Circumvention of Inherent or Acquired Cytotoxic Drug Resistance *In Vitro* Using Combinations of Modulating Agents

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Abstract. *Background/Aim: Modulating agents are used to circumvent drug resistance in the clinical setting. However achievable serum concentrations are often lower than those which are optimal in vitro. Combination of modulating agents with non-overlapping toxicities may overcome this obstacle. We have investigated combinations of three modulating agents (quinine, verapamil, and cinnarizine) to circumvent inherent or acquired resistance to the cytotoxic drugs doxorubicin, vincristine and paclitaxel. Materials and Methods: Dose-response curves to cytotoxic drugs in the presence/absence of modulating agents were determined using colony formation and cell proliferation assays. Doxorubicin accumulation into cell monolayers was measured by fluorescence spectrophotometry. Results: Greater (1.9-fold) sensitisation to particular cytotoxic drugs was observed for certain combinations of modulating agents compared to individual effects. The most effective combination was quinine-plus-verapamil with the cytotoxic drug doxorubicin. This increase in sensitivity was associated with increased doxorubicin accumulation. Such enhanced activity was, however, not observed for all combinations of modulating agents or for all studied cytotoxic drugs. Conclusion: The findings of the present study suggest certain combinations of modulating agents to have a clinical role in circumventing drug resistance. Particular combinations of modulating agents must be carefully chosen to suit particular cytotoxic drug treatments.*

The cytotoxic drugs doxorubicin hydrochloride (Adriamycin hydrochloride, DOX), vincristine sulphate (VC) and paclitaxel (taxol, PT) are used in the clinical setting to treat a variety of cancers, but it is becoming increasingly apparent that a major obstacle in their effective use is the presence or emergence of drug-resistant cells within the tumour (1).

DOX, VC and PT are all associated with the multidrug resistance phenotype (2-4), which has been observed in tumours made resistant by chronic treatment with cytotoxic drugs *in vitro* or *in vivo* (5-7). One characteristic of this phenotype is the presence of particular membrane proteins such as the P-glycoprotein (8-9) or multidrug resistance-associated protein (MRP) (10, 11) leading to altered membrane transport of a range of cytotoxic drugs. The clinical relevance of the multidrug resistance phenotype remains to be proven, but both P-glycoprotein and *MRP* have been shown to be expressed in a number of clinical tumour specimens (12-14).

One approach to investigate clinical relevance has been to study drug resistance in cell lines derived from clinical specimens, which have not received any prior drug treatment *in vitro*. While levels of resistance in this case are generally lower than those which can be derived in the laboratory, our previous studies of such inherent resistance in human glioma (15) and human non-small cell lung cancer (16) cell lines have shown similar patterns of cross-resistance between cytotoxic drugs as those observed in the multidrug resistance phenotype. The relevance of the multidrug resistance phenotype to clinical drug resistance is particularly important because such multidrug resistance can be circumvented by a number of modulating agents (17-19). The most thoroughly studied multidrug resistance-modulating agent is verapamil (VPM), which we have also shown to circumvent inherent resistance in human glioma and non-small cell lung cancer cell lines (16, 20).

Laboratory studies of multidrug resistance-modulating agents have lead to several clinical trials involving compounds such as VPM (21, 22) quinidine (23) amphotericin B (24) and cyclosporin A (25, 26). These trials have produced some encouraging results, but they have also shown that concentrations of modulating agents that can be achieved in the serum of patients are often less than those, which are optimal in reversing drug resistance *in vitro*. One way of effectively achieving higher serum (and hence tumour) concentrations might be to use combinations of

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modulating agents which have different toxicities so that each can be used at its maximum dosage in the presence of the other.

There are two additional reasons why combinations of modulating agents may be more effective than a single agent in circumventing drug resistance. Firstly, a variety of mechanisms (in an addition to changes in membrane permeability) have been shown to contribute to multidrug resistance in a variety of experimental models. Such mechanisms include changed DNA topoisomerase II activity (18, 27, 28) modified glutathione metabolism (29, 30) and altered intracellular drug binding (31). These mechanisms (and others) may contribute to inherent or acquired resistance, and they may be circumvented to different extents, by different modulating agents, such that combinations are more effective than single agents. Secondly, it has been shown that P-glycoprotein has multiple binding sites (32, 33), which have different affinities for different modulating agents. It is possible that P-glycoprotein would be completely inactivated in the presence of combinations of modulating agents, which bind to different sites rather than a single agent binding to a single site.

Previous *in vitro* studies have shown effective use of combinations of modulating agents including cyclosporin A, VPM and Tamoxifen in modulating the expression of the multidrug resistance phenotype (34, 35). In particular, significant synergism between tamoxifen and cyclosporine A on the sensitivity of DOX was seen at clinically administered concentrations within the hepatoma cell lines (35). However, further studies are warranted to identify which particular combinations of modulators at physiologically-achievable concentrations are most effective in overcoming the multidrug resitance phenotype.

In the present study we investigated the ability of the modulating agents quinine (QN), verapamil (VPM) and cinnarizine (CN) as single agents and in combination, in circumventing inherent resistance to DOX, VC and PT in the continuous murine cell line L929, its DOX resistant variant L929/A and the human breast cancer cell line MCF7. Each modulating agent was used at its maximum tolerated dose *in vivo* (ID_{50}/10: MTD). In each case this was a similar concentration to that achieved in plasma in patients (36-38). The modulating agents used in this study were chosen with a view to future clinical studies since they fulfil the criteria of having generally non-overlapping toxicities *in vivo*.

Materials and Methods

Cell lines. L929 (murine fibrosarcoma) and MCF7 (human breast adenocarcinoma) cell lines were obtained from ECACC (Porton Down, Wiltshire, UK). The DOX-resistant cell line, L929/A, was established during this work by exposure of parent L929 cells to increasing concentrations of DOX over a period of 3 months. Once established, the cell line was routinely sub-cultured and grown in medium containing 0.086 μM DOX. Stock cultures were maintained in 25-cm^2 tissue culture flasks and shown to be free of mycoplasma by staining with Hoechst 33258 (Sigma, Poole, Dorset, UK). Cells were grown at 37˚C as monolayer cultures in RPMI 1640 medium supplemented with 10% foetal bovine serum and, when the gas phase was ambient air, 10 mM HEPES. In some cases the medium was supplemented with 50 μg/ml gentamycin sulphate (Sigma). Cell suspensions were prepared by trypsinisation and viable cells (those excluding 0.2% trypan blue) were counted using a Neubauer haemocytometer.

Chemicals. The cytotoxic drugs DOX, VC and PT, the modulating agents QN, VPM and CN, and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenylterazolium (MTT) were obtained from Sigma. Stock solutions of DOX, VC, PT, VPM and QN (5 mg/ml, 1mg/ml, 0.5mg/ml, 50 mg/ml and 50mg/ml respectively) were prepared in 50% ethanol. Stock solutions of CN (50 mg/ml) were prepared in dimethyl sulphoxide (DMSO). All stock solutions were stored as aliquots in the dark at –20˚C, and were diluted with culture medium immediately before use. MTT (1 mg/ml) was dissolved in Dulbecco's phosphate buffered saline without calcium and magnesium (pH 7.2; PBS) (Sigma), prior to use and kept at 4˚C.

Colony formation assay. Five-hundred L929 cells in 10 ml culture medium were seeded into Greiner 90-mm tissue culture petri dishes (Philip Harris, Litchfield, Staffordshire, UK) in the presence of drugs or solvent (control). The dishes were incubated for 9-11 days with ambient air as the gas phase, fixed with methanol, stained with 0.1% crystal violet and colonies ≥0.5 mm diameter were scored visually. Colony number was expressed as a percentage of control and the concentration of drug causing a 50% reduction (ID_{50}) was determined graphically. Plating efficiency of controls was 45±3% (mean±S.E.M, n=60).

Sensitisation ratios as described by Merry *et al*. (1989) were calculated as: $ID_{50} - M / ID_{50} + M$ (15). Where M is the modulating agent (or combination of modulating agents) employed in that particular assay. Sensitisation ratios were calculated using the cytotoxicity curves obtained from duplicate petri dishes at each of 5 different concentrations all within a single experiment. All experiments were carried out at least in duplicate.

Cell proliferation assay. Fifty thousand L929, L929/A or MCF7 cells in 200 μl medium were seeded per well into Nunc 96-well tissue culture plates (Fisher Scientific, Loughborough, UK) in the presence of drugs or solvent (control). Cells were incubated for 7 days with a gas phase of 5% CO_2 in air before 20 μl MTT were added to each well and the plates were incubated for an additional 3 h (39). The supernatant was then aspirated from each well and 200 μl of DMSO were added to solubilize the coloured formazan product. After 20-30 min the absorbance (570 nm) of each well was determined (Anthos 2010 Microplate Reader, Anthos Labtech Instruments, Salzburg, Austria). Results were expressed as percent cytotoxicity relative to control wells.

Doxorubicin accumulation assay. One million L929 cells in 10 ml culture medium were seeded into Greiner 90-mm tissue culture petri dishes (Philip Harris), which were then incubated for 48 h with ambient air as the gas phase prior to the addition of 34 μM DOX. At 0, 30, 60, 90, 120 and 150 min the medium was poured from duplicate dishes and the cell monolayers were washed with ice cold

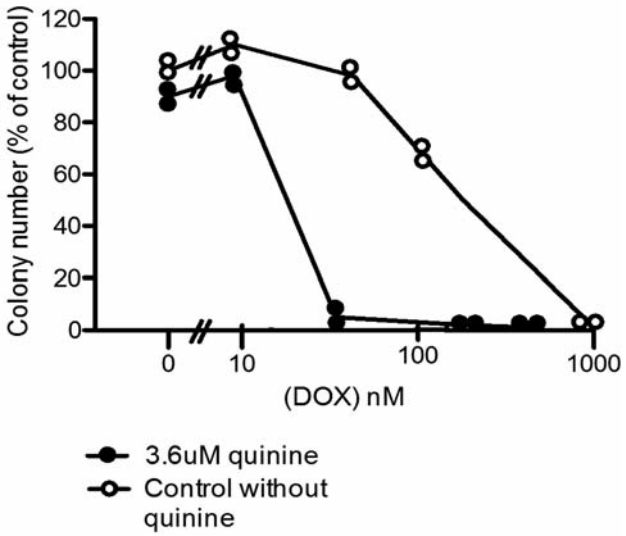

Figure 1. *The effect of quinine on the sensitivity of L929 cells to doxorubicin. The lines join the mean of duplicate determinations at each doxorubicin concentration.*

PBS (3x5 ml). Cell suspensions were prepared from the monolayers by treatment with 2 ml 0.25% trypsin/ 0.02% EDTA in PBS and an aliquot was removed for viable cell counting. A further 1.5-ml aliquot was homogenized (4 strokes of a Potter homogeniser) and DOX was extracted into 5 ml chloroform/isopropanol (2:1) and measured by fluorescence spectrophotometry (SFM 25 Spectrofluorometer, Kontron Instruments, Watford, UK) with excitation and emission wavelengths of 480 nm and 560 nm respectively (45). All experiments were carried out at least in triplicate. Preliminary experiments showed the absence of background fluorescence from untreated cell homogenates and DOX extraction efficiencies of 101±3% (mean±s.e.m, n=14).

Results

In the L929 model with colony formation as end-point the ID_{50} values for cytotoxic drugs DOX, VC and PT in the absence of modulating agents were found to be 124±21 nM, 87±20 nM and 160±25 nM respectively (mean±S.E.M, n=25, 20 and 13 respectively). Furthermore the ID_{50} values of the modulating agents QN, VPM, CN -alone were found to be 35.7 μM (range: 29.7-41.6 μM), 4.4 μM (range: 3.0-5.7 μM) and 2.7 μM (range: 2.6-2.8 μM) respectively. In subsequent cytotoxicity experiments these modulating agents were used at their maximum tolerated dose (MTD) *in vitro*, which was determined as 1/10 ID_{50}. For each modulator these are similar concentrations to those that have been achieved in plasma in patients (36-38) and, in all cases, the MTD inhibited colony formation *in vitro* by less than 6%.

Figure 1 shows the result of a typical colony formation assay. In this particular case QN is seen to produce a sensitisation ratio to DOX of 11.3 (ID_{50}-QN=186.2 nM, ID_{50}+QN=16.6 nM). Table I summarises the overall results of our L929 colony formation assays. It is seen that for all three cytotoxic drugs QN was the most effective modulating agent, VPM was less effective and CN showed little activity in this model. Furthermore, the size of the sensitisation ratio produced was dependent on the cytotoxic drug used. Sensitisation ratios were greatest for PT, less for DOX and smallest for VC.

Table I. *Summary of cytotoxicity results showing the effects of maximum tolerated doses of quinine, verapamil and cinnarazine on the sensitivity of L929 cells to doxorubicin, vincristine and paclitaxel.*

Modulating agent	Sensitisation ratio* (range, n=2)		
	DOX	VC	PT
QN	11.3-13.2	3.4-4.7	23.9-26.3
VPM	3.2-4.1	2.8-3.6	5.2-6.7
CN	1.5-1.7	0.9-1.1	1.4-1.4
QN plus VPM	21.1-25.8	5.9-6.9	15.8-18.8
QN plus CN	6.1-6.7	2.9-4.7	18.8-20.4
VPM plus CN	4.5-4.7	2.5-2.8	8.2-11.9

*Ratio=DOX ID_{50} without modulating agent(s)/DOX ID_{50} with modulating agent(s).

The combination of QN plus VPM produced greater sensitisation ratios than either modulating agent alone for DOX and for VC, but not for PT. Furthermore VPM plus CN produced greater sensitisation ratios than either modulating agent alone for DOX and PT, but not for VC, while QN plus CN produced lower sensitisation ratios than QN-alone for all three cytotoxic drugs. Toxicity (36±11%, mean±S.E.M., n=4) precluded the use of all three modulating agents together in our colony formation assay.

Figure 2 shows the effect of 3.6 μM QN (ID_{50}/10 MTD) on DOX accumulation into L929 cells. QN is seen to produce an increase in accumulation of 63% at 150 min (DOX accumulation-QN=655 pmol/10^6 cells, DOX accumulation+QN=1,069 pmol/10^6 cells). Furthermore the increase in DOX accumulation produced by QN was statistically significant at 60, 90, 120 and 150 min ($p<0.05$, $p<0.01$, $p<0.02$ and $p<0.05$, respectively) with a statistical trend ($p<0.1$) at 30 min. The accumulation of DOX both in the presence and absence of QN had not reached a plateau at 150 min, but experiments were terminated at this time point because longer incubations produced reductions in cell viability.

The overall results in our DOX accumulation experiments are summarised in Table II. In all cases the shape of the accumulation curves were similar to those of Figure 2. At 150 min QN and VPM increased accumulation by 63% and

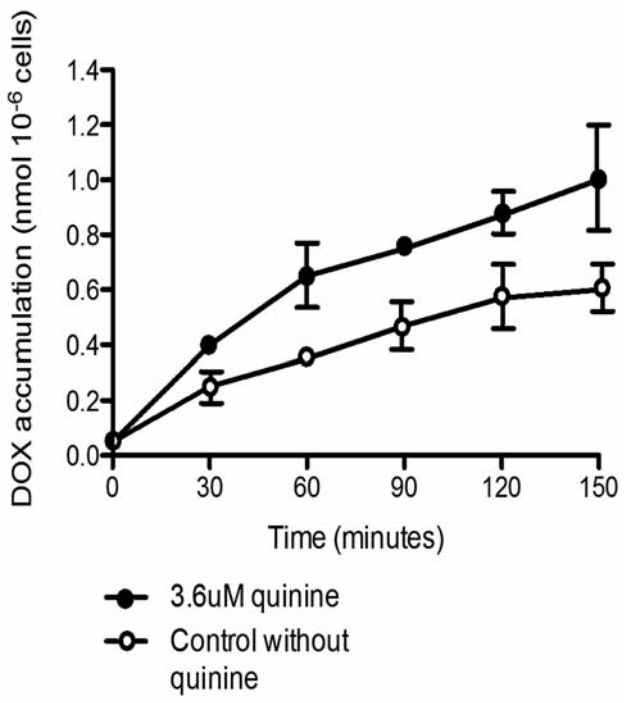

Figure 2. *A typical experiment showing the effect of quinine on doxorubicin accumulation into L929 cell monolayers. The lines join the mean of 3-4 determinations at each time point. Error bars indicate mean±S.E.M. and are omitted when their range is smaller than the size of the symbol used to indicate mean value.*

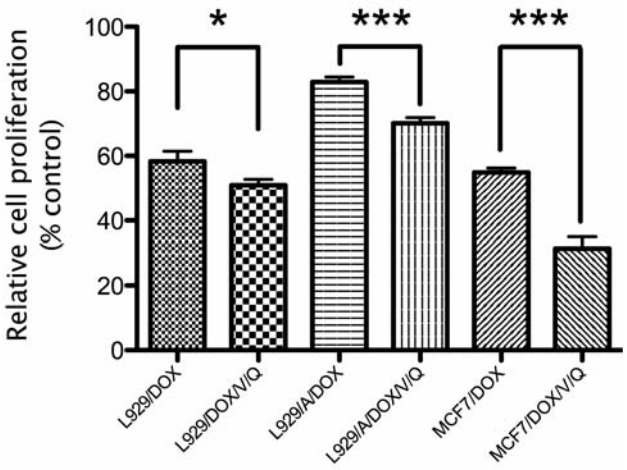

Figure 3. *Summary of the cell proliferation assay data showing the effect of maximum tolerated doses of the combined modulating agents quinine and verapamil on doxorubicin (100 nM) sensitivity. Error bars indicate mean±S.E.M. (n=20-60). *p<0.05, ***p<0.01.*

50% respectively, while CN produced a 16% reduction. The combination QN plus VPM increased DOX accumulation by 79%, whereas QN plus CN and VPM plus CN caused 3% and 8% reduction respectively. In additional experiments (data not shown) it was noted that 6.6 μM and 66 μM VPM produced increases of 74% and 71% respectively. Furthermore the combination QN plus VPM plus CN produced an increase of 53%.

Table III enables comparison of the effects of modulating agents on DOX resistance and DOX accumulation. It can be seen that QN and VPM enhanced accumulation both as single agents and in combination, and the increases are associated with increased DOX sensitivity with colony formation as end-point. This contrasts with the case of CN, which produced no increase in DOX accumulation both as a single agent and in combination with QN or VPM, even though CN, QN+CN and VPM+CN each produced increased sensitivity to DOX.

Figure 3 summarises results from cell proliferation assays. The effective modulator combination (VPM plus QN) identified for DOX in the colony formation assays also significantly increased DOX (100 nM) sensitivity ($p<0.05$, n=60) for L929 cells in cell proliferation assays. Similar sensitisation effects were additionally seen in the DOX-resistant variant L929/A ($p<0.0001$, n=60) and in the human breast cancer cell line MCF7 ($p<0.0001$, n=20).

Discussion

We have examined the ability of three drug resistance-modulating agents (QN, VPM and CN) both as single agents and in combination to circumvent inherent or acquired resistance to three cytotoxic drugs (DOX, VC and PT) in murine L929 and human MCF7 cells.

Initial experiments in this study utilised a cell line with inherent drug resistance mechanism (L929) since this may more truly reflect clinical drug resistance compared to multidrug resistant cell lines in which resistance has been derived by chronic treatment with cytotoxic drugs in the laboratory. Inherent drug resistance may result from a combination of mechanisms (see Introduction), which are not all equally amplified in multidrug-resistant cell lines derived in the laboratory. Subsequent experiments confirmed the relevance of observations concerning L929 to a variant cell line with acquired DOX resistance (L929/A) and to a human breast cancer cell line (MCF7).

The modulating agents were used at their MTD in this experimental model, which was determined as $ID_{50}/10$. These concentrations (3.6 μM QN, 0.4 μM VPM or 0.3 μM CN) were chosen since they are equally close to the toxicity threshold of the modulating agent. Furthermore those are similar concentrations to these that have been achieved in plasma in patients (36-38). Kunnumakkara *et al.* (2008) have argued for the utility of conventional rather than targeted

Table II. *Summary of Doxorubicin uptake data showing the effect of maximum tolerated doses of quinine, verapamil and cinnarizine on doxorubicin accumulation into L929 cells after150 min.*

Modulating agent	DOX accumulation (pmol 10^{-6} means±S.E.M. n=3-4)
None (control)	655±69
QN	1069±138*
VPM	983±52**
CN	552±17
QN plus VPM	1172±69 **
QN plus CN	638±52
VPM plus CN	603±69

*p<0.05, **p<0.02, ***p<0.01 using Student's t-test.

Table III. *Comparison of the effects of maximum tolerated doses of modulating agents on doxorubicin resistance and accumulation in L929 cells.*

Modulating agent (s)	Mean fold change in DOX sensitivity	Mean fold change in DOX accumulation at 150 min
QN	X 12.3	X 1.6
VPM	X 3.7	X 1.5
CN	X 1.6	X 0.8
QN + VPM	X 23.5	X 1.8
QN + CN	X 6.4	X 1.0
VPM + CN	X 4.6	X 0.9

'smart' drugs in cancer treatment since cancer typically involves the dysregulation of multiple pathways (44). The disruption of a single pathway by a 'smart' drug, they argue, is unlikely to be fully effective. QN, VPM and CN are each conventional drugs, which can be regarded as multi-targeted, and the range of affected pathways is further increased by their use in combination.

While the extent to which the modulating agents circumvented resistance in L929 cells varied between the three cytotoxic drugs. In all cases QN was the most effective single agent followed by VPM, and with CN showing only marginal activity. This may suggest that in this model there is a single mechanism (or group of mechanisms) that confers resistance to all three cytotoxic drugs. The use of combinations of modulating agents produced, in some cases, greater increases in sensitivity than either of the modulating agents alone with the most effective combination being QN plus VPM with the cytotoxic drug DOX. Such increased effectiveness of combinations was, however, not always observed and, when it was present, it did not apply to all cytotoxic drugs. This suggests that any combination of modulating agents used clinically would need to be first carefully chosen.

While combinations of modulating agents were sometimes more effective than single agents, with the possible exception of VPM plus CN with PT, synergistic effects were not observed. This is consistent with the presence of multiple mechanisms of inherent resistance in L929 cells since different modulating agents may well have different, but overlapping, abilities to circumvent each of the resistance mechanisms. The situations where combinations of modulating agents are less effective than one of the agents alone are more difficult to explain, but could result if a modulating agent had a mixture of activities which circumvent some resistance mechanisms, but enhance others.

Our DOX accumulation data support the previous suggestions. One useful example is the effect of CN on the circumvention of DOX resistance by QN. The enhanced DOX accumulation produced by QN as a single agent, was reversed by the addition of CN since QN plus CN produced no increase in DOX accumulation. QN plus CN, however, still circumvented DOX resistance, but to a lesser extent than QN alone. One possible interpretation of these results is that QN circumvented DOX resistance by additional mechanisms present in L929 cells as well as by enhancing DOX accumulation. When CN is present the enhanced accumulation is prevented, but the other resistance-circumventing activities of QN remain. In this interpretation of the data CN has contradictory effects. It actually enhances one mechanism of DOX resistance (*i.e.* reduced DOX accumulation), but circumvents other mechanisms of DOX resistance to a sufficient extent that it overall increases DOX sensitivity when used as a single agent.

Research into combining chemotherapeutic agents to allow increases in cytotoxic efficacy is always a topic of interest. However, experiments have mainly focused on attacking different biochemical targets (40) and there have been few previous studies investigating the effect of combinations of modulating agents on drug resistance. Three studies have used cell lines in which resistance was derived in the laboratory. Lehnert *et al*. reported that combinations of VPM and QN can be synergistic in overcoming DOX and vinblastine resistance in a multidrug-resistant human myeloma cell line (41). Hu *et al*. also reported a synergistic effect for combinations of VPM and cyclosporine A in circumventing DOX resistance in human leukaemia cell lines (34) whereas VPM and *trans*-flupenthixol was reported to have an additive effect in circumventing DOX resistance in a human breast cancer cell line (42). A fourth study by Ishida *et al*. showed that VPM and cyclosporine A, at some concentrations, had a

synergistic effect in overcoming inherent VC resistance in a human erythroleukaemia cell line (43). Our work also supports potential synergy in the utilisation of combinations of modulators and suggests viability in their ability to circumvent drug resistance.

Furthermore, these studies and our data on inherent and acquired resistance indicate that the complex interactions between modulating agents, could possibly be exploited to circumvent drug resistance in the clinic and that combinations of modulating agents could be more effective than single agents. However the optimum combination of modulating agents may differ for particular cytotoxic drugs. Further studies are required.

In conclusion, our data show that some combinations of modulating agents are more effective in circumventing inherent drug resistance than single agents. This increased effectiveness was not seen in all cases and the most effective combination differed for different cytotoxic drugs. The effects of modulating agents on drug resistance could not be totally explained by changes in drug accumulation. Further studies are required to identify the precise clinical applications of these findings.

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