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The Multidrug-Resistant Human Lung Tumour Cell Line, DLKP-A10, Expresses Novel Drug Accumulation and Sequestration Systems

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ABSTRACT. Drug accumulation studies with the anticancer agents adriamycin and vincristine were carried out on the MDR variant of the human lung cell lines DLKP, DLKP-A10 which overexpresses the MDR associated P-glycoprotein efflux pump. Reduced cellular accumulation of both agents was observed in the resistant variant. The subsequent addition of verapamil and cyclosporin A resulted in partial restoration of cellular accumulation of both drugs in the DLKP-A10 resistant variant while complete restoration of cellular drug levels was observed in the SKMES-1/ADR cell line. These results suggested that the accumulation defect observed in the SKMES-1/ADR cell line was P-glycoprotein mediated and that accordingly, the cells exhibited characteristics consistent with the classical MDR phenotype. In contrast, while P-glycoprotein also appears to mediate a reduction in cellular drug accumulation in the DLKP-A10 cells, an alternative transport mechanism may also be present. No significant increase in the expression of either the MRP or LRP transport proteins was observed in the resistant cells. Metabolic inhibition by antimycin A (but not sodium azide or 2-deoxy-D-glucose) resulted in complete restoration of drug accumulation suggesting the presence of an alternative energy dependent transport mechanism. Fluorescent microscopy studies indicated different cellular localisation of the drug within the parental and resistant cells despite equivalent intracellular concentrations. These studies also revealed the presence of an ATP-dependent, vesicular sequestration mechanism which may be involved in the reduction of nuclear adriamycin accumulation in the DLKP-A10 cell line. This was indicated by observation of the disruption of cytoplasmic vesicles by antimycin A and also inhibition of cytoplasmic drug sequestration by the carboxylic ionophores, monensin and nigericin, accompanied by increased adriamycin accumulation and redistribution of the drug from the cytoplasm to the nucleus. BIOCHEM PHARMACOL 53;10:1493–1502, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. multidrug resistance; P-glycoprotein; drug accumulation; subcellular distribution; antimycin A; monensin

RESISTANCE to chemotherapy is an important factor in the failure of many cancer treatments. While resistance to chemotherapeutic agents is inherent in many human tumours, other tumours, initially responsive to chemotherapy, also develop resistant variants. With acquired drug resistance, a population of cells, initially sensitive to the drug, develops resistant characteristics. Typically, while there is an initial decrease in tumour mass, tumour growth eventually resumes, despite continued treatment. Combination chemotherapy was introduced in an attempt to overcome this problem. However, many tumours can also develop multidrug resistant (MDR) variants. Classical MDR is characterised by cross resistance to a range of chemically unrelated drugs. In general, the cytotoxic agents involved are usually large molecules, containing hydrophobic and hydrophillic regions and include the anthracyclines, vinca alkaloids, taxol, epipodophyllotoxins, actinomycin D and colchicine. Given the widespread lack of sustained response to chemotherapy in human tumours, there is considerable interest in understanding the mechanism of MDR. In attempting to identify the factors giving rise to MDR, a number of mechanisms have been described. These include an increase in drug efflux related to overexpression of a membrane glycoprotein, reduced expression of the enzyme topoisomerase II [1, 2], and alterations in the metabolising enzymes, glutathione-S-transferases (for review see [3]). The most frequent determinant of MDR in many tumour cell lines, however, appears to be related to the ability of the cells to greatly decrease the cellular accumulation of drug. This usually results from the overexpression of the *mdr-1* gene, which encodes the transmembrane protein P-glycoprotein. This protein acts as an ATP-dependent efflux pump for a variety of cytotoxic agents, thus reducing the cellular concentration of drug [4, 5, 6, 7]. Several adriamycin and vincristine resistant sublines have been

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Abbreviations: MDR, multidrug resistance; MRP, multidrug resistance-associated protein; LRP, lung resistance-related protein; Adr, adriamycin; TBS, tris buffered saline.

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found to exhibit reduced cellular uptake of anticancer agents. Many of these resistant variants express higher levels of the P-glycoprotein membrane pump relative to the parental wild type cell and also exhibit cross resistance to the classical MDR drugs. In addition to a reduction in drug accumulation, many MDR cell lines also exhibit altered cellular distribution and compartmentalisation of drug into vesicles away for the target site [8, 9]. This has led to numerous studies investigating drug distribution in wild type and MDR cells. Other resistant cell lines also exhibiting a drug accumulation defect have been shown to be negative for P-glycoprotein expression but overexpress the MRP or LRP proteins. MRP is believed to be an efflux pump for drug conjugates, with a substrate spectrum similar but not identical to that of P-glycoprotein [10] while the role of LRP in resistance remains to be clarified [11]. This current work was undertaken to determine the role of P-glycoprotein in the altered drug accumulation and distribution patterns observed in the human lung carcinoma cell lines DLKP-A10 and also to investigate alternative mechanisms that may play a role in drug accumulation and distribution in this cell line.

MATERIALS AND METHODS

Cell Lines

The DLKP cell line was derived from a poorly differentiated squamous cell carcinoma of the lung [12]. The DLKP-A10 variant was established by continuous exposure of an adriamycin resistant variant of the DLKP cells, DLKP-A [13], to increasing concentrations of adriamycin, to a final concentration of 17.25 μM. The cells were cultured in 50% DMEM/50% Hams F12 medium supplemented with 5% foetal calf serum and 1% L-glutamine. The human lung squamous carcinoma cell line SKMES-1 was obtained from the American Tissue Culture Collection. The SKMES-1/ADR resistant variant was established by continuous exposure of the SKMES-1 cells to increasing concentrations of adriamycin to a final concentration of 0.43 μM adriamycin. The SKMES-1 variants were cultured in MEM medium supplemented with 5% foetal calf serum, 1% L-glutamine, 1% sodium pyruvate and 1% non essential amino acids.

Drugs and Chemicals

Adriamycin (doxorubicin) and 5-fluorouracil were obtained from Farmitalia (UK), carboplatin and VP16 (etoposide) from Bristol-Myers Pharmaceuticals, vincristine from David Bull Laboratories (UK) and radiolabelled vincristine (^{3}H-vincristine) from Amersham. Cyclosporin A was obtained from Sandoz (Switzerland). Verapamil, sodium azide, 2-deoxy-D-glucose, antimycin A and monensin were obtained from Sigma (UK). The C219 antibody was obtained from Centocor Diagnostics and the LRP-56 and MRP antibodies from TCS Biologicals (UK).

Cytotoxicity Assay

The cytotoxicity of a number of anticancer agents was determined by an acid phosphatase procedure [14]. Briefly, single cell suspensions were prepared by trypsinization and 1×10^4 cells per mL were seeded in 96-well microtitre plates. Following incubation for 24 hr at 37°C, cultures were exposed to various concentration of the anticancer agent and the plates incubated for a further 5 days. The medium was then decanted and the cells washed twice in phosphate buffered saline. The assay substrate (10 mM p-nitrophenyl phosphate, 0.1 M sodium acetate and 0.1% triton X-100) was added and the plates were incubated for 2 hr at 37°C until suitable colour development was visible. The addition of 1N NaOH caused an enhancement of the colour and also stopped the enzymatic reaction. The absorbance was measured using a dual beam ELISA plate reader at a wavelength of 405 nm (reference wavelength 620 nm).

Western Blotting for P-Glycoprotein and MRP Detection

Western blotting for P-glycoprotein detection was preformed using plasma membrane fractions [15] while crude whole cell lysates were prepared for MRP detection. The protein concentration of each fraction was estimated by the Pierce Bincinchoninic acid (BCA) protein assay [16]. The proteins were separated using 7.5% SDS-polyacrylamide gels in a discontinuous buffer system according to the method described by Laemmli [17]. Western blotting was performed by the method of Towbin *et al.*, [18] using Hybond C-super nitrocellulose sheets (Amersham, UK). Following protein transfer the nitrocellulose sheets were placed in blocking buffer (5% non-fat dried milk in Tris buffered saline (20 mM Tris, 500 mM NaCl pH 7.5)) for 2 hr prior to the addition of the primary antibody. Two detection methods were used for protein detection; either an alkaline phosphatase-conjugated secondary antibody was applied to the blots which were then developed using an alkaline phosphatase substrate (nitroblue tetrazolium, 5-bromo-4-chloro-3-indoxyl phosphate, NBT/BCIP) or a horseradish peroxidase-linked secondary antibody was added which was detected by enhanced chemiluminescence (ECL).

Immunocytochemistry for LRP Detection

LRP expression was detected on cytospins using the ABC method. Briefly, the cells were fixed in ice cold acetone for 10 min and allowed to air dry. The cells were then preincubated in 0.6% (v/v) hydrogen peroxide in methanol for 5 min (to block endogenous peroxidase activity) and then blocked in 20% normal rabbit serum for 20 min. The LRP antibody was applied for 2 hr at room temperature. After a thorough wash, the cells were incubated for 30 min with the biotinylated rabbit anti-mouse IgG secondary antibody followed by a 30 min incubation with the

strepABC/HRP complex. The slides were again washed thoroughly and the cells incubated with the horseradish peroxidase substrate 3,3-diaminobenzidine tetrahydrochloride (DAB) until suitable colour had developed. The cells were then counterstained with hematoxylin (Bayers), dehydrated in graded alcohols and mounted with DPX (BDH, UK).

Intracellular Adriamycin and Vincristine Accumulation

Intracellular adriamycin accumulation was measured by a modification of the method described by Ganapathi and Grabowski [19]. Exponentially growing cells were trypsinized and a single cell suspension prepared. The cells were plated in 6 well cluster plates at a concentration of 1×10^5–5×10^5 cells per well (concentration dependent on the cell line) and incubated for 48 hr at 37°C in 5% CO_2. The growth medium was then decanted and replaced by fresh medium containing adriamycin (10 μM). After specified time periods the drug containing medium was removed and the cells were rinsed twice in ice-cold phosphate buffered saline. Adriamycin was extracted directly by the addition of 0.3 N hydrochloric acid-50% methanol and the fluorescence measured using a Perkin Elmer LC50 luminescence spectrometer at an excitation wavelength of 470 nm and an emission wavelength of 585 nm. The cellular uptake of vincristine was determined by measuring the quantity of radiolabelled vincristine accumulated in the cells. Exponentially growing cells were trypsinized and a single cell suspension prepared. Cells were seeded in 6 well cluster plates and incubated for 48 hr. Following incubation, the medium was decanted and medium containing ^{3}H-vincristine (30 nM) was added to the cells. After specified time intervals the medium was aspirated and the cells washed twice in ice-cold phosphate buffered saline. The plates were blotted dry and the cells solubilized by overnight incubation in 0.2 N NaOH. The cell lysates were neutralized by the addition of 0.2 N HCl and the radioactivity of each sample determined by liquid scintillation counting (Beckman LS 6500). The cellular concentration of adriamycin and vincristine was quantitated from a standard curve prepared from known concentrations of both agents. For all metabolic inhibition studies the cells were preincubated in glucose free medium (Gibco) for 2 hr prior to the assay.

Intracellular Adriamycin Distribution

Cells at the exponential phase of growth were trypsinized and a single cell suspension prepared. Cells were plated onto sterile coverslips in petri dishes at a concentration of 1×10^5 cells per petri dish and incubated overnight at 37°C in 5% CO_2. The cells were exposed to adriamycin (10 μM) for specified time periods and then washed twice with ice-cold phosphate buffered saline. The coverslips were inverted onto slides and sealed with silicone grease to protect against dehydration. The cells were viewed for fluorescence under UV illumination using a Nikon microscope equipped with a mercury lamp. The UV illumination induces an orange fluorescence at the site of adriamycin accumulation.

TABLE 1. IC_{50} values (±standard deviation) for the DLKP and DLKP-A10 cell lines (a) and the fold resistant levels of the DLKP-A10 variants (b) where N = 3

A.

IC_{50} (nM)	DLKP	DLKP-A10
Adriamycin	8.75 ± 0.77	6696 ± 453
Vincristine	0.728 ± 0.062	2184 ± 132
VP16	22.9 ± 18.5	1448 ± 98.2
5-fluorouracil	7923 ± 57.7	7308 ± 522
Carboplatin	3243 ± 278	2676 ± 198

B.

Drug	DLKP-A10
Adriamycin	765.3
Vincristine	3000
VP16	63.3
5-fluorouracil	0.92
Carboplatin	0.825

RESULTS

Cytotoxicity Profiles

The cytotoxicity of adriamycin, vincristine, VP16, 5-fluorouracil and carboplatin to the DLKP and DLKP-A10 cell lines was assessed (Table 1). While the resistant variant exhibited cross resistance to the classical MDR chemotherapeutic agents adriamycin, vincristine and VP16, the cells displayed the greatest resistance to vincristine. No significant resistance or sensitivity was observed with 5-fluorouracil or carboplatin.

Western Blot Analysis of P-Glycoprotein and MRP Expression

Western blot analysis was carried out on membrane fractions of the cells using the anti-P-glycoprotein monoclonal antibody, C219. The results indicated a low level of P-glycoprotein in the parental cell lines. In contrast strong immunoreactivity was evident in the control SKMES-1/ADR and in the DLKP-A10 cells, indicating high expression of P-glycoprotein in the resistant cells (Fig. 1). No detectable level of MRP was observed in either the parental or resistant cells while the MRP-positive control HL60/ADR cells showed strong positive staining with the MRP antibody (data not shown).

Immunocytochemistry for LRP Expression

LRP was found to be localised predominately in the cytoplasmic regions of the cells. However significant differences in the staining pattern or intensity of staining was not

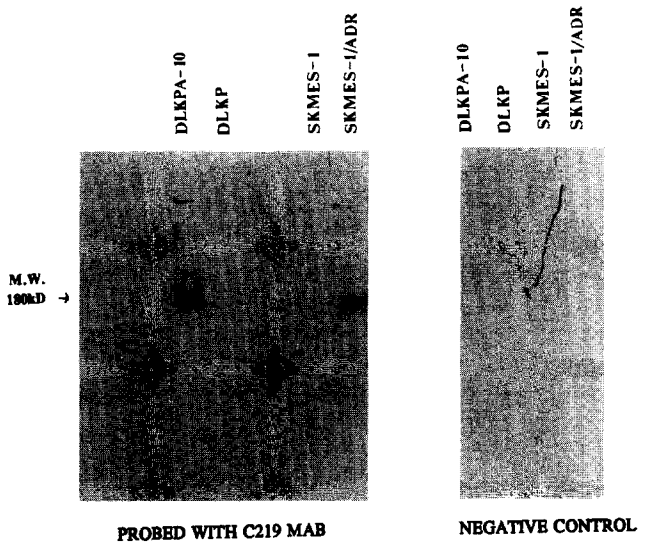

FIG. 1. Western blot detection of P-glycoprotein in cellular membrane preparations of the DLKP, DLKP-A10, SKMES-1 and SKMES-1/ADR cell lines with the C219 monoclonal antibody.

noted between the parental DLKP and resistant DLKP-A10 cells (data not shown).

Drug Accumulation Studies

The intracellular accumulation of both adriamycin and vincristine was markedly lower in the resistant variant than in the parental cells (Fig. 2). The rate of drug accumulation in the resistant variant was greatest within the first hour following the addition of drug, after which time a steady rate of drug accumulation was observed with both drugs. When the control SKMES-1/ADR cells were exposed to adriamycin in the presence of verapamil (60 μM), cyclosporin A (8.3 μM) or metabolic inhibitors a significant increase in drug accumulation was observed with the intracellular drug levels comparable to the levels observed in the parental cells. Although treatment with verapamil and cyclosporin A also resulted in an increase in the cellular drug concentration in the DLKP-A10 cell line, the maximum level of adriamycin and vincristine accumulation was only approximately 45% and 30% for verapamil and 55% and 40% for cyclosporin A of the level observed in the parental cells respectively. The drug accumulation defect could not be reversed following longer incubation periods or at higher concentrations of the circumvention agent (concentrations up to 100 μg/mL were studied). Similar to the results obtained for verapamil and cyclosporin A, treatment with sodium azide (10 mM) and 2-deoxy-D-glucose (60 μM) resulted in only partial restoration of drug levels in the resistant variant, however, antimycin A (10 μM) proved to be very effective at restoring cellular levels of both adriamycin and vincristine in the DLKP-A10 cells (Fig. 3). The time course of drug accumulation was also studied in the presence of the carboxylic ionophore, monensin. Although no significant increase in the cellular drug accumulation was observed in the sensitive cells following treatment with monensin, it proved to be effective at partially restoring levels in the DLKP-A10 cells. Cotreatment with monensin (10 μM) resulted in an 6-fold and 3.2-fold increase in the maximum cellular levels of adriamycin and vincristine respectively in the DLKP-A10

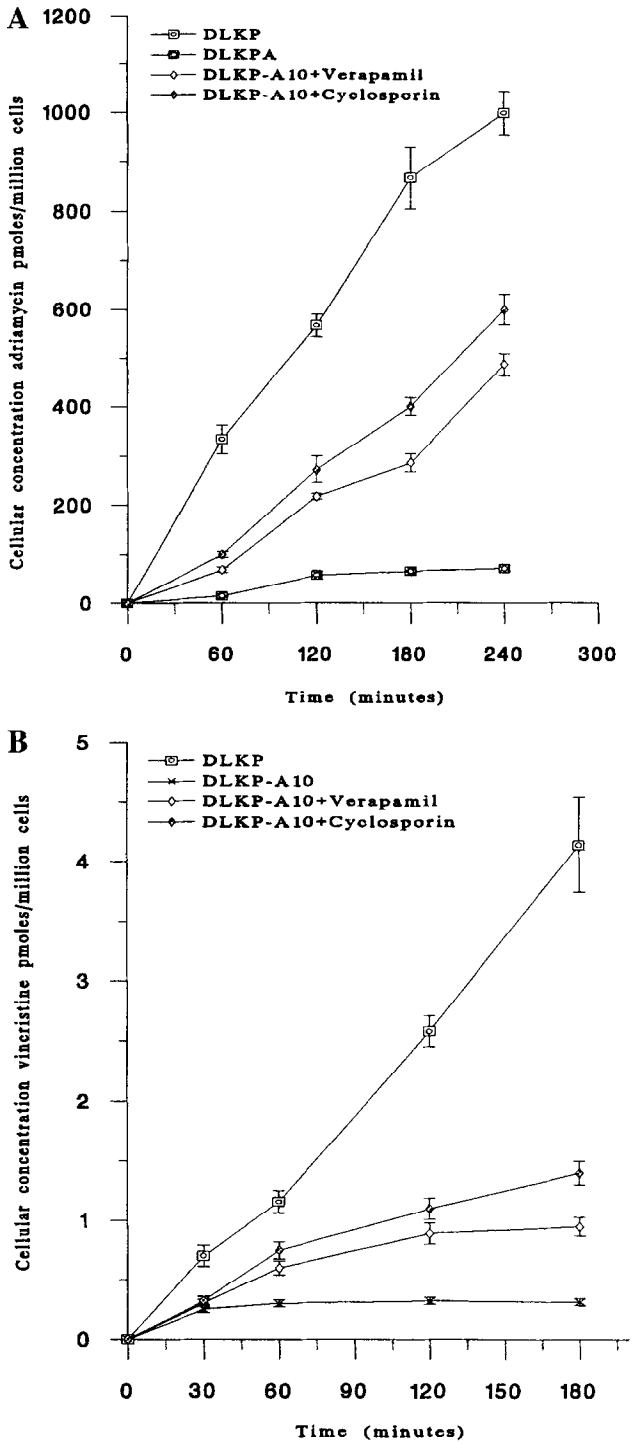

FIG. 2. The effect of verapamil and cyclosporin A on the time course of adriamycin and vincristine accumulation (± standard deviation) in the DLKP and DLKP-A10 cell lines (A, B).

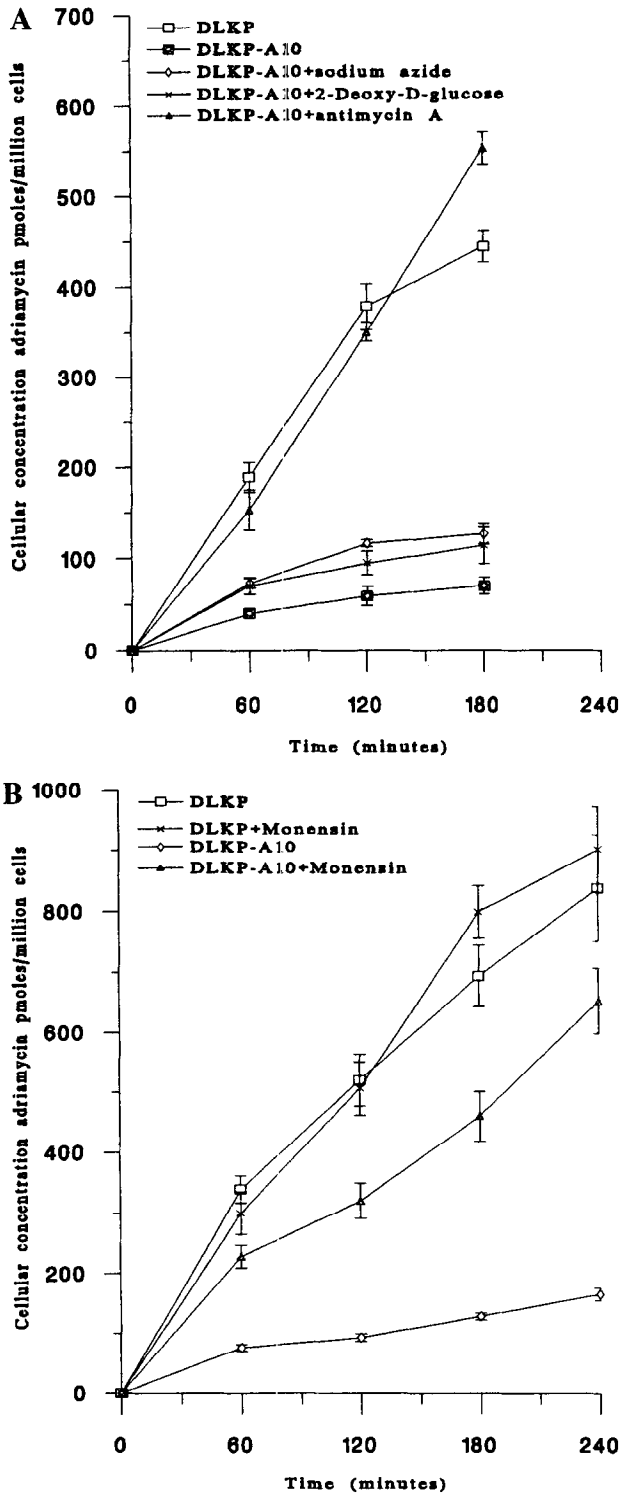

FIG. 3. The effect of sodium azide (10 mM), 2-deoxy-D-glucose (25 μg/mL) and antimycin A (10 μM) on the time course of adriamycin accumulation (± standard deviation) in the DLKP-A10 cell line (A). The effect of monensin (10 μM) on the time course of adriamycin accumulation in the DLKP and DLKP-A10 cell lines (B).

cells (Fig. 3). Although pretreatment with monensin resulted in a further enhancement of the maximum cellular drug levels in the DLKP-A10 cells complete restoration of drug levels was not observed.

Adriamycin Subcellular Localisation Studies

The subcellular adriamycin distribution pattern was studied in the parental and resistant cells following 2 hr exposure to adriamycin (10 μM, Fig. 4). Intense nuclear fluorescence was clearly distinguishable in the parental cells with distinct areas of more intense fluorescence visible within the nuclei. In contrast, the majority of the resistant cells displayed only faint nuclear fluorescence although speckles of more intense fluorescence were visible throughout the cytoplasmic region. Increasing the concentration of adriamycin (to a maximum of 50 μM) or prolonged drug exposure resulted in an increase in the intensity of cytoplasmic fluorescence but no corresponding increase in nuclear fluorescence. To determine if nuclear drug exclusion was a consequence of reduced cellular drug concentration, drug distribution was studied in the DLKP variants at equivalent intracellular concentrations. Quantitative studies revealed the cellular concentration of adriamycin in the DLKP-A10 cells after 4 hr was equivalent to the concentration of drug in the DLKP cells after exposure for just 5 min. However, the drug distribution studies following these incubation periods indicated that the drug was still localised within the nucleus of the DLKP cells and within the cytoplasm of the DLKP-A10 cells (Fig. 4) thus indicating that different sequestration mechanisms are operating within the two cell lines. Cotreatment of the DLKP-A10 cells with verapamil or cyclosporin A resulted in a marked increase in nuclear fluorescence, however the intensity of the fluorescence was substantially less than that observed in the parental DLKP or control SKMES-1/ADR cells. Pretreatment with the circumvention agent for 2 hr prior to the addition of adriamycin did not significantly alter the intensity of nuclear fluorescence in either the parental or resistant cells (Fig. 5). Treatment with sodium azide or 2-deoxy-D-glucose resulted in an increase in cytoplasmic fluorescence in the resistant cells but did not significantly alter the intensity of nuclear fluorescence. Pretreatment of the DLKP-A10 cells with these agents resulted in an increase in nuclear fluorescence, although the intensity was less than that observed in the parental cells. Treatment with antimycin A, however, resulted in an increase in nuclear fluorescence in DLKP-A10 cells with the intensity of fluorescence comparable to that observed in the DLKP cells. Coincubation with antimycin A also resulted in a decrease in cytoplasmic fluorescence in the resistant cells (Fig. 6). The effect of antimycin A on adriamycin distribution was also investigated in the DLKP-A10 cells following preloading with adriamycin (10 μM) in glucose free medium. The cells were exposed to adriamycin for 2 hr and then incubated in drug free medium containing antimycin A. When the cells were viewed after 2 hr exposure to adriamycin, the typical cytoplasmic fluorescent pattern was observed. Following treatment with antimycin A (in the absence of extracellular adriamycin) faint nuclear fluorescence was observed in the majority of cells viewed. A decrease in cytoplasmic fluorescence was also distinguish-

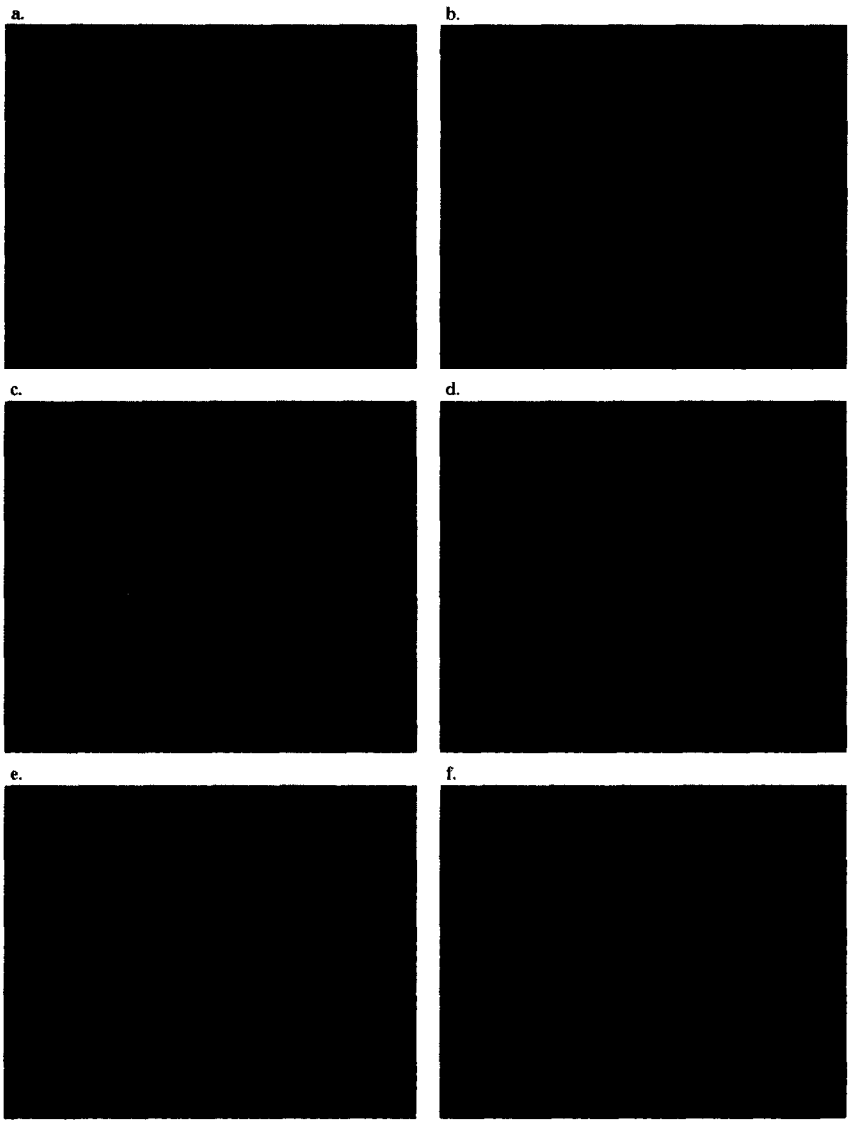

FIG. 4. The subcellular adriamycin distribution pattern in the SKMES-1 (a), SKMES-1/ADR (b), DLKP (c) and DLKP-A10 (d) following 2 hr exposure to the drug (10 μM), in the DLKP-A10 cells following 4 hr exposure (e) and in the DLKP cells following 5 min exposure (f).

able in particular in the quantity of fluorescent vesicles scattered through the cytoplasmic region (Fig. 6). This result indicates that antimycin A sensitive processes are involved in drug sequestration and/or nuclear drug exclusion in the DLKP-A10 cells. The effect of a number of agents, that have previously been shown to alter the subcellular distribution of adriamycin in MDR cell lines, was investigated in the DLKP and DLKP-A10 cells. The agents studied included the Golgi apparatus disrupting agent brefeldin A, the vacuolar ATPase inhibitor bafilomycin A1, the tyrosine kinase inhibitor genestein, the carboxylic ionophores monensin and nigericin, the calmodulin inhibitor trifluoroperazine and the lysosomal disrupting agents chloroquine and methylamine. Of the agents tested, only the ionophores monensin and nigericin appear to significantly alter the subcellular distribution of adriamycin in the DLKP-A10 resistant cells. However, the intensity of nuclear fluorescence was less than that observed in the parental cells. Fig. 6 illustrates the subcellular distribution pattern of adriamycin in the DLKP-A10 following cotreatment with monensin. A marked increase in the intensity of nuclear fluorescence and a decrease in cytoplasmic fluorescence was observed in all the cells viewed. Distinct regions of intense fluorescence were still visible within the cytoplasmic regions, although the quantity and intensity of the regions of fluorescence was less than that observed in the absence of monensin. The effect of monensin on DLKP-A10 cells that were preloaded with adriamycin was also investigated to determine if monensin could cause redistribution of the drug from the cytoplasm to the nucleus. However, unlike antimycin A, monensin did not significantly alter the drug distribution pattern in the cells and the typical cytoplasmic fluorescence was still evident in all the cells viewed.

DISCUSSION

Decreased accumulation of anticancer agents has been implicated as a mechanism of resistance in many MDR cell lines and it has been demonstrated that the reduction in

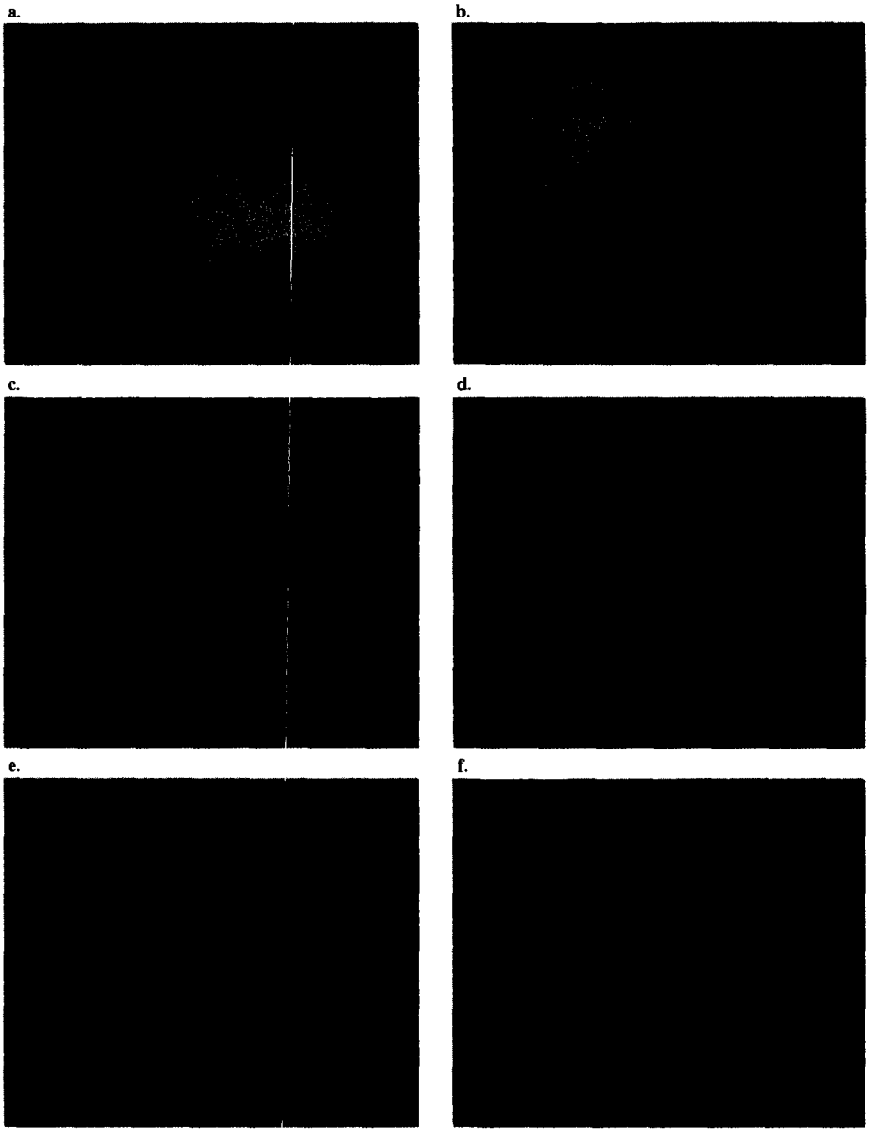

FIG. 5. The subcellular adriamycin distribution pattern in the SKMES-1/ADR cells following two hr exposure to the drug in the presence of cyclosporin A (a) and sodium azide (b) and in the DLKP-A10 cells in the presence of cyclosporin A (c), 2-deoxy-D-glucose (d) sodium azide (e) and following pretreatment with sodium azide (f).

intracellular drug levels usually occurs as a result of overexpression of the transmembrane pump, P-glycoprotein. Studies have shown that P-glycoprotein can bind diverse cytotoxic agents and extrude them from the cells in an energy requiring reaction, thus reducing the cellular concentration of the drug [6]. The work described here was carried out to investigate the role of P-glycoprotein in the human lung carcinoma cell line DLKP and its MDR variant, DLKP-A10, that was shown to be cross resistant to the MDR drugs vincristine and VP16 and to overexpress P-glycoprotein. The SKMES-1/ADR cell line was used throughout these studies as a positive control cell line since this cell lines displays characteristics consistent with the classical MDR phenotype. The initial findings from these studies also suggested that as with classical MDR cell lines, overexpression of P-glycoprotein was associated with the observed reduction in drug accumulation in the DLKP-A10 cells since a significant decrease in cellular drug levels and rapid drug efflux when glycolysis was re-initiated was observed. However, further studies using verapamil and cyclosporin A (agents which are effective at restoring cellular drug levels in P-glycoprotein positive MDR cell lines [8, 20, 21]) revealed that these agents could only partially restore drug levels thereby indicating that overexpression of P-glycoprotein can not fully explain the accumulation defect in these cells. No significant increase in the expression of MRP or LRP was observed by Western blotting and immunocytochemistry respectively suggesting that these transport mechanisms were not involved in the altered accumulation pattern. Other mechanisms must, therefore, play a role in mediating resistance to account for the apparent non-P-glycoprotein accumulation defect observed in this cell line. In accordance with previous reports on MDR cell lines, microscopic studies revealed that adriamycin was predominately localised in vesicles within the cytoplasmic region of the DLKP-A10 cells, whilst the drug was predominately localised within the nucleus of the parental cells. To determine if nuclear drug exclusion in the DLKP-A10 cells was a consequence of reduced cellular drug concentration, drug distribution was studied in the parental

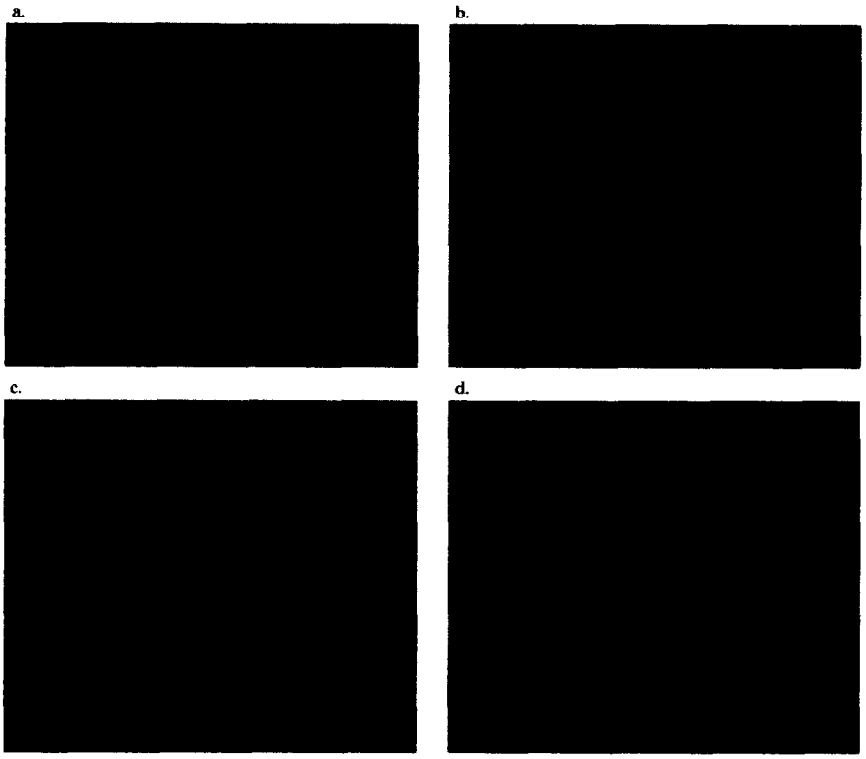

FIG. 6. The subcellular adriamycin distribution pattern in the DLKP-A10 cells following exposure to the drug in the presence of antimycin A (a) and monensin (c). The effect of antimycin A (b) and monensin (d) on drug sequestration in the DLKP-A10 cells (the cells were preloaded with adriamycin for 2 hr, washed and incubated with antimycin or monensin for a further 2 hr).

and resistant cells at equivalent intracellular concentrations. However, different distribution patterns were still observed indicating that reduced intracellular drug levels were not principally responsible for the decrease in nuclear fluorescence and that alternative mechanisms are involved in nuclear drug exclusion. This was further supported by the finding that exposing the DLKP-A10 cells to higher drug concentrations or for longer time periods (up to 48 hr) did not significantly alter the drug distribution pattern even though higher cellular levels were obtained. Cotreatment with verapamil or cyclosporin A resulted in only partial redistribution of the drug from the cytoplasm to the nucleus in the resistant cells, again suggesting that, while P-glycoprotein is probably associated with altered drug distribution other non-P-glycoprotein mechanisms must also play a role. Initial studies with sodium azide and 2-deoxy-D-glucose implicated a non-ATP dependent mechanism since both agents were ineffective at restoring nuclear drug levels. However, further studies using antimycin A showed that this agent could effectively restore cellular drug levels and inhibit drug sequestration in cytoplasmic regions. Additional studies revealed that following preloading of the cells, antimycin A was effective at disrupting the cytoplasmic vesicles and causing redistribution of the drug from the vesicles to the nucleus. The mechanism(s) by which antimycin A can apparently disrupt the cytoplasmic vesicles and thus prevent the localisation of drug within the cytoplasm is unclear although it is possible that it could be due to inhibition of P-glycoprotein (presumably by ATP inhibition) and thus redistribution of the drug to the nucleus. Alternatively it could be due to pH alterations within the cells since studies have shown that shifts in intracellular pH leads to alterations in drug accumulation and distribution in MDR cells [22]. It is possible also that antimycin A could have a direct inhibitory effect on some current unknown vesicular transport system. Although numerous studies have reported increased cellular drug levels in MDR cell lines with the metabolic inhibitors, sodium azide and 2-deoxy-D-glucose, literature searches revealed no information on the use of antimycin A in the study of MDR. The results obtained from these studies indicate that of the three compounds tested, antimycin A was the most effective at inhibiting drug efflux and cytoplasmic localisation of the drug. These findings could be attributable to the different sites of action, since each of the compounds act at different stages of ATP production. 2-deoxy-D-glucose is an inhibitor of glycolysis, while both sodium azide and antimycin A are inhibitors of the electron transport chain. Since antimycin A acts at an earlier stage of the electron transport chain (inhibits electron transport from ubiquinone to cytochrome C) than sodium azide (inhibits reduction of oxygen catalysed by cytochrome oxidase) it could more effectively deplete cellular ATP levels within the limited time period studied. Inhibition of glycolysis may also be ineffective at depleting cellular levels of ATP since other metabolic pathways would still be functional and could ultimately feed into the electron transport chain and lead to ATP production. A number of studies have also demonstrated that ionophores, including monensin and nigericin, can alter the cellular concentration and distribution of anticancer agents [23, 24, 25, 26]. In our studies monensin proved to be an effective agent at

enhancing drug accumulation and causing a redistribution of adriamycin from the cytoplasm to the nucleus in the resistant DLKP-A10 cells. Considerable confusion exists regarding the exact mechanism by which ionophores exert their effect in resistant cells. Studies have shown that ionophores act by inhibiting P-glycoprotein mediated drug efflux and thereby increasing cellular levels of the drug [27, 28]. However since monensin proved to be more effective than cyclosporin A at enhancing nuclear adriamycin accumulation and appears to be specific for the resistant cells (while treatment with verapamil or cyclosporin resulted in an increase in drug accumulation in the parental cells, no significant effect was observed in these cells following treatment with monensin) it would appear that monensin also exerts its effect in the DLKP-A10 cells by a non-P-glycoprotein mechanism. Studies have also shown that ionophores can alter drug distribution in MDR cells by disrupting intracellular vesicular traffic. The involvement of an acidic vesicular transport system in MDR cell lines was first described independently by Sehested *et al.* and Beck [29, 30], who suggested that the lysosomal system in MDR cells was involved in drug efflux and that drugs such as adriamycin and vinblastine, which are weak bases, could become trapped in these acidic compartments. According to this proposal, these lysosomal vesicles migrate to the plasma membrane where they fuse and extrude their contents. The mechanism by which ionophores disrupt intracellular vesicular traffic is unclear, although it has been proposed that these agents insert into membranes and facilitate the exchange of Na^+ or K^+ for protons and abolish the Na^+K^+ gradient. This would ultimately lead to an increase in the pH environment and subsequent disruption of the acidic vesicles. It has also been proposed that an alternative membrane trafficking system, based on the trapping of drug within the Golgi apparatus, could exist in MDR cells resulting in an increase in drug efflux [5, 9, 31]. However, the results obtained from our studies suggest that adriamycin is not associated with either system in the DLKP-A10 cells since treatment with the Golgi disrupting agent, brefeldin A or the lysosomotropic agents chloroquine and methylamine did not significantly alter subcellular drug distribution in the cells. The adriamycin distribution pattern observed in the DLKP-A10 cells does however implicate the involvement of an alternative acidic vesicular transport system. Fluorescent microscopy studies clearly demonstrated the presence of fluorescent vesicles within the cytoplasm which is consistent with the localisation of the drug within cytoplasmic acidic vesicles. Disruption of these drug containing vesicles by antimycin A results in redistribution of the drug to the nucleus. While monensin does not appear to disrupt the cytoplasmic vesicles, the results indicate that it prevents sequestration of the drug into the vesicles, inhibits drug efflux and thus, as in the case with antimycin A, causes redistribution of the drug from the cytoplasm to the nucleus of the cell. The results suggest that in addition to P-glycoprotein mediated efflux an alternative energy-dependent drug sequestration system exists in the DLKP-A10 cells which appears to play a role in drug exclusion from the nucleus. If this mechanism exists also *in vivo* in human tumours, it may limit the effectiveness of circumvention therapy with P-glycoprotein and MRP antagonists such as verapamil and cyclosporin A. It may also be relevant that DLKP-A10 is considerably more resistant to adriamycin than is the SKMES-1/ADR cells (IC_{50} 1205 nm) and may represent a late stage in the evolution of multidrug resistance. It would therefore be of interest, in future experiments, to examine drug accumulation and distribution along with the emergence of P-glycoprotein overexpression in cell lines at various stages during the emergence of resistance.

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