It must always be borne in mind that the relative accumulation of Rh123 in parental and resistant cell is likely to be influenced by any differences in mitochondrial number or membrane potential between the cell types in addition to effects of active transporters. Furthermore, we believe that the results presented in this paper must introduce serious reservations as to the interpretation of Rh123 efflux data. Firstly, the parental EMT6/P cells efflux Rh123 in a highly efficient manner. These cells do contain Pgp but at very low levels which are not detected by conventional immunohistochemistry or western blotting. The efflux must, therefore, reflect a highly efficient operation of these low Pgp levels or else a co-existing mechanism the details of which we are unaware. This mechanism may be inhibited by reduced temperature or by CsA.

Secondly, it is clear from our data that selective efflux of Rh123 also occurs in an MDR cell line where there is no Pgp but

where resistance is associated with MRP (whether or not MRP is directly responsible for the resistance remains the subject of continuing investigation). Transport mechanisms other than that based on P-gp can, therefore, use Rh123 as a substrate and interpretation of Rh123 data must take this fact into account. This is particularly so in studies of human haemopoietic cells where high levels of MRP gene expression have been shown [6].

In contrast to the large effect seen in Pgp-expressing cells, there was little effect of CsA in the non-Pgp MDR cell line COR-L23/R. Use of such modifiers in conjunction with Rh123 efflux may, therefore, provide a tool for discrimination between Pgp and non-Pgp-based mechanisms. However, the considerable efflux seen in EMT6 parental cells, which could be inhibited by CsA, means that such efflux is by no means a specific test for cells with moderate to high levels of Pgp.

*Note added in proof: It has now been shown by Grant *et al.* [26] that transfection of the MRP gene results in cells with an MDR phenotype. This evidence confirms that the MRP gene is directly involved in the mechanism of resistance.

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