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THE EFFLUX OF ANTHRACYCLINES IN MULTIDRUG-RESISTANT CELL LINES

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Abstract—In order to address the association of enhanced drug efflux with the multidrug-resistant (MDR) phenotype, we have studied the cellular pharmacokinetics of anthracyclines in the P-glycoprotein (Pgp)-positive MDR cell lines H69/LX4 (human small cell lung cancer) and EMT6/AR1.0 (mouse mammary tumour). Both doxorubicin (DOX) and daunorubicin (DNR) were accumulated to a lesser extent and effluxed at a higher rate by MDR cells than by their drug-sensitive counterparts. In contrast, the 9-alkyl substituted compound, aclacinomycin A (ACL), was accumulated and effluxed from parent and MDR cells at an identical rate. In experiments designed to examine energy-dependent efflux, DOX and DNR were shown to be efficiently effluxed against the concentration gradient in the presence of glucose. However, in the same experiments the analogues ACL and Ro 31-3294 (9-alkyl and morpholinyl substituted), which have previously been shown to retain activity against MDR cell lines, were accumulated and effluxed at identical rates in parent and MDR EMT6 cells. Hence, 9-alkyl and morpholinyl substituted compounds appear to behave less favourably as substrates for energy-driven drug efflux by Pgp-positive MDR cells than do DOX or DNR. Resistance modifiers verapamil and cyclosporin A appeared to abolish energy-dependent efflux for DOX and DNR in both the EMT6 and H69 MDR lines whereas they had no effect on the cellular efflux of ACL. The altered cellular pharmacology in MDR cell lines may provide a rational basis for the use of modified anthracycline analogues (e.g. 9-alkyl and morpholinyl substituted) and resistance of modifying agents in the treatment of tumours expressing a Pgp-mediated phenotype.

A causal link between the multidrug resistant (MDR‡) phenotype and membrane P-glycoprotein (Pgp) hyperexpression is well established [1, 2]. Characteristically, MDR cell lines show extensive cross-resistance to structurally and mechanistically unrelated anticancer agents which tend to be amphipathic and relatively high molecular weight natural products.

Enhanced drug efflux in MDR cell lines was first demonstrated by Dano [3] and by Biedler and Riehm [4], and was thought to explain, at least in part, the reduced drug accumulation seen for these cells. The presence of an energy-dependent efflux process was first demonstrated in experiments incorporating metabolic inhibition using glucose-free medium and uncouplers of oxidative phosphorylation [5, 6]. The putative structure of Pgp comprises two ATP binding sites per molecule duplex, strongly indicative of an energy requirement for the putative membrane efflux pump complex [7].

There now exist at least two fundamental approaches which may result in the circumvention of the MDR phenotype. Firstly, numerous *in vitro* experiments and a smaller number of *in vivo* studies have demonstrated the efficacy of specific modulating agents, e.g. verapamil (VRP) and cyclosporin A (CYA), known collectively as resistance modifiers [8–10]. Competitive binding of resistance modifying agents to the Pgp during export protein has been demonstrated [11–13]. The second approach is to use structurally modified analogues of MDR-related compounds which are capable of partially or completely overcoming the MDR phenotype [14–18]. It is envisaged that the retention of activity of the structurally modified anthracyclines is a result of a reduced affinity for Pgp.

The work described in this paper was designed to elucidate principally the behaviour of modified anthracyclines and resistance modifiers in terms of effects on cellular drug efflux. We have used two pairs of parental cell lines: the EMT6 mouse mammary tumour cell line and the NCI-H69 human small cell lung cancer line alongside their doxorubicin (DOX)-resistant sublines [19, 20]. The modified anthracyclines aclacinomycin A (ACL) and Ro 31-3294 have previously been shown by us to retain almost complete activity in these MDR cell lines [21]. Ro 31-3294 contains both a 9-alkyl substitution on the A-ring and a morpholino sugar. ACL also has the 9-alkyl substitution and a substantially different sugar residue (comprising -rhodosamine-deoxyfucose-cinerulose). Drug accumulation studies previously carried out on ACL have shown levels to be virtually identical in parental and MDR cells [22].

We now demonstrate differences in the cellular

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‡ Abbreviations: MDR, multidrug resistance/multidrug-resistant; Pgp, P-glycoprotein; VRP, verapamil; CYA, cyclosporin A; ACL, aclacinomycin A; DOX, doxorubicin; DNR, daunorubicin; CEM, complete Eagle's medium; PBS, phosphate-buffered saline; RF, resistance factor; MR-DOX, morpholinyl DOX.

pharmacokinetics of compounds such as ACL and Ro 31-3294 in comparison with DOX and daunorubicin (DNR). In addition, we show how resistance modifying agents, VRP and CYA, can bring about circumvention of MDR by inhibiting energy-dependent drug efflux. The effects of VRP and CYA on anthracycline accumulation have been previously reported by us [22]. We now describe the capacity of the cell lines to efflux anthracyclines with or without resistance modifiers and we have further investigated the involvement of energy-dependent mechanisms which may be related to Pgp.

MATERIALS AND METHODS

Cell culture and conditions. The parental drug-sensitive human small cell lung cancer cell line NCI-H69/P (obtained from Drs A. Gazdar and D. Carney, NCI, U.S.A.) is hereafter designated as H69/P. The MDR subline known as H69/LX4 was derived by *in vitro* exposure to DOX [20]. The murine mammary tumour parental cell line EMT6/Ca/VJAC, hereafter referred to as EMT6/P, originated in a mouse alveolar tumour nodule and was successively transplanted between animal and *in vitro* culture before growth in continuous culture [23]. The drug-resistant variant of this cell line, EMT6/AR1.0, was derived by *in vitro* exposure to DOX [19].

The H69 cell lines were grown as floating aggregates in RPMI 1640 medium (Gibco Biocult, Paisley, U.K.) with 10% foetal calf serum (Seralab, Crawley Down, U.K.), penicillin and streptomycin (at concentrations of 100 U/mL and 100 μg/mL, respectively). Stock cultures were maintained at 37° in an atmosphere of 92% air, 8% CO_2. EMT6 cell lines were maintained as monolayers in Eagle's minimal essential medium with Earles' salts and with 20% new born calf serum (Gibco Biocult; CEM) under the same conditions as for H69. Drug-resistant cell lines were maintained in DOX but the drug was removed for at least two passages prior to use in experiments. All cells were harvested in the exponential phase of growth.

H69 cell cultures for use in experiments were reduced to a suspension containing small groups of cells by pipetting. EMT6 cultured 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°. A single cell suspension was obtained by resuspension of cells in CEM by pipetting.

Drug preparation. DOX was obtained from Farmitalia Carlo Erba (Milano, Italy); DNR was obtained from May and Baker (Welwyn Garden City, U.K.); ACL was obtained from Dr David Allen, Lundbeck (Luton, U.K); Ro 31-3294 was obtained from Dr Joe Martin, Roche Products (Welwyn Garden City, U.K). VRP was obtained from Abbot Laboratories (Queenborough, U.K.); CYA was kindly supplied by Sandoz (Basel, Switzerland).

Anthracyclines were dissolved in sterile distilled water at 500 μg/mL, filter sterilized (pore size 0.2 μm) and stored at −20°. VRP was obtained as a 250 μg/mL aqueous solution in sealed ampoules and diluted in PBS. CYA was initially dissolved in absolute ethanol and diluted in PBS immediately prior to use. The final concentration of ethanol did not exceed 0.1% (v/v).

Drug accumulation studies. Single cell suspensions in CEM (EMT6) or RPMI (H69) containing 2×10^5 cells/mL (as determined by haemocytometer counting) in 5 mL aliquots were allowed to equilibrate for 10 min at 37°. The drug solutions were added to produce final concentrations of 10 μg/mL for DOX, DNR and ACL (corresponding to 18.4, 19.0 and 12.3 μM) and 1 μg/mL Ro 31-3294 (1.8 μM), as used by others [24, 25]. During the incubation period the tubes were agitated at 10 min intervals. At the appropriate time points the tubes were centrifuged rapidly at 4° (300 *g* for 2 min) and the cells were washed twice with ice-cold PBS.

Drug extraction and quantitation was carried out by the method described by Schwartz [26]. A volume of 0.2 mL of ice-cold silver nitrate solution (33% w/v) was then added to each cell pellet and the tubes shaken for 10 min at 4°. At the end of this time 4 mL of isoamyl alcohol was added followed by a further 10 min shaking period and centrifugation for 5 min at 200 *g*. Alcohol layers were then transferred to 4 mL borosilicate glass tubes and the fluorescence due to drug content measured. Standards were prepared by the addition of appropriate amounts of anthracyclines to tubes containing untreated cells followed by immediate addition of sodium lauryl sulphate and silver nitrate. Fluorescence was shown to be linear with concentration. Fluorimetric analyses were carried out using an MPF-4 fluorescence spectrophotometer (Perkin Elmer, CT, U.S.A.). Wavelengths (in nm) for the spectrophotometric analysis were as follows: DOX ex 490, em 595; DNR ex 483, em 588; Ro 31-3294 ex 467, em 535; ACL ex 450, em 570.

Drug efflux studies. Single cell suspensions in CEM, containing 2×10^5 cells/mL (counted by haemocytometer counting) in 5 mL aliquots, were loaded with appropriate anthracycline compounds. Loading concentrations were as for accumulation experiments (see above). During the loading period (1 hr at 37°) the cell suspensions were agitated at 10 min intervals. Cell suspensions were then rapidly centrifuged, subjected to a single wash with ice-cold PBS, resuspended in prewarmed CEM and sampled at the time points indicated. Aliquots were washed twice with ice-cold PBS. Drug extraction and quantitation was carried as above.

To study efflux in drug-containing medium all anthracyclines were used at a concentration of 5 μM (in accordance with Friche *et al.* [27]). Single cell suspensions were prepared in glucose-free Eagle's minimal essential medium with the addition of 10 mM sodium azide and incubated for 10 min to achieve temperature equilibration. At time zero the appropriate anthracycline was added and the initial drug accumulation measured at the time points indicated by sampling of the cell suspension as before. At 30 min either 20 mM glucose in PBS or the same volume of PBS (100 μL) was added and the cell suspensions again sampled at the time points indicated. Cell pellets were washed and processed

Table 1. Drug accumulation and resistance factors in parental vs MDR cells

Compound	Drug accumulation at 120 min (pg/cell)				RF	
	EMT6/P	EMT6/AR1.0	H69/P	H69/LX4	EMT6	H69
DOX	3.20	0.30	3.10	1.13	33.9 (4.1)	131 (5.2)
DNR	10.10	3.60	5.20	2.30	25.0 (4.7)	67.5 (5.9)
ACL	11.20	11.20	9.20	9.20	4.7 (1.1)	5.8 (1.4)
Ro 31-3294	0.38	0.33	ND	ND	1.1 (0.1)	1.9 (0.1)

RF (see below) data taken from [15] (DOX, ACL) and [21] (Ro 31-3294).
Accumulation data for DOX and ACL taken from [22], and are taken from representative experiments (each data point performed in duplicate).
Figures in parentheses denote SD for three or more replicate analyses.
Calculation for resistance factor: IC_{50} resistant line/IC_{50} parental line.
ND, not done.

as before. This method is similar to that employed by Inaba *et al.* [6].

Effects of resistance modifiers on drug efflux. The method was followed as above except that either VRP at 3.3 μg/mL (6.6 μM), CYA at 5 μg/mL (4.2 μM) or solvent alone was added, together with the appropriate anthracycline at the start of the loading period. Upon washing and resuspension the resistance modifier was added to the drug-free medium at the same concentrations as before.

The effects of resistance modifiers on energy-dependent drug efflux were investigated as above except that the resistance modifying agent (in a 100 μL aliquot dissolved in PBS) was added to one set of cell suspensions at 30 min in combination with 20 mM glucose.

RESULTS

Drug accumulation

Table 1 summarizes differences in drug accumulation in parental versus resistant cells for DOX, DNR, ACL and Ro 31-3294. Resistance factors are also shown for comparison. The 120 min time point is used as this corresponds to the steady state drug concentration, judged by the plateauing of the accumulation curves [22]. It is clear that the largest differential in cellular accumulation in both the cell line pairs studied is seen for DOX. A somewhat smaller differential is seen for DNR whereas for ACL and Ro 31-3294 cellular accumulation is essentially the same for parental and MDR cell lines. These smaller differentials for ACL and Ro 31-3294 are in line with the lower resistance factors compared to DOX and DNR.

Anthracycline efflux

Figure 1A and B shows typical DOX efflux data obtained for the EMT6 and H69 cell line pairs, respectively. In these experiments cells were preloaded and resuspended in drug-free medium, as described in Materials and Methods. It can be seen that the differential between parental and resistant cell line efflux is somewhat smaller for H69 than EMT6, as the closeness of the efflux curves indicates. Figure 2A shows DNR efflux for the EMT6 cell lines. This occurred at a faster rate than the efflux of DOX (Fig. 1A) in both parental and resistant cells. A high proportion of the preloaded DNR was extruded within the first 10 min. Approximately 55% and 25% of the loading dose was retained at 60 min for EMT6/P and EMT6/AR1.0, respectively.

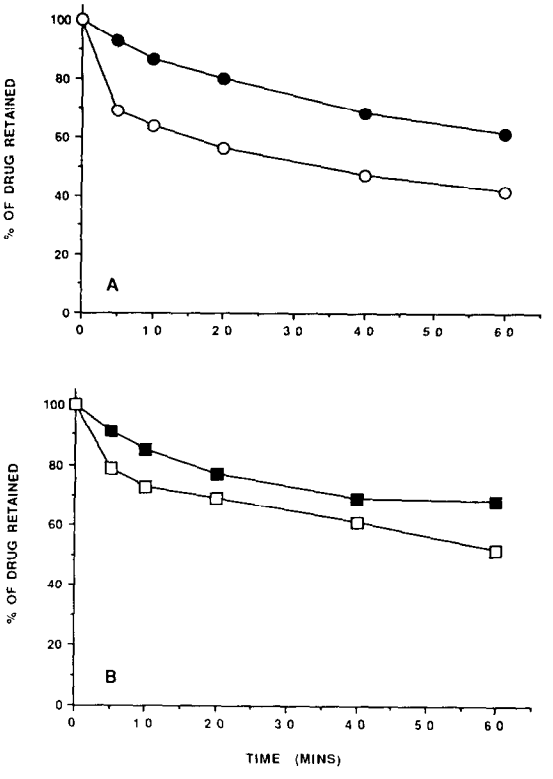

Fig. 1. Efflux of DOX from (A) EMT6/P (closed circles), EMT6/AR1.0 (open circles), and (B) H69/P (closed squares), H69/LX4 (open squares) cells lines, preloaded at 10 μg/mL for 1 hr. Note: points represent mean values from two independent experiments, each with duplicate data points with no more than 10% variation between them.

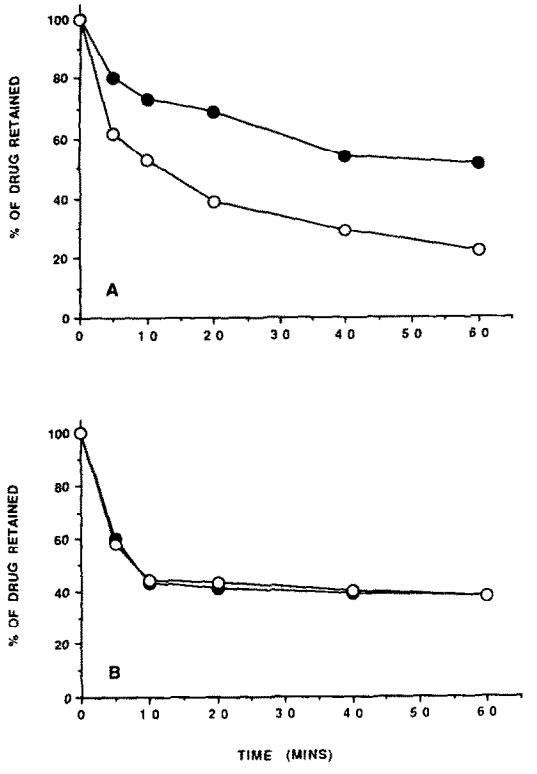

Fig. 2. Efflux of (A) DNR from EMT6/P (closed circles), EMT6/AR1.0 (open circles) and (B) aclacinomycin A, same cell lines. Note as for Fig. 1.

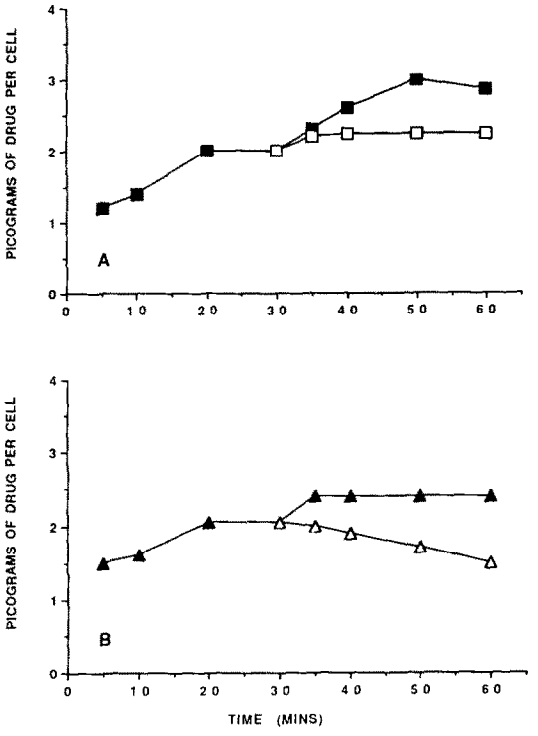

Fig. 3. Cellular content of DOX in (A) H69/P (squares) and (B) H69/LX4 (triangles) cell lines, in DOX-containing medium with sodium azide and glucose-free medium throughout (closed symbols); exogenous glucose added at 30 min (open symbol). Note: data points are mean values from duplicate samples (with less than 10% difference between them) from a single, representative experiment. The trends were confirmed in at least one, and usually two or three, independent repeat experiments.

Figure 2B shows the efflux of ACL in preloaded EMT6 cell lines, again after resuspension in drug-free medium. The initial rate of efflux was somewhat faster than that seen for both DOX (Fig. 1A) and for DNR (Fig. 2A). Most importantly, there was no difference in the efflux of ACL in the parent compared to the resistant cell lines. Similar results were obtained with ACL in the H69 cell lines (data not shown).

Energy-dependent efflux

Figures 3–6 and Table 2 show the results of drug efflux experiments in which cells were suspended in glucose-free medium plus azide, with or without the addition of glucose at 30 min. In contrast to the experiments described earlier, extracellular anthracycline was present throughout. In each case, the data shown in Table 2 are taken from different, independent experiments to those shown in the Figures. The data in the Table are, however, taken from full time-course experiments which, in each case, confirm the trends shown in the Figures. Figure 3 shows the efflux of DOX in the H69 cell lines whilst Fig. 4 shows the efflux of DNR in the EMT6 cell lines. It can be seen that from these data and those in Table 2 that the addition of glucose led to a plateauing of drug levels in EMT6/P and to a decline in drug levels in EMT6/AR1.0. Similar results were obtained for the H69 cell lines (Table 3). It can also be seen that DNR was effluxed (in an energy-dependent manner) to a greater extent than DOX.

For ACL in both parent and resistant EMT6 cell lines the cellular drug levels had reached a plateau at 20 min and addition of glucose at 30 min had only a very small effect (Fig. 5). Some glucose stimulation of drug efflux was demonstrated for the morpholinyl compound Ro 31-3294. However, the effect was small and the extent was equivalent for both parent and resistant cell lines as shown in Fig. 6 and Table 2. By comparison with the corresponding data obtained for DOX (Fig. 3, Table 2) a difference can be pinpointed. Whilst the effect of glucose stimulation at 60 min was virtually the same in the parent and resistant lines using Ro 31-3294 (as indicated by the fold change values, Table 2), there was a greater difference seen between the two cell lines at the same data point when using DOX. Thus for those compounds which gave low resistance factors in the EMT6 cell lines (see Table 1 and Refs 15, 21), glucose had little effect on their energy-dependent efflux.

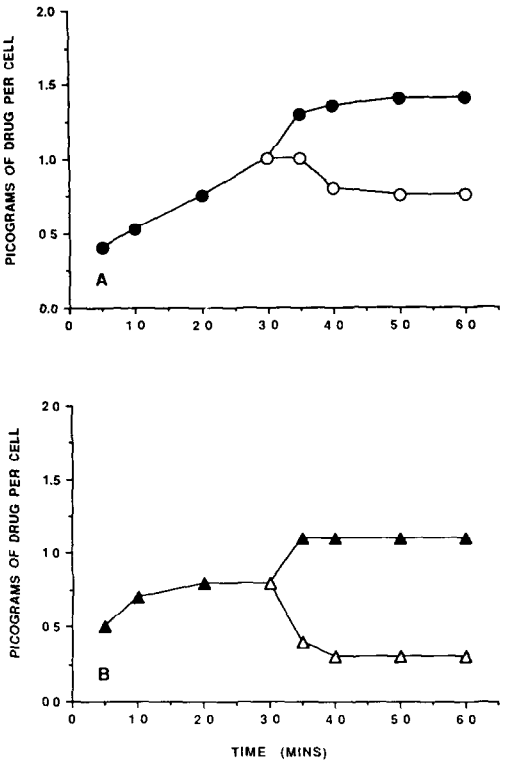

Fig. 4. Cellular content of DNR in (A) EMT6/P (circles) and (B) EMT6/AR1.0 (triangles) cell lines, in DNR-containing medium with sodium azide and glucose-free medium throughout (closed symbols); exogenous glucose added (open symbols) at 30 min. Note as for Fig. 3.

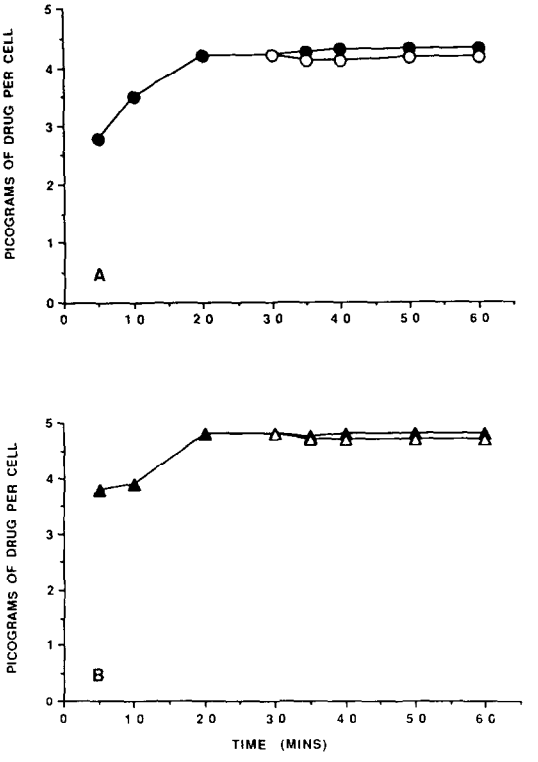

Fig. 5. Cellular content of ALL in (A) EMT6/P (circles) and (B) EMT6/AR1.0 (triangles) cell lines in ALL-containing medium with sodium azide and glucose-free medium throughout (closed symbols); exogenous glucose added (open symbols) at 30 min. Note as for Fig. 3.

The effects of resistance modifiers on cellular drug efflux

Figure 7 shows the efflux of DOX in preloaded EMT6/P and EMT6/AR1.0 cell lines in the absence or presence of VRP. Note that DOX was absent from the efflux medium in this experiment. The initial rate of DOX efflux was retarded for both EMT6/P and for EMT6/AR1.0 in the presence of VRP. In fact, the inhibition of DOX efflux by VRP was more pronounced in the parent line than in the resistant line, where only a small increase in cellular DOX levels was seen. The efflux of DOX in the presence of CYA was very similar to that seen in the presence of VRP (data not shown). Again, the major effect was on the efflux of DOX in the EMT6/P cell line.

The inhibition of the energy-dependent efflux of DOX by VRP is illustrated in Table 3. As previously, the data in Table 3 are from typical time-course experiments similar to those shown in Figs 7 and 8. In each case the trends were confirmed in independent, repeat experiments. The effect was noted for both the H69/P and H69/LX4 lines as well as the EMT6/P and EMT6/AR1.0 lines. Inhibition of DNR efflux by VRP is shown for the EMT6 cell lines in Fig. 8, where VRP did not totally abolish the stimulatory effect of glucose on efflux. Nevertheless there was a marked inhibition of drug efflux noted for the MDR line EMT6/AR1.0. The relatively small amount of energy-dependent efflux in EMT6/P was also completely inhibited by VRP. In addition, Table 3 shows that CYA had a similar effect to VRP on DNR efflux in the EMT6 cell lines. In this case the greatest effect was seen for the resistant cell line. In similar experiments with ACL in the EMT6 cells, the incorporation of VRP was shown to have no effect on the cellular levels of ACL (Table 3).

DISCUSSION

The efflux of selected anthracyclines has been characterized using parental and MDR sublines of two cultured cell lines. Both MDR lines show typical cross-resistance patterns and hyperexpression of membrane Pgp, characteristic of the classical MDR phenotype. A variety of experimental conditions were used in an attempt to elucidate the cellular pharmacological basis for strategies which result in circumvention of the MDR phenotype.

It is important to stress that in the two types of efflux experiments employed in this study the drug concentration gradients across the cell membrane are quite different. For efflux measured in preloaded cells suspended in drug-free medium, movement of drug molecules occurs down the concentration

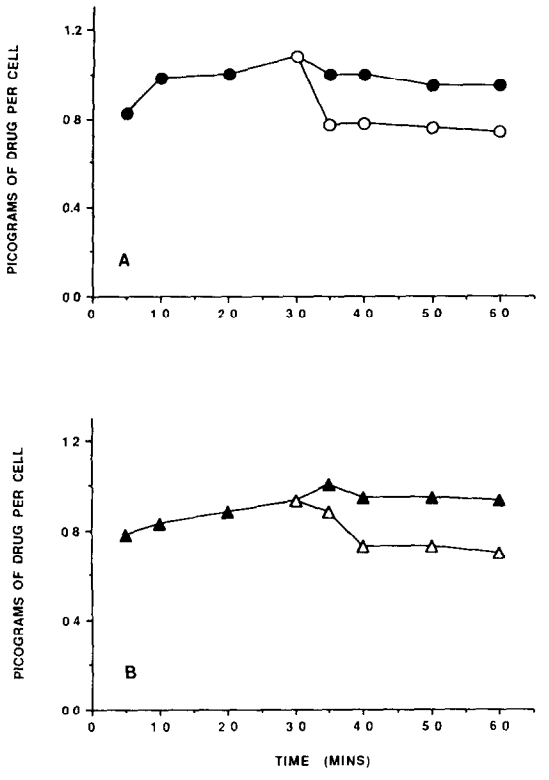

Fig. 6. Cellular content of Ro 31-3294 in (A) EMT6/P (circles) and (B) EMT6/AR1.0 (triangles) cell lines in Ro 31-3294-containing medium with sodium azide and glucose-free medium throughout (closed symbols); exogenous glucose added (open symbols) at 30 min. Note as for Fig. 3.

gradient, i.e. from a high intracellular drug concentration to essentially zero concentration extracellularly. Other experiments were designed to assess energy-dependent efflux with drug transported against a concentration gradient by using drug-containing medium. In these experiments azide was employed as a "pump poison" which inhibits oxidative phosphorylation and thereby decreases cellular ATP levels. Cells were in glucose-free medium. Subsequent glucose addition served to reactivate the pump by acting as a substrate for glycolysis as an alternative energy source. Energy-dependent membrane transport, such as that proposed for the Pgp efflux pump, is able to operate against a concentration gradient and it was envisaged that the above experimental conditions would optimize the identification of such Pgp-associated active mechanisms.

In an earlier study by Dano [3] it was suggested that the extrusion of DNR in a DNR-resistant cell line was against an electrochemical gradient and was carrier-mediated and energy-dependent, as judged from experiments designed to examine steady-state levels of the drug. The results of the present study, where experiments on cellular drug efflux have been carried out, are in agreement with that of Dano [3].

The anthracyclines DOX and DNR were shown to be accumulated to a lesser extent in the EMT6/AR1.0 and H69/LX4 cell lines than in their parental counterparts (Table 1), in line with them acting as substrate for Pgp in those cells. These two structurally related compounds appear to be extruded from MDR cells in a similar manner, as shown both by the results presented herein and by previous studies incorporating the use of metabolic inhibitors [6, 24]. However, it is clear that DNR is a better substrate for energy-dependent efflux than is DOX, even when extended time points beyond those shown here

Table 2. Data showing energy-dependent drug efflux in parental and MDR cell lines

Cell line	Drug	Cellular drug content (pg/cell) 30 min	60 min − glucose	60 min + glucose
EMT6/P	DOX	0.50	0.90 (1.8)	0.65 (1.3)
EMT6/AR1.0		0.30	0.55 (1.8)	0.20 (0 7)
H69/P	DOX	2.10	3.30 (1.6)	2.30 (1.1)
H69/LX4		2.00	2.30 (1.2)	1.50 (0.8)
EMT6/P	DNR	1.00	1.60 (1.6)	0.95 (1 0)
EMT6/AR1.0		1.00	1.20 (1.2)	0.30 (0.3)
H69/P	DNR	1.50	2.40 (1.6)	1.60 (1.1)
H69/LX4		1.20	1.60 (1.3)	0.55 (0.5)
H69/P	ACL	3.80	4.20 (1.1)	3.80 (1.0)
H69/LX4		4.20	4.20 (1.0)	4.00 (1.0)
EMT6/P	ACL	3.30	3.30 (1.0)	3.20 (1.0)
EMT6/AR1.0		3.40	3.20 (0.9)	3.10 (0.9)
EMT6/P	Ro 31-3294	1.10	0.95 (0.9)	0.70 (0.6)
EMT6/AR1.0		0.95	0.90 (0.9)	0.70 (0.7)

Figures in parentheses represent the fold change from the 30 min value (at which point glucose was added).

Data from typical experiments with duplicate samples at each point; see also Results.

Table 3. Data showing energy-dependent drug efflux in parental and MDR cell lines in the presence of resistance modifiers

Cell line	Drug	Cellular drug content (pg/cell) 30 min	60 min − glucose	60 min + glucose	60 min + glucose + RM
EMT6/P	DOX	0.60	1.00 (1.7)	0.85 (1.4)	0.93 (1.6)*
EMT6/AR1.0		0.55	0.70 (1.3)	0.42 (0.8)	0.60 (1.1)*
H69/P	DOX	0.45	0.61 (1.4)	0.42 (0.9)	0.61 (1.4)*
H69/LX4		0.43	0.48 (1.1)	0.32 (0.7)	0.48 (1.1)*
EMT6/P	DNR	1.10	1.60 (1.5)	0.85 (0.8)	1.20 (1.1)*
EMT6/AR1.0		0.85	1.30 (1.5)	0.30 (0.4)	1.05 (1.2)*
EMT6/P	DNR	1.20	1.60 (1.3)	1.00 (0.8)	1.10 (0.9)†
EMT6/AR1.0		1.05	1.60 (1.3)	0.45 (0.3)	1.60 (1.5)†
EMT6/P	ACL	5.60	5.60 (1.0)	5.6 (1.0)	5.6 (1.0)*
EMT6/AR1.0		5.50	5.40 (1.0)	5.5 (1.0)	5.5 (1.0)*

Legend as for Table 2.
RM, resistance modifier; * VRP; † CYA.

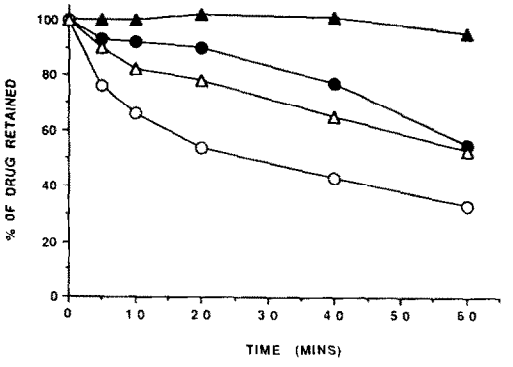

Fig. 7. Efflux of DOX from EMT6/P (closed symbols) or EMT6/AR1.0 (open symbols) cell lines, preloaded at 10 μg/mL for 1 hr, in the absence (circles) or presence (triangles) of VRP at 3.3 μg/mL. Note as for Fig. 1.

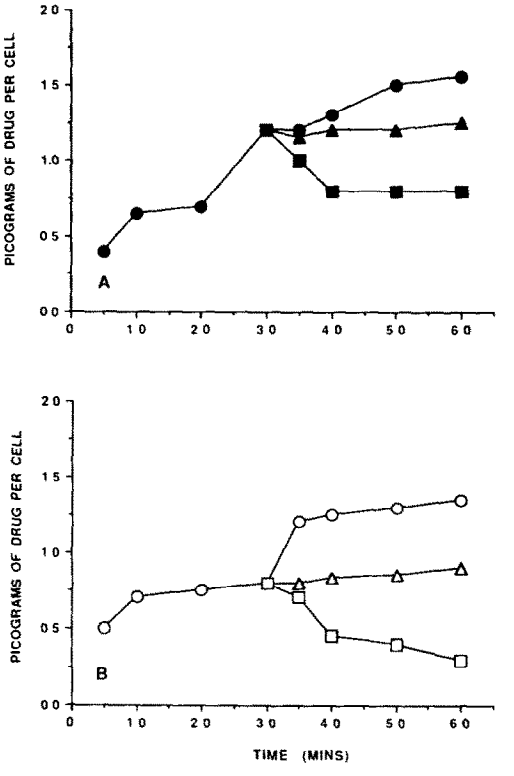

Fig. 8. Cellular content of DNR in (A) EMT6/P or (B) EMT6/AR1.0 cell lines, with sodium azide and glucose-free medium throughout (circles); with exogenous glucose added (squares; with exogenous glucose and VRP added (triangles). Note as for Fig. 3.

are examined (unpublished data). This agrees with the report by Inaba and Johnson [5] using sensitive and resistant P388 leukaemia lines, in which efflux of DNR was shown to be greater than that of DOX itself in the DOX-resistant MDR counterpart.

Since both the EMT6/AR1.0 and the H69/LX4 cell lines have lower levels of resistance to DNR than to DOX (resistance factors are 34 and 25 in EMT6 and 131 and 68 in H69, for DOX and DNR, respectively) the efflux and accumulation data are somewhat paradoxical. Kinetic studies have shown the K_m (an index of membrane carrier affinity) and V_{max} (a measure of membrane transport capacity) for DNR to be considerably higher than for DOX in a DOX-resistant Ehrlich ascites cell line [25]. In addition, a recent report by Spoelstra *et al.* [28] suggests that the saturation kinetics for DNR (in a wild-type human colon carcinoma cell line with intrinsic drug resistance) provide evidence for an active efflux of the drug across the cell membrane. The authors suggest that this may occur at two or more sites on Pgp, possibly involving the two halves of the protein molecule.

The efflux of ACL down its own concentration gradient appears to be rapid and virtually identical for both EMT6/P and EMT6/AR1.0, and for

H69/P and H69/LX4 cell lines. However, the energy-dependent efflux for ACL (measured against a concentration gradient) was minimal in both pairs of cell lines. The cellular handling of ACL probably involves predominantly passive transport processes, as there was no effect mediated by sodium azide. This is in agreement with the results of Fourcade *et al.* [29] using a Friend leukaemia cell line, but not with those of Zenebergh *et al.* [30] who noted significant inhibition of ACL efflux using metabolic inhibitors in the L-1210 cell line. On the basis of the results obtained herein it is proposed that ACL acts as a poor substrate for the putative efflux pump. This idea was also proposed by Seeber *et al.* [31] on the basis of drug transport studies using DOX-sensitive and -resistant Ehrlich ascites tumour cell line. The cellular pharmacokinetic behaviour of ACL in MDR cell lines may relate to the highly lipophilic character of this drug [32].

The results for the 9-alkyl morpholinyl DOX (MR-DOX) analogue Ro 31-3294 (Table 1) are in accordance with the cytotoxicity data reported previously [21]. Thus, this compound is equally cytotoxic in drug-resistant and in drug-sensitive cells and has virtually identical cellular pharmacokinetics in those cells. There appears to be a small amount of energy-dependent efflux of Ro 31-3294 but this is virtually identical for both cell lines. This contrasts with the data obtained for DOX and DNR. Streeter *et al.* [16] reported enhanced efflux of the related MR-DOX in a P388 DOX-resistant line compared to the parental line, despite a resistance factor of only 1.5. These data are suggestive of different cellular pharmacokinetics for MR-DOX compared to Ro 31-3294, possibly due to the presence of the 9-alkyl group in the latter.

It seems that mechanisms which regulate cellular drug levels are not solely responsible for the cytotoxic potential of the morpholinyl anthracyclines, and indeed the other anthracycline compounds in this study. In the case of the morpholinyl anthracyclines, the ionization of the amino nitrogen in the daunosamine moiety of this class of compounds may be an important factor for cellular retention and binding to intracellular sites. Whilst the pK_a of the amino group on DOX has been reported to be approximately 8.2 [33] the pK_a values of the morpholinyl derivatives are thought to be higher because of the presence of the electron-releasing groups [34]. In addition, Ro 31-3294 has two structural features that render it more lipophilic than the MR-DOX compound: firstly, the 9-alkyl group and secondly the loss of the weakly polar 4-methoxy group on the anthracenedione ring portion of the molecule. This chemistry could render Ro 31-3294 more favourable for membrane uptake than MR-DOX, although their accumulation profiles are very similar (unpublished data). A recent report by Watanabe *et al.* [18] describes the cellular pharmacokinetics of the 9-alkyl morpholinyl anthracycline MX2 [21] which bears very close structural resemblance to Ro 31-3294. The authors concluded that Pgp-mediated efflux is not rate limiting for MX2, which is in agreement with the hypothesis presented here that these particular anthracyclines represent poor substrates for this drug transport system.

The results for the experiments which incorporated the use of cells suspended in medium containing the anthracycline and resistance modifying agents are generally consistent with the expected role for an inhibition of energy-dependent drug efflux for the MDR cell lines. Conversely, the marked inhibition of efflux seen in the parent EMT6/P line as opposed to that seen for the EMT6/AR1.0 line was not expected. VRP and CYA have been shown previously to be effective resistance modifying agents in combination with DOX [15] where RF values were reduced to values ranging from 15.0 to 36.6 in the H69 cell lines. Interestingly, Fig. 7 shows VRP to be more effective in the inhibition of DOX efflux in the parental drug-sensitive cell line, as opposed to that seen for the MDR variant. In agreement with this, data previously published [15] show the marked VRP-induced DOX sensitization of the EMT6/P line to be greater than that seen for EMT6/AR1.0. This unexpected result may in part be explained by the presence of membrane Pgp in the EMT6/P cell line in addition to hyperexpression in the MDR counterpart [19]. From the data presented it appears that both the cytotoxic and the resistance modifying agents have to be present together on the external membrane surface to cause inhibition of cytotoxic drug efflux in the MDR cell line.

There was no effect on cellular ACL content mediated by VRP or CYA. However, it should be noted that the presence of resistance modifiers produced a modest enhancement of ACL activity for H69/LX4 and EMT6/AR1.0 in cytotoxicity experiments employing continuous drug exposure [15], e.g. resistance factor (RF) values reduced from 4.0 to 1.7 and 6.6 to 3.1 in H69 and EMT6, respectively. Resistance modifying agents have been shown to alter patterns of intracellular drug accumulation [35, 36]. Our subsequent studies will examine changes in intracellular localization of anthracyclines such as DOX and DNR under various experimental conditions, by use of confocal fluorescence microscopy.

A report by Hamada and Tsuruo [37] has indicated that VRP stimulates ATPase activity associated with Pgp at concentrations comparable to those which inhibit drug resistance in MDR cell lines. It is incongruous, therefore, that VRP (and trifluoperazine) which apparently inhibits efflux of chemotherapeutic drugs should enhance ATPase activity (and presumably encourage the pumping activity) of Pgp, the candidate molecule for the putative drug efflux pump. In support of this, a report by Broxterman *et al.* [38] showed that VRP increased cellular ATP consumption in Pgp-positive MDR cells. Hence, it appears likely that resistance modifying agents may have a locus (or loci) of action that is dependent upon changes in cellular ATP levels in MDR cells. Other possible effects include changes in membrane fluidity [39] and an enhanced VRP-induced decrease in membrane trafficking and turnover in a P388 DNR-resistant cell line compared to the parental line [40]. Hence, membrane trafficking and drug efflux could be related processes, as suggested by Beck [41].

The results obtained from the present study provide further support for the concept that a specific mechanism for overcoming the MDR phenotype involves enhancement of cellular drug concentrations by use of either structural analogues or resistance modifying agents. However, the question of whether the decreased drug retention seen in MDR cell lines is due to either the action of the energy-dependent drug efflux pump or to a diminished binding of drug to cellular organelles and proteins has not been fully answered by this study. We have demonstrated differences between parental and MDR cell lines in terms of drug efflux. It should be stressed that our findings may represent one of the many subtle changes apparent upon comparing such cell lines. Indeed, the importance of such findings in terms of their contribution to the MDR phenotype remains unclear. Nevertheless, evidence has been provided which supports the notion that energy-dependent membrane transport processes are implicated in MDR. More importantly we have suggested that compounds with effective cytotoxic activity against MDR cells may act as poor substrates for Pgp pump activity. Our findings are in agreement with those of Friche *et al.* [42] who concluded that the lipophilic analogue 4′-deoxy-4′-iododoxorubicin (an amino sugar-substituted anthracycline) is not a substrate for Pgp as it did not compete with DNR or vincristine for efflux processes. In addition, 4′-deoxy-4′-iododoxorubicin was shown by us to be effective against the two MDR lines used in the present study [15].

There have been a number of studies which have attempted to elucidate the structural and pharmacological requirements necessary to circumvent successfully the MDR phenotype. These include structure–activity relationships using either resistance modifying agents [42–46] or, as discussed earlier, using structurally altered analogues of commonly used anticancer agents. We propose that the 9-alkyl and morpholinyl-substituted anthracyclines are effective compounds against cells bearing the MDR phenotype, possibly due to their lack of affinity for the membrane Pgp drug transporter. Such compounds warrant further clinical investigation with regard to their usefulness in the treatment of MDR-associated tumour groups.

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