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Identification of anthracyclines and related agents that retain preferential activity over Adriamycin in multidrug-resistant cell lines, and further resistance modification by verapamil and cyclosporin A

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Summary. A range of anthracyclines and related compounds were evaluated for activity against murine and human cell lines exhibiting multidrug resistance (MDR). Cell lines used were the NCI-H69 human small-cell lung cancer line and the EMT6 murine mammary tumour line, together with their multidrug-resistant counterparts produced by in vitro exposure to Adriamycin (ADM). Chemosensitivity testing was carried out using the tetrazolium (MTT) dye assay. Results were expressed as the ratio of the ID_{50} for the resistant line to that obtained in the parent, i.e. the resistance factor (RF). Compounds exhibiting much lower RF values than ADM in both resistant cell lines were identified as those anthracyclines with 9-alkyl substituents and those with certain changes to the amino sugar residue at position 3' and 4', together with the anthracenedione mitoxantrone (MIT). In a further attempt to overcome resistance, we used four of these compounds, Ro 31-1215, 4'-deoxy-4'-iodo-ADM (iodo-ADM), aclacinomycin A (ACL) and MIT (all yielding low RF values), in combination with the resistance modifiers verapamil (VRP) and cyclosporin A (CYA). Additional enhancement of chemosensitivity was achieved in the ADM-resistant sublines, as shown by the further decrease in RF values. At the concentrations used, the largest effects were generally seen with CYA, and the combination of this modifier with ACL and MIT was particularly effective. For the H69/LX4 resistant line, the latter combinations gave RF values approaching unity. These findings point to the use of analogues with the 9-alkyl substituent and/or specific changes to the sugar residue in combination with resistance modifiers as a therapeutic strategy for circumvention of the MDR phenotype.

Introduction

Acquired resistance to cancer chemotherapy is a complex process involving multiple mechanisms [6, 30]. The precise molecular mechanism underlying pleiotropic or multidrug resistance (MDR) remains unclear, although the presence of P-glycoprotein appears to be an important correlate in experimental models [11, 24]. This membrane protein may play a role in the enhanced drug efflux commonly associated with the MDR phenotype [8, 11, 26].

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Attempts to overcome the problem of MDR involve two main approaches. Much attention has been focused on analogues of cytotoxic agents in current use in an attempt to discover compounds that retain relatively good activity in multidrug-resistant cells. Adriamycin (ADM), used extensively in chemotherapeutic regimens, readily induces resistance, as shown by numerous in vitro studies using tumour cell lines [17, 40]. Several analogues of ADM and other anthracyclines are in various stages of preclinical development or clinical trial [2, 43]. A range of these analogues and related agents have formed the focus of the present study, the first part of which attempts to identify structural features required for activity against multidrug-resistant cells.

A second major approach to the circumvention of MDR has been the use of resistance modifiers (RMs), agents that have been shown to reduce the degree of drug resistance in MDR cell lines [22, 32, 35, 36, 42]. We therefore evaluated the activity of two candidate RMs: verapamil (VRP), a calcium channel blocker, and cyclosporin A (CYA), a calmodulin-binding, immunosuppressive agent [12]. VRP has previously been demonstrated to alter cellular drug accumulation in MDR cell lines [35], presumably by the inhibition of efflux processes. Conversely, CYA has been reported to have no effect on cellular pharmacokinetics in similar cell lines [20]. We therefore decided to compare these agents in the light of their potentially differing mechanisms of resistance modification, in hopes that a combination of the improved relative cytotoxic activity found for certain analogues together with the effects of a resistance modifier might possibly completely overcome the MDR phenotype.

The experiments described in this paper were carried out using the tetrazolium (MTT) dye reduction assay, which represents an efficient, reproducible means of screening large numbers of cytotoxic agents in multiple cell lines [1, 5, 9, 23]. This technique relies on the conversion of MTT [3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyl tetrazolium bromide] to a coloured formazan product by reductive enzymes present only in metabolically viable cells [23, 33]. Hence, the quantitation of formazan production may be used to determine the ability of a test compound to inhibit cell proliferation.

We describe the use of the MTT assay to determine the effectiveness of selected ADM analogues and related compounds used together with resistance modifiers in two cultured cell lines and their respective ADM-resistant sub-

lines. The EMT6 murine mammary tumour cell line and its ADM-resistant subline (EMT6/AR1.0) were used together with the NCI-H69 human small-cell lung cancer (SCLC) line and its resistant subline H69/LX4. The drug-resistant sublines were derived in our laboratory by continuous exposure to ADM [40, 41] and have clearly demonstrable P-glycoprotein hyperexpression ([38]; JG Reeve and PR Twentyman, personal communication) as well as a spectrum of cross-resistance consistent with their classification as typical MDR lines.

Materials and methods

Cell lines and culture conditions. The NCI-H69 human small-cell lung cancer line (hereafter referred to as H69/P) and its in vitro-derived ADM-resistant variant (H69/LX4) were grown as floating aggregates in RPMI 1640 medium (Gibco Biocult, Paisley, UK) with 10% foetal calf serum (Seralab, Crawley Down, UK), penicillin and streptomycin (at concentrations of 100 IU/ml and 100 μg/ml, respectively). Stock cultures were maintained in 15 ml medium in 75-cm^2 tissue-culture flasks at 37° C in an atmosphere of 92% air and 8% CO_2. The H69/LX4 variant [40] was maintained in 0.4 μg/ml ADM, but the drug was removed at least 2 days before use in experiments. Cells were harvested from cultures in the exponential growth phase.

The murine mammary-tumour parent cell line EMT6/Ca/VJAC (hereafter referred to as EMT6/P) and its ADM-resistant variant EMT6/AR1.0 [41] were maintained as monolayers in Eagles' minimal essential medium with 20% newborn calf serum (Gibco Biocult) in 75-cm^2 flasks with the same antibiotics and gas and temperature conditions as given above for the H69 cell lines. The resistant variant EMT6/AR1.0 was routinely maintained in 1.0 μg/ml ADM until 2 days before experimental use. Again, harvested cells were in the exponential phase of growth.

H69 cell-line cultures for use in experiments were reduced by pipetting to a suspension containing small groups of cells. Cell counts were carried out by taking an aliquot of the suspension and incubating it with trypsin (0.4%) and versene (0.02%) in phosphate-buffered saline (PBS) for 15 min at 37° C. The single-cell suspension was counted manually using a haemocytometer counting chamber and diluted as appropriate.

The EMT6 cultured cell-line monolayers were subjected to two rinses with 0.1% trypsin in PBS followed by a 15-min incubation at 37° C. A single-cell suspension was obtained by resuspension of cells in full Eagles' medium with mechanical disaggregation and counted and diluted as for the H69 cell lines.

Drugs and chemicals. We are grateful for the gifts of the following compounds: cytorhodin HLB 817 (CTR) from Behring Ltd. (Marburg, FRG); anthrapyrazole C1941 (APZ) from Warner Lambert Ltd. (Ann Arbor, Mich, USA); morpholinyl ADM (MRA) from Dr E. Acton, M. D. Anderson Hospital and Tumor Institute (Houston, Tex, USA); 4'-epi-Adriamycin (4'-epi-ADM), 4'-deoxy-4'-iodo-Adriamycin (iodo-ADM), 4'-deoxy-Adriamycin (4'-deoxy ADM) and 4-demethoxy daunorubicin (4-demethoxy-DNR) from Farmitalia (Milan, Italy); Ro 31-1215 from Roche Products Ltd. (Welwyn Garden City, UK); mitoxantrone (MIT) from Lederle Ltd. (London, UK); aclacinomycin A (ACL) from Lundbeck Ltd. (Luton, UK); cyclosporin A (CYA) from Sandoz Ltd. (Basel, Switzerland). ADM was obtained from Sigma (Poole, UK) and verapamil (VRP) was supplied by Abbot laboratories (Queenborough, UK). Structures of the anthracyclines and related compounds are shown in Fig. 1.

All cytotoxic drugs except ACL were dissolved in distilled water at 500 μg/ml, filtered via a Millipore membrane (pore size, 0.2 μm) and stored in aliquots at −20° C. ACL was dissolved in 0.1% propylene glycol. Drugs were

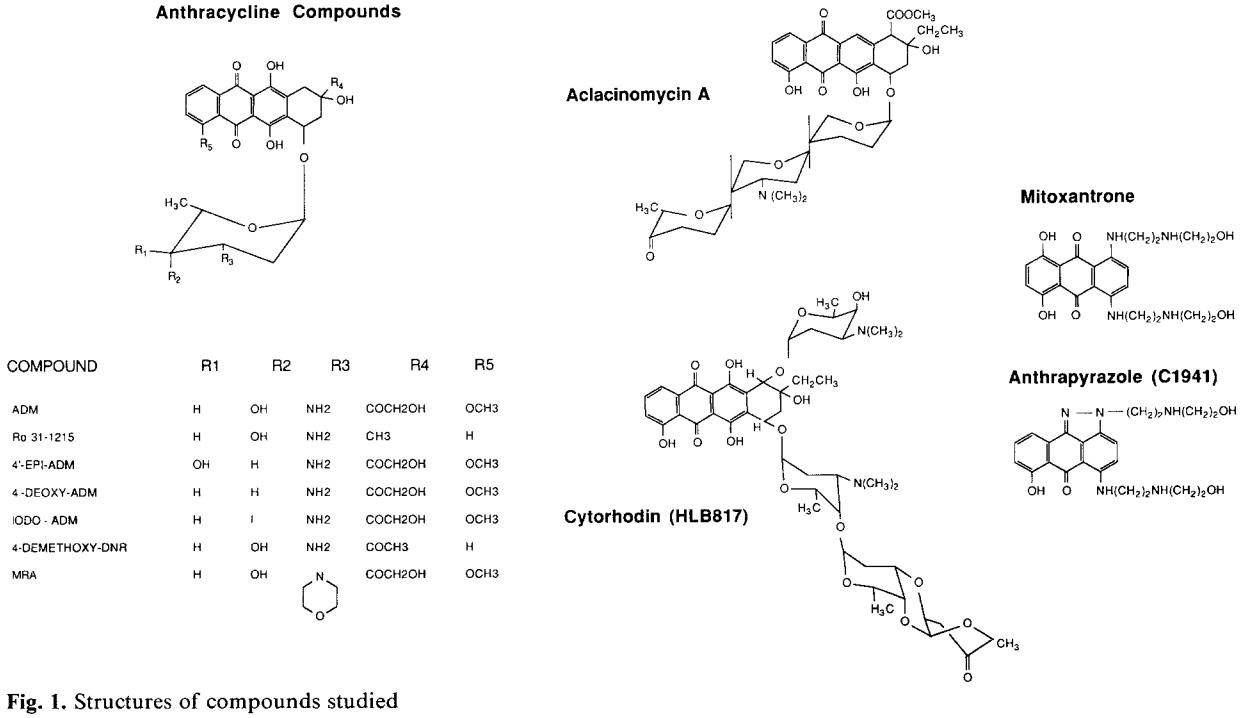

Fig. 1. Structures of compounds studied

thawed and diluted in distilled water immediately before use. 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 just prior to use. The final concentration of ethanol (0.1% v/v) does not affect cell growth or drug sensitivity.

MTT assay. The MTT assay was based on that described by Mosmann [23], with a number of modifications [1, 5, 39]. Cell suspensions were prepared as previously described and dispensed in 200-μl aliquots into 96-well tissue-culture plates at a concentration giving 1×10^3 and 2×10^3 cells/well for EMT6/P and AR1.0 and 6×10^3 and 1×10^4 cells/well for H69/P and H69/LX4, respectively. The cells were allowed to settle for 2–3 h. Drugs were then added to the wells in a 20-μl volume to give the required final concentration. The range of drug concentration was selected in preliminary experiments with the drug diluted over 4–5 orders of magnitude. The selected range encompasses drug doses that produce a decrease in optical density (due to formazan) to 10% of the control value (i.e. cell suspension in the absence of the cytotoxic agent).

The cells were continuously exposed to the drugs at 37° C in an atmosphere of 8% CO_2 and 92% air for a period equivalent to their achieving a 10- to 20-fold increase in cell number for untreated controls. These exposure times were 3 and 6 days for the EMT6 and H69 cell lines, respectively. Following this incubation, MTT solution (5 mg/ml in PBS) was added to each well at a volume of 20 μl. The plates were incubated for a further 5 h at 37° C. The enzyme reaction was terminated by the removal of supernatant from each well by gentle aspiration in the case of the EMT6 cell lines (which grow attached to the plastic surface) or by centrifugation of the plates (200 *g* for 5 min at 4° C) followed by careful aspiration for the floating population of H69 cells. The crystalline deposit of MTT formazan was then dissolved in 200 μl dimethyl sulphoxide (DMSO; BDH Chemicals, UK) and allowed to agitate gently for 10 min on a plate shaker.

Absorbances were read on a Titertek Multiskan MCC ELISA plate reader (Flow Laboratories; Helsinki, Finland) at a wavelength of 540 nm. The absorbance values obtained were expressed as a fraction of those obtained for the control wells. In all experiments, 3–6 replicate wells were used for each concentration. Each assay was carried out independently at least twice, ADM being included in each experiment as a standardisation control.

Resistance modifiers. RMs were added to cells at a volume of 10 μl to give a final concentration of 3.3 μg/ml (6.6 μ*M*) for VRP and 5 μg/ml (4.2 μ*M*) for CYA. The RMs were added to all wells, including those designated as controls (i.e. without cytotoxic drug). The plates were left at 37° C for a further 2–3 h before the addition of the cytotoxic agents, as described above.

The RM concentrations used have previously been shown to have a similar effect on ADM sensitivity in the H69/LX4 cell line, giving rise to 7- to 10-fold reductions in ID_{50} values [38]. In addition, both drug concentrations represent 2–3 times the clinically achievable peak plasma levels [16, 25].

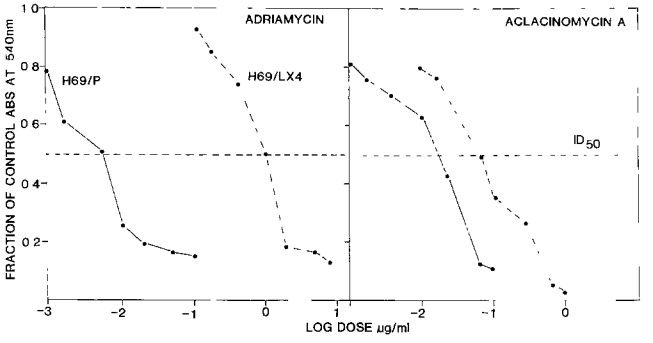

Fig. 2. Response of H69/P (*solid lines*) and H69/LX4 (*broken lines*) cell lines to Adriamycin or aclacinomycin A obtained using the MTT assay with continuous exposure. The ID_{50} values are read from the intersection of the horizontal broken line with the lines through the data points as indicated. These data are from a single typical experiment and the data points represent the mean values obtained from 3–6 replicate wells. The coefficient of variation was typically 11%

Results

Cross-resistance properties

Figure 2 shows typical dose-response curves seen for ADM and ACL with the H69 cultured cell lines. From such data ID_{50} values were estimated (indicated by the broken line), being the drug concentration required to reduce the final optical density to 50% of the control values. From ID_{50} values for parent and respective resistant cell lines, resistance factors (RF) were given by

$$RF = \frac{ID_{50} \text{ for the resistant line}}{ID_{50} \text{ for the parent line}}$$

Table 1 lists the ID_{50} and RF values for the various anthracyclines and related compounds obtained in the parent EMT6/P murine mammary tumour and EMT6/AR1.0 ADM-resistant lines, and Table 2 gives the corresponding data for the parent H69/P small-cell lung cancer line and its H69/LX4 ADM-resistant derivative. It may be seen that the RF for ADM was considerably higher in the H69 lines than in the EMT6 lines. Whereas the ID_{50} values of the two resistant lines were similar, the H69/P parent line was around 7-fold more sensitive to ADM than the EMT6/P parent line.

Table 1. ID_{50} and RF values for ADM, various analogues and related compounds in the EMT6/P (parent) and EMT6/AR1.0 (ADM-resistant) murine mammary tumour cell lines

Compound	Mean ID_{50} EMT6/P	EMT6/AR1.0 (μg/ml)	Experiments (*n*)	RF (mean ± SEM)
ADM	0.083	2.38	15	33.9 ± 4.1
Ro 31–1215	0.200	0.37	8	8.1 ± 1.5
ACL	0.028	0.09	7	4.7 ± 1.1
4′-Epi-ADM	0.058	0.54	3	10.1 ± 1.7
4′-Deoxy-ADM	0.006	0.264	5	47.0 ± 7.6
Iodo-ADM	0.028	0.11	6	4.4 ± 0.6
4-Demethoxy DNR	0.007	0.073	5	14.2 ± 3.8
APZ	0.25	1.56	5	9.2 ± 2.9
MIT	0.064	0.38	4	7.5 ± 1.9
CTR	0.006	0.047	6	8.2 ± 0.6
MRA	0.033	0.072	3	2.7 ± 0.8

RF, resistance factor

Table 2. ID_{50} and RF values for ADM, various analogues and related compounds in the H69/P (parent) and H69/LX4 (ADM-resistant) human small-cell lung carcinoma cell lines

Compound	Mean ID_{50} H69/P	H69/LX4 (μg/ml)	Experiments (*n*)	RF (mean ± SEM)
ADM	0.012	1.57	12	97.3± 5.2
Ro 31–1215	0.024	0.075	6	12.4± 1.6
ACL	0.034	0.14	6	5.8± 1.4
4'-Epi-ADM	0.002	1.18	4	528 ±79.0
4'-Deoxy-ADM	0.0055	0.37	3	63.5± 8.8
Iodo-ADM	0.0009	0.015	5	18.9± 1.7
4-Demethoxy DNR	0.00009	0.023	2	287 ±95.0
APZ	0.021	5.6	4	142 ±52.0
MIT	0.054	0.41	5	15.4± 6.6
CTR	0.037	0.18	3	5.4± 1.8
MRA	0.028	0.07	2	2.4± 0.2

RF, resistance factor

Although differing markedly in their sugar residues as well as at the 10-position of the A-ring (CTR), the three analogues Ro 31-1215, ACL and CTR share two common features: (1) all lack the 4-methoxy substituent in the D-ring, and (2) all possess a 9-alkyl substituent in the A-ring (Fig. 1). These three compounds gave low RF values compared with those for ADM in both the H69 and EMT6 lines. The RF values for Ro 31-1215 were 4-fold and 8-fold lower than for ADM in the EMT6 and H69 lines, respectively; those for CTR were 4-fold and 18-fold lower, and those for ACL, 7-fold and 17-fold lower. These dramatic effects cannot be attributed to the absence of the 4-methoxy residue, as 4-demethoxy DNR did not show a consistent change in RF compared with ADM. In the case of the EMT6 lines, a small reduction of 50% was seen, compared with a 3-fold increase for the H69 lines. However, it was clear from the ID_{50} values that 4-demethoxy DNR was a more potent drug than ADM, particularly in the two H69 cell lines.

Table 3. RF und SR values obtained with VRP used in combination with ADM and selected compounds in the EMT6/P and EMT6/AR1.0 cell lines

Compound	SR		RF	
	EMT6/P	EMT6/AR 1.0	−VRP	+VRP
ADM	14.2	2.3	22.9	143.0
	2.9	1.4	38.0	74.2
	7.5	5.3	35.2	60.0
	>25.0	6.4	35.6	140.0
Ro 31–1215	2.6	3.6	6.8	5.0
	1.5	4.7	7.9	2.4
	1.4	9.5	13.0	2.1
Iodo-ADM	1.0	1.5	4.6	3.0
	3.6	>2.8	7.0	<9.1
ACL	0.8	1.6	6.6	3.1
	1.2	3.6	9.9	3.0
MIT	0.8	1.6	13.3	6.0
	3.6	7.2	7.2	3.6

SR, sensitisation ratio; RF, resistance factor in the absence (–) or presence (+) of VRP

There was no overall trend within the data for the analogues substituted in the 4' position of the daunosamine sugar to show either increased or decreased RF values. The RF for 4'-epi-ADM was 3 times lower than that for ADM in the EMT6 cell lines but 5-fold higher in the H69 lines. In the EMT6 lines, 4'-deoxy-ADM gave RF values greater than those obtained for ADM. However, in H69/LX4 this analogue was capable of partially overcoming drug resistance, as shown by the 1.5-fold decrease in RF. The 4'-deoxy-4'-iodo analogue, iodo-ADM, showed a substantial decrease in RF in both the EMT6 and the H69 cell lines compared with ADM (8-fold and 5-fold, respectively), with lower ID_{50} values for all of the individual cell lines.

MRA possesses a morpholinyl ring that incorporates the 3'-amino nitrogen of the daunosamine sugar unit. This compound exhibited a substantial reduction in RF for both cell lines, with decreases of 13- and 40-fold for EMT6 and H69, respectively. The novel anthrapyrazole APZ gave a marked reduction of 75% in RF for the EMT6 cell lines and a 45% increase for the H69 cell lines. The anthracenedione MIT exhibited reductions in RF values of 4- and 6-fold for EMT6 and H69, respectively.

Resistance modifiers

On the basis of their low RF values and their distinct structural features, four compounds were chosen for evaluation alongside ADM in combination with RMs: Ro 31-1215, iodo-ADM, ACL and MIT. VRP- and CYA-mediated effects were evaluated in the parent and resistant cell lines for both H69 and EMT6. The results for individual experiments are summarised in Tables 3–6, in the form of RF values in the presence and absence of the modifiers and as the derived sensitisation ratios (SR) given by

$$SR = \frac{ID_{50} \text{ in the absence of RM}}{ID_{50} \text{ in the presence of RM}}$$

Table 4. RF und SR values obtained with VRP in combination with ADM and selected compounds in the H69/P (parent) and H69/LX4 (ADM-resistant) cell lines

Compound	SR		RF	
	H69/P	H69/LX4	−VRP	+VRP
ADM	1.3	8.3	100.0	15.0
	0.8	4.5	88.8	15.9
	1.1	6.1	209.0	36.6
	1.6	6.3	100.0	26.7
Ro 31–1215	1.6	3.2	12.0	6.1
	1.3	1.9	10.0	7.5
Iodo-ADM	1.9	6.0	21.5	7.1
	1.3	2.5	15.6	8.0
	1.1	8.0	18.6	2.6
ACL	0.9	2.1	3.8	1.7
	1.3	1.7	7.6	5.5
	0.9	10.2	11.9	1.0
	1.0	2.4	4.0	1.7
MIT	1.8	2.9	3.1	1.9
	1.5	1.2	7.6	4.0

SR, sensitisation ratio; RF, resistance factor in the absence (–) or presence (+) of VRP

Table 5. RF and SR values obtained with CYA in combination with ADM and selected cytotoxic compounds in the EMT6/P (parent) and EMT6/AR1.0 (ADM-resistant) cell lines

Compound	SR		RF	
	EMT6/P	EMT6/AR 1.0	−CYA	+CYA
ADM	>11.2	>15.8	28.2	–
	9.1	22.9	22.9	9.1
	2.3	>15.9	35.2	< 5.0
Ro 31–1215	3.6	> 0.1	9.7	< 3.8
	3.2	17.3	8.6	1.7
	2.1	> 2.2	11.2	<10.4
Iodo-ADM	1.0	1.6	4.6	2.8
	1.4	1.8	7.0	5.7
ACL	1.3	4.0	6.4	2.0
	2.0	1.0	9.3	4.6
MIT	1.8	5.6	13.3	4.1
	3.1	1.8	7.2	1.3

SR, sensitisation ratio; RF, resistance factor in the absence (–) or presence (+) of CYA

Table 6. RF und SR values obtained with CYA in combination with selected compounds in the H69/P (parent) and H69/LX4 (ADM-resistant) cell lines

Compound	SR		RF	
	H69/P	H69/LX4	−CYA	+CYA
ADM	2.5	24.7	143.0	14.5
	4.0	27.3	88.8	13.0
	1.0	>7.9	209.0	<25.0
Ro 31–1215	0.9	6.3	13.2	1.8
	1.6	3.6	12.0	5.3
	1.3	2.1	10.0	6.3
Iodo-ADM	1.3	7.3	24.2	4.4
	1.1	5.3	18.6	3.9
ACL	0.6	2.4	3.8	1.0
	0.7	5.3	7.6	1.0
	0.6	9.0	11.9	1.3
	1.0	2.6	4.0	1.5
MIT	0.9	3.1	3.1	0.9
	0.1	5.5	7.6	1.0

SR, sensitisation ratio; RF, resistance factor in the absence (–) or presence (+) of CYA

An unusual effect of VRP was seen in the experiments with ADM in the EMT6 parent and resistant cell lines (Table 3). Although a clear enhancement of ADM sensitivity was observed in the EMT6/AR1.0 resistant line, an even greater effect was seen with the EMT6/P parent line. In contrast, for all other agents the SR values for the resistant counterpart exceeded those obtained in the parent line (Table 3). In addition, the preferential sensitising effect shown by VRP on the ADM sensitivity of the EMT6/P line was not seen for CYA; for all compounds the SR values were generally higher in the resistant line (Table 5). In corresponding experiments using the H69 cell lines (Tables 4 and 6), a different pattern of effects was seen. VRP had negligible effect on the ADM response in the H69/P line, as indicated by SR values of around 1.0, but increased the sensitivity in the H69/LX4 line, with SR values varying from 4.5 to 8.3. Clear, preferential enhancement of sensitivity was also seen in the resistant line with CYA; SR values varied from 0.95 to 4.0 in the H69/P line compared with values of around 25 for the H69/LX4 resistant line.

When VRP was used in the EMT6 parent and resistant cell lines (Table 3), a moderate decrease in RF was seen for Ro 31-1215, for example from a control value of 7.9 down to 2.4. Similarly, iodo-ADM, ACL and MIT all showed decreases in RF values in the presence of VRP, typically in the region of 50%. In similar experiments with the H69 lines (Table 4), RF values were consistently reduced, approaching 1.0 for MIT and ACL, indicating an almost complete circumvention of MDR. For CYA in the EMT6 cell lines, the largest decreases in RF were seen with Ro 31-1215 and MIT, with smaller but significant decreases occurring for ACL and iodo-ADM (Table 5).

In general, the effects of CYA on RF values were even more marked in the H69 cell lines, with the exception of Ro 31-1215, where moderate decreases were seen (Table 6). CYA produced around a 5-fold reduction in RF values for iodo-ADM in the H69 cell lines as opposed to the 40% and 20% reductions seen with the EMT6 cell lines. The most striking results were seen with ACL and MIT, where RF values were consistently reduced to values approaching unity (Table 6).

To summarise this large series of results with VRP and CYA, the overall effect was to reduce the RF values, with the exception of VRP with ADM in the EMT6 cell lines. CYA appeared to be the more effective modifier at the concentrations used, and its combination with ACL or MIT emerges as a particularly successful means of circumventing MDR, especially in the H69/LX4 resistant line.

Discussion

We describe the use of the MTT chemosensitivity assay to select rapidly from a large series of compounds those to which ADM-resistant cell lines show minimal cross-resistance. This has enabled us to characterize certain key structural features and to study the effects of VRP and CYA as RMs.

Previous studies by ourselves [41] and Scott et al. [27] have identified the 9-alkyl substitution as a distinct molecular feature in anthracyclines, the incorporation of which leads to a marked decrease in RFs in rodent and human MDR lines. We confirm that ACL and Ro 31-1215 retained activity in ADM-resistant H69/LX4 and EMT6/AR1.0 cells compared with ADM, and similar results were also obtained for the 9-alkyl analogue CTR, as noted elsewhere [18]. Although these three compounds lack the 4-methoxy substituent present in ADM, such activity was not seen with 4-demethoxy DNR, suggesting that this is unlikely to be an important molecular feature for activity against MDR cells. The EMT6/AR1.0 cell line showed a degree of cross-resistance with 4-demethoxy DNR, whereas the H69/LX4 line was fully cross-resistant to it. Although both ACL and CTR contain markedly different and more complex sugar residues than ADM, Ro 31-1215 contains a daunosamine sugar identical to that of ADM, consistent with the view that the 9-alkyl substitution can predominate over certain modifications to the sugar for activity against MDR cells [27, 41].

On the other hand, our studies have additionally shown that two compounds also yielding low RF values,

namely, iodo-ADM and MRA, have alterations in the sugar residue but lack the 9-alkyl substitution. Thus, changes at the adjacent 3' or 4' positions of the sugar can also confer activity against MDR cell lines. For both EMT6 and H69 cell lines, MRA exhibited the lowest RF values of any of the compounds investigated, in agreement with the previous observation [34] for morpholinyl anthracyclines in ADM-resistant P388 cells in vitro.

With iodo-ADM we consistently obtained lower RF values than with ADM in both the EMT6 and H69 cell lines. Potency was greater by 100-fold in the H69/LX4 and 20-fold in the EMT6/AR1.0 lines, compared with the parent anthracycline. Previous reports have shown this compound to be 1.5- to 2-fold more cytotoxic than ADM in P388 ADM-resistant cells in vitro [3]. Two human non-small-cell lung cancer lines with varying degrees of inherent resistance to ADM in vitro similarly showed a relative lack of cross-resistance to iodo-ADM as well as to Ro 31-1215 [21].

The importance of the 4' position on the sugar residue was further illustrated by our observation that 4'-epi-ADM was more effective against the EMT6/AR1.0 line than was ADM in terms of the RF values obtained. However, the complexity of the situation is evident from our finding that this agent exhibited much higher RF values than ADM in the H69 cell lines. In addition, a P388 ADM-resistant line has been reported to be fully cross-resistant to 4'-epi-ADM in vivo [27]. Furthermore, 4'-deoxy-ADM was shown to be moderately effective against the H69 cell line, with a 1.5-fold decrease in RF, whereas the EMT6 cell line was fully cross-resistant. Hill et al. [13] have reported a lack of cross-resistance to 4'-deoxy-ADM in the murine lymphoma L5178Y ADM-resistant subline in vitro. It should be noted, however, that this latter line exhibited a low degree (2.5-fold) of resistance compared with the lines used in the present study. Scott et al. [27] have reported that the 4'-deoxy anthracycline analogue Ro 31-2118 exhibited a reduced RF value compared with ADM for both the CCRF human lymphoblastoid leukaemia and P388 leukaemia ADM-resistant lines in vitro.

In our studies the anthrapyrazole APZ gave rise to considerably lower RF values than ADM in EMT6 but not in H69 cell lines. Merry et al. [21] have reported a degree of cross-resistance to ADM in the two human non-small-cell lung cancer lines mentioned above. In addition, a lack of cross-resistance with ADM has been demonstrated for APZ and related compounds in a series of in vivo murine tumour systems [19]. We also showed that the anthracenedione MIT gave lower RF values than ADM in both the EMT6/AR1.0 and H69/LX4 lines. Scott et al. [27] have reported a similar lack of cross-resistance in the ADM-resistant U266BL lymphoblastoid leukaemia line in vitro, whereas cross-resistance has been observed in the ADM-resistant P388 tumour in vivo [7].

For the four selected compounds exhibiting low RF values (ACL, Ro31-1215, iodo-ADM and MIT), a direct relationship can be seen between the degree of cross-resistance and the extent of sensitisation by VRP in both EMT6/AR1.0 and H69/LX4. Thus, lower sensitisation ratios were obtained for the agents to which cross-resistance with ADM is reduced. With CYA the same relationship holds true for EMT6/AR1.0 but not H69/LX4. Overall, these results are in good agreement with those previously reported by Tsuruo et al. [37].

It is important to emphasise that although the degree of sensitisation by VRP and CYA was lower than that by ADM for the compounds to which the MDR cell lines showed minimal cross-resistance, the overall effect of combining these agents with the RMs appears promising. With MIT and ACL, for example, resistance in the H69/LX4 subline was fully circumvented. In contrast, Gibby et al. [10] have reported no significant enhancement by VRP in the in vitro cytotoxicity of MIT for a L5178Y murine lymphoma with 4-fold in vitro resistance to ADM.

The precise molecular mechanisms by which MDR may be overcome through the use of structurally modified anthracyclines or resistance-modifying agents remain unclear and may be multifactorial. We have demonstrated markedly reduced ADM accumulation in both the EMT6/AR1.0 (unpublished data) and H69/LX4 lines [40], in agreement with other studies [4, 14, 15, 31]. In clear contrast, similar experiments using Ro 31-1215, ACL and iodo-ADM have shown a much smaller differential in cellular uptake between parent and resistant lines (unpublished data), suggesting a difference in cellular pharmacokinetics for these compounds. Moreover, we have recently found that both VRP and CYA can increase the accumulation of ACL, Ro 31-1215 and iodo-ADM in the resistant lines to levels similar to those in the parent counterparts (unpublished data). These observations are in good agreement with most other studies on the effects of various RMs on MDR cells [32, 35, 36], but they conflict with a report by Slater et al. [32] using CYA.

Since both the EMT6/AR1.0 and H69/LX4 ADM-resistant lines exhibit gene amplification and hyperexpression of P-glycoprotein mRNA together with increased expression of the protein itself, the results are consistent with a model in which an up-regulation of P-glycoprotein is causally involved in the reduced cellular accumulation and enhanced efflux of MDR-type drugs. The potential for P-glycoprotein to act as an "efflux pump" is supported by its structural homology to the bacterial haemolysin transport protein [8, 11, 24]. Alterations in membrane properties, in particular, increased membrane endocytotic processing or "trafficking", has been reported in drug-resistant cell lines [28], which could facilitate drug trapping and extrusion to the extracellular medium [28], and it is conceivable that P-glycoprotein and/or other membrane or cytosolic proteins may be involved in this mechanism. VRP has been shown to inhibit membrane traffic in MDR cell [29] and to bind to P-glycoprotein [26]. Our current studies are designed to elucidate the relationship between the expression of P-glycoprotein, on the one hand, and membrane traffic and subcellular drug handling, on the other.

References

1. Alley MC, Scudiero DA, Monks A, Hursey ML, Czerwinski MJ, Fine DL, Abbot BJ, Shoemaker RH, Boyd MR (1988) Feasibility of drug screening with panels of human tumour cell lines using a microculture tetrazolium assay. Cancer Res 48: 589–601
2. Arcamone F (1985) Properties of antitumour anthracyclines and new developments in their application: Cain Memorial Award Lecture. Cancer Res 45: 5995–5999
3. Barbieri B, Suarto A, Penco S, Geroni C, Bellini O, Fumagli A, Casazza AM, Guiliana FC (1984) Biologic activity of 4'-haloanthracyclines. Proc Am Assoc Cancer Res 25: 305

4. Biedler JL, Riehm H (1970) Cellular resistance to actinomycin D in Chinese hamster cells in vitro: cross-resistance, radioautographic and cytogenetic studies. Cancer Res 30: 1174–1184
5. Carmichael J, De Graff WG, Gazdar AF, Minna JD, Mitchell JB (1987) Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. Cancer Res 47: 936–942
6. Chabner B (1986) The oncologic end game (Karnofsky Memorial Lecture). J Clin Oncol 4: 625–638
7. Chandrasekaran B, Dimling J, Capizzi RL (1987) Cross-resistance to menogaril and mitoxantrone in a subline of P388 leukaemia resistant to doxorubicin. Cancer Treat Rep 71: 195–196
8. Chen CJ, Chin JE, Ueda K, Clark DP, Pastan I, Gottesman MM, Roninson IB (1986) Internal duplication and homology with bacterial transport proteins in the MDR 1 (P-glycoprotein) gene from multidrug resistant cells. Cell 47: 381–389
9. Cole SP (1986) Rapid chemosensitivity testing of human lung tumour cells using the MTT assay. Cancer Chemother Pharmacol 7: 259–263
10. Gibby EM, Boyse O, Hill BT (1987) Selective interactions of verapamil with anthraquinones in Adriamycin-sensitive and -resistant murine and human tumour cell lines. Cancer Chemother Pharmacol 20: 5–7
11. Gros P, Croop J, Houseman D (1986) Mammalian multidrug resistance gene: complete cDNA sequence indicates strong homology to bacterial transport proteins. Cell 47: 371–380
12. Hess AD, Colombani PM (1986) Mechanism of action: in vitro studies. Prog Allergy 38: 198–221
13. Hill BT, Dennis LY, Li X, Whelan RDH (1985) Identification of anthracycline analogues with enhanced cytotoxicity and lack of cross-resistance to Adriamycin using a series of mammalian cell lines in-vitro. Cancer Chemother Pharmacol 14: 194–201
14. Inaba M, Johnson RK (1978) Uptake and retention of Adriamycin and daunomycin by sensitive and anthracycline resistant sublines of P388 leukemia. Biochem Pharmacol 27: 2123–2130
15. Inaba M, Kobayashi H, Sakurai Y, Johnson RK (1979) Active efflux of daunorubicin and Adriamycin in sensitive and resistant sublines of P388 leukemia. Cancer Res 39: 2200–2203
16. Kahan BD, Reid M, Newburger J (1983) Pharmacokinetics of cyclosporine in human renal transplantation. Transplant Proc 15: 446–451
17. Kaye S, Merry S (1985) Tumour cell resistance to anthracyclines – a review. Cancer Chemother Pharmacol 14: 96–103
18. Kraemer HP, Berscheid HG, Ronneberger H, Zilg H, Sedacek HH (1986) Preclinical evaluation of cytorhodin S, a new anthracycline, with activity in a human tumor based screening system. Proceedings of the 5th NCI EORTC symposium on new cancer therapy, Amsterdam
19. Leopold WR, Nelson JM, Plowman J, Jackson RC (1985) Anthrapyrazoles, a new class of intercalating agents with high-level, broad spectrum activity against murine tumours. Cancer Res 45: 5532–5539
20. Meador J, Sweet P, Stupecky M, Wetzel M (1987) Cyclosporin A enhances daunorubicin efficacy in Ehrlich ascites carcinoma and murine hepatoma 129. Cancer Res 47: 6216–6219
21. Merry S, Courtney ER, McCormick C, Kaye SB, Freshney I (1986) In-vitro cross-resistance patterns of anthracyclines in human non-small cell lung cancer lines with differing inherent resistance to Adriamycin. Br J Cancer 55: 336–337
22. Merry S, Courtney ER, Fetherson CA, Kaye SB, Freshney I (1987) Circumvention of drug resistance in human non-small cell lung cancer in vitro by verapamil. Br J Cancer 56: 401–405
23. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival. Application to proliferation and cytotoxicity assays. J Immunol Methods 65: 55
24. Riordan JR, Ling V (1985) Genetic and biochemical characterisation of multidrug resistance. Pharmacol Ther 28: 51–75
25. Rogan AM, Hamilton TC, Young RC, Klecker RW Jr, Ozols RF (1984) Reversal of Adriamycin resistance by verapamil in human ovarian cancer. Science 224: 994–996
26. Safa AR, Glover CI, Meyers MB (1986) Vinblastine photoaffinity labelling of a high molecular weight surface membrane glycoprotein specific for multidrug-resistant cells. J Biol Chem 261: 6137–6140
27. Scott CA, Westmacott D, Broadhurst MJ, Thomas GJ, Hall MJ (1986) 9-Alkyl anthracyclines. Absence of cross-resistance to Adriamycin in human and murine cell cultures. Br J Cancer 53: 595–600
28. Sehested M, Skovsgaard T, Deurs B van, Winther-Nielsen H (1987) Increase in nonspecific adsorptive endocytosis in anthracycline and vinca alkaloid-resistant Ehrlich ascites tumor cell lines. J Natl Cancer Inst 78: 171–177
29. Sehested M, Skovsgaard T, Deurs B van, Winther-Nielsen H (1987) Increased plasma membrane traffic in daunorubicin resistant P388 leukaemia cells. Effect of daunorubicin and verapamil. Br J Cancer 56: 747–751
30. Selby P (1984) Acquired resistance to cancer chemotherapy. Br Med J 288: 1252–1253
31. Skovsgaard T (1978) Carrier mediated transport of daunomycin, Adriamycin and rubidazone in Ehrlich ascites tumour cells. Biochem Pharmacol 27: 1221–1227
32. Slater LM, Sweet P, Stupecky M, Wetzel MW, Gupta S (1986) Cyclosporin A corrects daunorubicin resistance in Ehrlich ascites carcinoma. Br J Cancer 54: 235–238
33. Slater TF, Sawyer B, Stauli U (1963) Studies on succinate-tetrazolium reductase systems. Biochim Biophys Acta 77: 383
34. Streeter DG, Johl JS, Gordon GR, Peters JH (1986) Uptake and retention of morpholinyl anthracyclines by Adriamycin-sensitive and -resistant P388 cells. Cancer Chemother Pharmacol 16: 247–252
35. Tsuruo T, Iida H, Tsukagoshi S, Sakurai Y (1982) Increased accumulation of vincristine and Adriamycin in drug resistant P388 tumour cells following incubation with calcium antagonists and calmodulin inhibitors. Cancer Res 42: 4730–4733
36. Tsuruo T, Iida H, Tsukagoshi S, Sakurai Y (1983) Potentiation of vincristine and Adriamycin effects in human haemopoietic cell lines by calcium antagonists and calmodulin inhibitors. Cancer Res 43: 2267–2272
37. Tsuruo T, Kawabata H, Nagumo N, Iida H, Kitatani Y, Tsukagoshi S, Sakurai Y (1985) Potentiation of antitumor agents by calcium channel blockers with special reference to cross-resistance patterns. Cancer Chemother Phrmacol 15: 16–19
38. Twentyman PR (1988) Modification of cytotoxic drug resistance by non-immunosuppressive cyclosporins. Br J Cancer 57: 254–258
39. Twentyman PR, Luscombe M (1987) A study of some variables in a tetrazolium dye (MTT) based assay for cell growth and chemosensitivity. Br J Cancer 56: 279–285
40. Twentyman PR, Fox NE, Wright K, Bleehen NM (1986) Derivation and preliminary characterisation of Adriamycin resistant lines of human lung cancer cells. Br J Cancer 53: 529–537
41. Twentyman PR, Fox NE, Wright KA, Workman P, Broadhurst MJ, Martin JA, Bleehen NM (1986) The in-vitro effects and cross-resistance patterns of some novel anthracyclines. Br J Cancer 53: 585–594
42. Twentyman PR, Fox NE, White DJG (1987) Cyclosporin A and its analogues as modifiers of Adriamycin and vincristine resistance in a multi-drug resistant human lung cancer cell-line. Br J Cancer 56: 55–57
43. Weiss RB, Saosy G, Clagett-Carr K, Russo M, Leyland-Jones B (1986) Anthracycline analogs: the past, present and future. Cancer Chemother Pharmacol 18: 185–197

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