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# Differential effectiveness of a range of novel drug-resistance modulators, relative to verapamil, in influencing vinblastine or teniposide cytotoxicity in human lymphoblastoid CCRF-CEM sublines expressing classic or atypical multidrug resistance

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**Abstract.** A series of five potential modulators of resistance were tested for their relative ability, as compared with verapamil, to sensitize CEM lymphoblastoid leukemia drug-resistant tumor sublines expressing either the classic or the atypical multidrug-resistance (MDR) phenotype to vinblastine or teniposide. Maximal non-cytotoxic concentrations of each modulator were tested and sensitization indices (SIs) were derived by comparing the drug concentration required to inhibit growth by 50% in their presence or absence. Like verapamil (10 μ*M*) itself, three of the other modulators tested, namely, S9788 (4 μ*M*), flunarizine (20 μ*M*) and quinidine (30 μ*M*), resulted in 2- to 3-fold sensitization of vinblastine against the parental CEM cells, and comparable effects were noted in the CEM/VM-1 cells, which were not cross-resistant to vinblastine. In contrast, cyclosporin A (0.5 μ*M*) and B859-35 (2 μ*M*) did not enhance vinblastine growth inhibition in these lines. However, the greatest sensitization with all the modulators was noted in the classic MDR VBL1000 cells, with SIs ranging from 40- to 350-fold, except for cyclosporin A, which proved ineffective at the concentration tested (SI, 2.6). The greatest extent of differential sensitization of these VBL1000 tumor cells occurred with quinidine or B859-35, which proved significantly more effective than verapamil alone. Combinations of modulators resulted in additive effects, with B859-35 plus cyclosporin A proving superior to B859-35 plus verapamil. In contrast, none of these compounds proved effective as a sensitizer to teniposide. The growth-inhibitory effects of this drug were not modified significantly in either the 92-fold teniposide-resistant VM-1 cells or in the parental cells. Addition of verapamil itself also failed to modulate teniposide growth inhibition in the VBL1000 cells, which express significant cross-resistance to this drug (36-fold). However, SI values of 3- to 5-fold were obtained using quinidine or B859-35. These results serve (a) to emphasise the need to monitor the effects of modulators not only on drug-resistant cells but also on their drug-sensitive counterparts so as to ensure differential sensitization such that normal sensitive tissues are not likely to be adversely influenced and (b) to highlight the observation that the extent of modulation differs depending not only on the antitumor drug used but also on the mechanism of drug resistance expressed. This in vitro model system appears to provide a useful screening system for resistance modulators and certainly could be used in attempts to identify alternative agents that may influence teniposide sensitivity in these drug-resistant sublines.

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## Introduction

Strategies to reverse drug resistance are of great theoretical interest and are potentially of practical importance. The major impetus for identifying agents that might circumvent drug resistance has come from the demonstration that one major type of resistance, namely, multidrug resistance (MDR), is mediated by the cell-surface glycoprotein, P-glycoprotein (Pgp), a finding first established in experimental laboratory model systems and now recognized as a clinical phenomenon (cf. [8, 17–19, 22, 54]). The initial identification of verapamil as an agent capable of reversing the cytotoxicity of one of the drugs implicated in the MDR phenotype, namely, vinblastine, in a murine lymphoid leukemia-lymphoma in vivo was reported by Tsuruo et al. in 1981 [48]. Subsequently it was shown by photoaffinity-labeling studies that verapamil could bind to Pgp and presumably interfere with its drug-efflux functions [1].

There are now many reports describing how MDR can be overcome, to varying extents, by a series of agents termed "resistance modulators", including such diverse compounds as calcium antagonists and calmodulin inhibitors [6, 28, 49], phenothiazines [39], anti-malarial drugs [4, 61], anti-arhythmics [50, 55, 58], steroid hormone

[38, 60] and the cyclosporins [9, 43, 52]. Many in vitro tests of these modulators have provided encouraging positive data (cf. recent reviews [15, 16, 47, 52]), but when moved into the clinical setting they have either proved less effective or their administration has been associated with major toxic side effects, including, especially with verapamil, life-threatening cardiotoxicity [32, 36, 41, 54, 59]. New examples of effective modulators are therefore being sought.

In our study we evaluated a series of five compounds in relation to the in vitro effectiveness of verapamil. Two of the modulators, namely, cyclosporin A and quinidine, have produced encouraging in vitro results in other laboratories [9, 33, 35, 43, 53, 57, 58] and have been evaluated in preliminary clinical studies [3, 45]. The three newer compounds, namely, S9788, a novel triazinoaminopiperdine derivative; flunarizine, or 1-[bis(4-fluorophenyl)methyl]-4-13-phenyl-Z-propenyl) piperazine hydrochloride, a calcium-entry blocker without cardiac toxicity; and B859-35, the sterically pure dihydropyridine compound dexniguldipine hydrochlodride, have only recently been tested in vitro [20, 21, 23, 37], but all are either under consideration for or entering phase I/II clinical trials.

We used for screening purposes the "classic MDR" subline CEM/VBL1000 [26] and the "atypical (at-) MDR" subline CEM/VM-1 [13]. In this way we aimed to establish whether these compounds would modulate only Pgp-mediated MDR or whether they could also circumvent other resistance mechanisms that may be operating, for example, in clinical tumors with their known heterogeneity. In addition, since numerous groups have frequently only evaluated the efficacy of modulators in reversing resistance to one particular anti-tumor agent and then tended to extrapolate their results as if these would apply to all drugs associated with MDR, we compared the modulatory effects of these various agents when combined not only with vinblastine but also with teniposide. Furthermore, we examined the efficacy of some combinations of modulators.

## Materials and methods

*Cell lines and culture techniques.* The CCRF-CEM parental lymphoblastoid T-cell leukemia cell line originally described by Foley et al. [14] and a vinblastine-resistant subline (VBL1000) derived by intermittent treatment followed by continuous exposure to vinblastine as detailed elsewhere [26] were kindly provided by Dr. V. Ling, Ontario Cancer Institute (Toronto, Canada). The VM-1 subline derived by intermittent exposure to teniposide [13] was obtained from Dr. W. T. Beck, St. Jude Children's Hospital and Research Center (Memphis, Tenn., USA). All three lines were maintained as suspension cultures in RPMI 1640 medium plus 10% foetal calf serum at 37° C in a humidified atmosphere containing 5% $CO_2$. Media and sera were supplied by Gibco-Biocult (Paisley, UK) and disposable plasticware (Falcon) was purchased from Marathon (London, UK).

*Anti-tumor drugs and modulators.* Vinblastine, verapamil and quinidine were purchased from Sigma Chemicals (Poole, Dorset, UK). Teniposide was kindly provided by Lederle Laboratories (Gosport, Hants, UK). The various novel modulators were donated for these studies: S9788, by Institut de Recherches Internationales Servier, Courbevoie, France; flunarizine, from Janssen Research Foundation via Dr. H. Hendriks (New Drug Development Office, European Organization for Research and Treatment of Cancer, Amsterdam, The Netherlands); cyclosporin A, by Sandoz (Leeds, UK); and B859-35, by Byk-Gulden (Konstanz, Germany). S9788 was prepared as a stock solution in water (50 μg/ml) and was stored at 4° C for up to 2 months or freshly prepared. Flunarizine and B859-35 were freshly prepared as stock solutions in dimethyl sulphoxide (DMSO, 10 mg/ml) and subsequent dilutions were made in tissue-culture medium, with the final DMSO concentration in cultures never exceeding 1%. Cyclosporin A was dissolved in absolute ethanol and subsequently diluted in tissue-culture medium.

**Table 1.** Modulator concentrations used and their associated cytotoxicities

| Modulator | Concentration | % Growth relative to untreated controls[a] | | |
|---|---|---|---|---|
| | | CEM/P cells | VM-1 cells | VBL1000 cells |
| Verapamil | 10 μ*M* | 98.4 ± 0.3 | 97.9 ± 1.2 | 81.7 ± 3.0 |
| S9788 | 4 μ*M* | 107.0 ± 7.2 | 105.4 ± 3.3 | 90.1 ± 3.9 |
| Flunarizine | 20 μ*M* | 98.0 ± 10.0 | 103.1 ± 3.3 | 70.9 ± 2.3 |
| Cyclosporin A | 0.5 μ*M* | 90.3 ± 0.4 | 93.4 ± 5.5 | 82.6 ± 0.7 |
| B859-35 | 2 μ*M* | 87.2 ± 1.4 | 93.2 ± 0.8 | 86.0 ± 1.9 |
| Quinidine | 30 μ*M* | 104.3 ± 0.9 | 92.1 ± 2.8 | 87.7 ± 3.2 |
| B859-35 plus cyclosporin A | 2 μ*M* 0.5 μ*M* | 92.8 ± 2.9 | 89.7 ± 2.1 | 80.6 ± 3.0 |
| B859-35 plus verapamil | 2 μ*M* 10 μ*M* | 88.1 ± 3.5 | 90.0 ± 1.5 | 78.2 ± 6.0 |

[a] Tumor cells were exposed continuously for 5 days to each modulator, after which total cell numbers were counted. Each value represents the mean ± SE of at least 3 repeated experiments

*Cytotoxicity assays.* Tumor cells ($2 \times 10^4$/ml and 5 ml/tube) were exposed continuously for 5 days to a range of concentrations of either vinblastine or teniposide in the presence or absence of modulator. Cell numbers were then determined using a Coulter Counter Model ZM. Full dose-response curves were established from which $GI_{50}$ values (drug concentration required to reduce the number of cells in non-drug-treated control cultures by 50%) were calculated. A comparison of the $GI_{50}$ value obtained in the absence of modulator with that obtained in the presence of modulator yielded a sensitization index (SI). The ratio of the SI obtained for the sensitive parental cells versus that obtained for the resistant subline was defined as the differential SI (dSI). Each experiment was repeated on at least three independent occasions using duplicate samples for each assay point.

## Results

### *Selection of modulator concentrations and derivation of relative resistance indices of the CEM sublines*

Initial studies were aimed at identifying the maximal "non-cytotoxic" concentrations (i.e. those that inhibited the growth of treated cultures relative to untreated controls by $\leq 10\%$) of each modulator to be used in combination with each anti-tumor drug selected for study, namely, vinblastine and teniposide. The modulator concentrations selected from full dose-response curve data and their associated growth-inhibitory effects are listed in Table 1. It should be noted that whereas the selection criteria for a $\leq 10\%$ inhibitory concentration was identified for the parental cells and the VM-1 resistant subline, in all cases the

**Table 2.** $GI_{50}$ values and relative resistance indices derived from full dose-response curves

| Cell line | Vinblastine | | Teniposide | |
|---|---|---|---|---|
| | $GI_{50}$ (ng/ml)[a] | Resistance index[b] | $GI_{50}$ value (ng/ml)[a] | Resistance index[b] |
| CEM/P | 2.7±0.8 | 1 | 7.9±0.2 | 1 |
| CEM/VM-1 | 2.7±0.8 | 1 | 727±35 | 92 |
| CEM/VBL1000 | 525±141 | 194 | 284±24 | 36 |

[a] Tumor cells were exposed continuously for 5 days to a range of drug concentrations. Each value represents the mean ± SE of at least 4 repeated experiments

[b] Calculated by comparing the $GI_{50}$ value of the subline with that of the parental cells

VBL1000 resistant cells proved more sensitive to each of the modulators tested. This collateral sensitivity to the cytotoxic effects of modulators such as verapamil or cyclosporin A has been noted in other MDR tumor systems [29, 51, 56]. For these comparative studies, however, we opted to use the same concentration of each modulator on each of the three cell lines and, as shown in Table 1, these resulted in growth inhibition of 10%–30% being noted in the VBL1000 cells.

$GI_{50}$ values for vinblastine and for teniposide were derived from full dose-response curves and the results are listed in Table 2. These data confirm the marked resistance of VBL1000 cells to vinblastine (194-fold) and of VM-1 cells to teniposide (92-fold). As reported previously [13, 20], the "at-MDR" VM-1 cells were not cross-resistant to vinblastine, although the classic MDR VBL1000 cells proved to be 36-fold cross-resistant to teniposide in this study.

### *Growth-inhibitory effects of vinblastine with or without modulation*

Table 3 provides a summary of the mean and differential SIs for vinblastine growth inhibition obtained with each modulator. These data show that the effects of each of the modulators tested was essentially the same in the CEM/P and the CEM/VM-1 cells, with either no significant modulation being shown with, for example, cyclosporin A or B859-35 or a 2- to 3-fold enhancement of cytotoxicity being noted with, for example, the addition of either verapamil, S9788, flunarizine or quinidine. Examples of these differential effects of the various modulators on the CEM/P cells are provided in Fig. 1 so as to emphasize the definite extent of enhancement of growth inhibition in these parental drug-sensitive cells by verapamil and by quinidine. However, all the modulators proved significantly more effective in enhancing growth inhibition in the highly resistant VBL1000 cells (see Table 3 and Fig. 1), with SIs falling in the range of 40- to 350-fold, except for cyclosporin A, which resulted in only minor modulation (2.6-fold) at the concentration used. In these VBL1000 cells, a straight comparison of SIs suggests that only one of the modulators tested produced significantly more sensitization than verapamil, namely, quinidine, and indeed it appeared essentially completely to reverse vinblastine resistance in these cells.

**Table 3.** Summary of the mean and differential dose-modification factors for vinblastine growth inhibition obtained with each modulator tested

| Modulator | CEM/P cells | VM-1 cells | | VBL1000 cells | |
|---|---|---|---|---|---|
| | SI[a] | SI[a] | [dSI][b] | SI[a] | [dSI][b] |
| Verapamil | 2.8±0.1 | 2.8±0.6 | [1.0] | 48.7±0.2 | [17.4] |
| S9788 | 2.4±0.3 | 2.3±0.5 | [1.0] | 68.7±21.2 | [28.6] |
| Flunarizine | 2.4±0.4 | 2.2±0.7 | [0.9] | 47.8±5.3 | [19.9] |
| Cyclosporin A | 1.1±0.1 | 1.2±0.1 | [1.1] | 2.6±0.3 | [2.4] |
| Quinidine | 2.9±0.1 | 2.6±0.3 | [0.9] | 355.7±8.0 | [122.7] |
| B859-35 | 1.1±0.1 | 1.1±0.1 | [1.0] | 47.4±5.9 | [43.1] |
| B859-35 plus cyclosporin A | 1.2±0.2 | 1.1±0.1 | [0.9] | 96.4±5.0 | [80.3] |
| B859-35 plus verapamil | 3.0±0.3 | 3.2±0.8 | [1.1] | 100.1±9.5 | [33.3] |

[a] Tumor cells were exposed continuously for 5 days to a range of vinblastine concentrations in the absence or presence of each modulator. SI values were calculated by comparing the $GI_{50}$ values derived from full-dose-response curves from at least 3 repeated experiments evaluating vinblastine alone versus vinblastine plus modulator

[b] Calculated by dividing the SI of the resistant subline by that of the parental line

A comparison of dSIs, however, served to emphasize superiority over verapamil also for B859-35, since this modulator was without sensitizing effects of vinblastine cytotoxicity in the parental sensitive cells. With B859-35, however, reversal of vinblastine resistance was only partial. In an attempt to increase the extent of modulation, a combination of the two modulators that had not affected the sensitive parental cells, namely, B859-35 and cyclosporin A, was tested and appeared to result in additive sensitization of vinblastine growth inhibition in these VBL1000 cells, producing an SI of 80, which was closer to that of 123 achieved with quinidine alone. B859-35 plus verapamil also resulted in additive sensitization in these VBL1000 cells, yielding an SI of 100, but the dSI was only 33 since 3-fold sensitization occurred with the parental cells.

### *Growth-inhibitory effects of teniposide with or without modulation*

The two most effective modulators of vinblastine growth inhibition in the VBL1000 cells were also tested, in comparison with verapamil, for their interaction with teniposide in these three CEM cell lines. The results summarized in Table 4 indicate that (a) in the parental drug-sensitive cells, none of the modulators significantly influenced the growth-inhibitory effects of teniposide; (b) in the VM-1 cells, a very slight sensitization of teniposide growth inhibition was noted with verapamil and with quinidine, but none was achieved with B859-35 (see Fig. 2); (c) in the VBL1000 cells, verapamil did not modulate teniposide, contrasting with the sensitization obtained

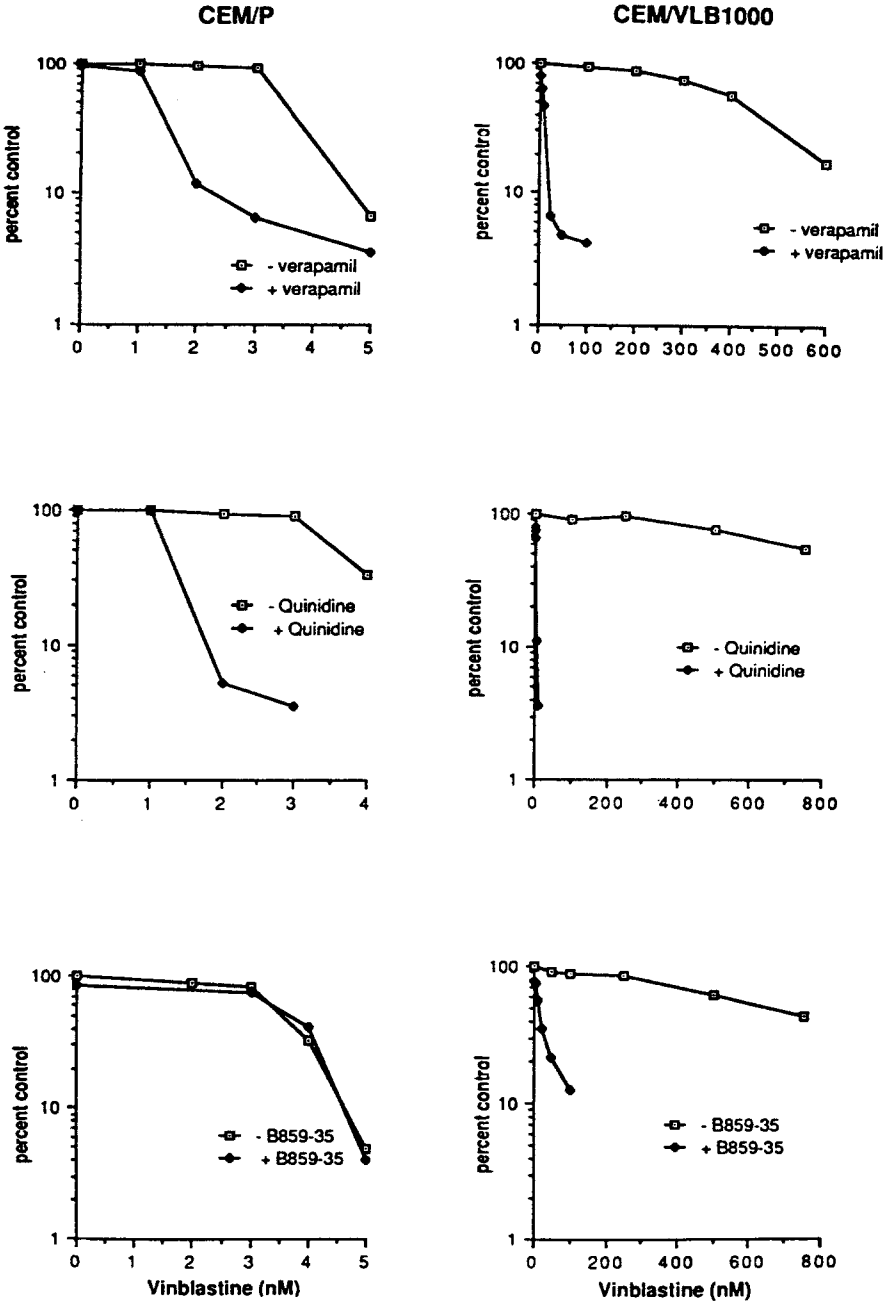


**Fig. 1.** Comparative growth-inhibitory effects of vinblastine ± modulator (verapamil, quinidine or B859-35) on the CEM/P and CEM/VBL1000 cell lines. Tumor cells were exposed continuously for 5 days to a range of drug concentrations in the presence or absence of each modulator

**Table 4.** Summary of the mean and differential dose-modification factors for teniposide growth inhibition obtained with each modulator tested

| Modulator | CEM/P cells | VM-1 cells | | VBL1000 cells | |
|---|---|---|---|---|---|
| | SI[a] | SI[a] | [dSI][b] | SI[a] | [dSI][b] |
| Verapamil | 1.14±0.01 | 1.57±0.03 | [1.4] | 1.30±0.12 | [1.1] |
| Quinidine | 0.81±0.23 | 1.24±0.03 | [1.5] | 5.15±0.92 | [6.3] |
| B859-35 | 1.04±0.14 | 1.03±0.01 | [1.0] | 3.40±0.01 | [3.3] |
| B859-35 plus verapamil | 1.25±0.18 | 1.61±0.09 | [1.3] | 4.80±1.46 | [3.8] |

[a] Tumor cells were exposed continuously for 5 days to a range of concentrations of teniposide in the absence or presence of each modulator. SI values were calculated by comparing the $GI_{50}$ values derived from full dose-response curves from at least 3 repeated experiments evaluating teniposide alone versus teniposide plus modulator

[b] Calculated by dividing the SI of the resistant subline by that of the parental line

with B859-35 and quinidine, which produced SIs of 3 and 5, respectively (see Fig. 2); and (d) the combination of B859-35 and verapamil was not significantly more effective at sensitization than B859-35 alone. However, under these conditions a clearly effective reversal of teniposide resistance by the modulators tested in these two drug-resistant sublines, expressing 92- and 36-fold levels of resistance, respectively, was not obtained.

## Discussion

These data serve to illustrate that the use of maximal non-cytotoxic concentrations of certain of these modulators can result in a 2- to 3-fold enhancement of vinblastine cytotoxicity in the parental drug-sensitive CEM cells. Beck et al.

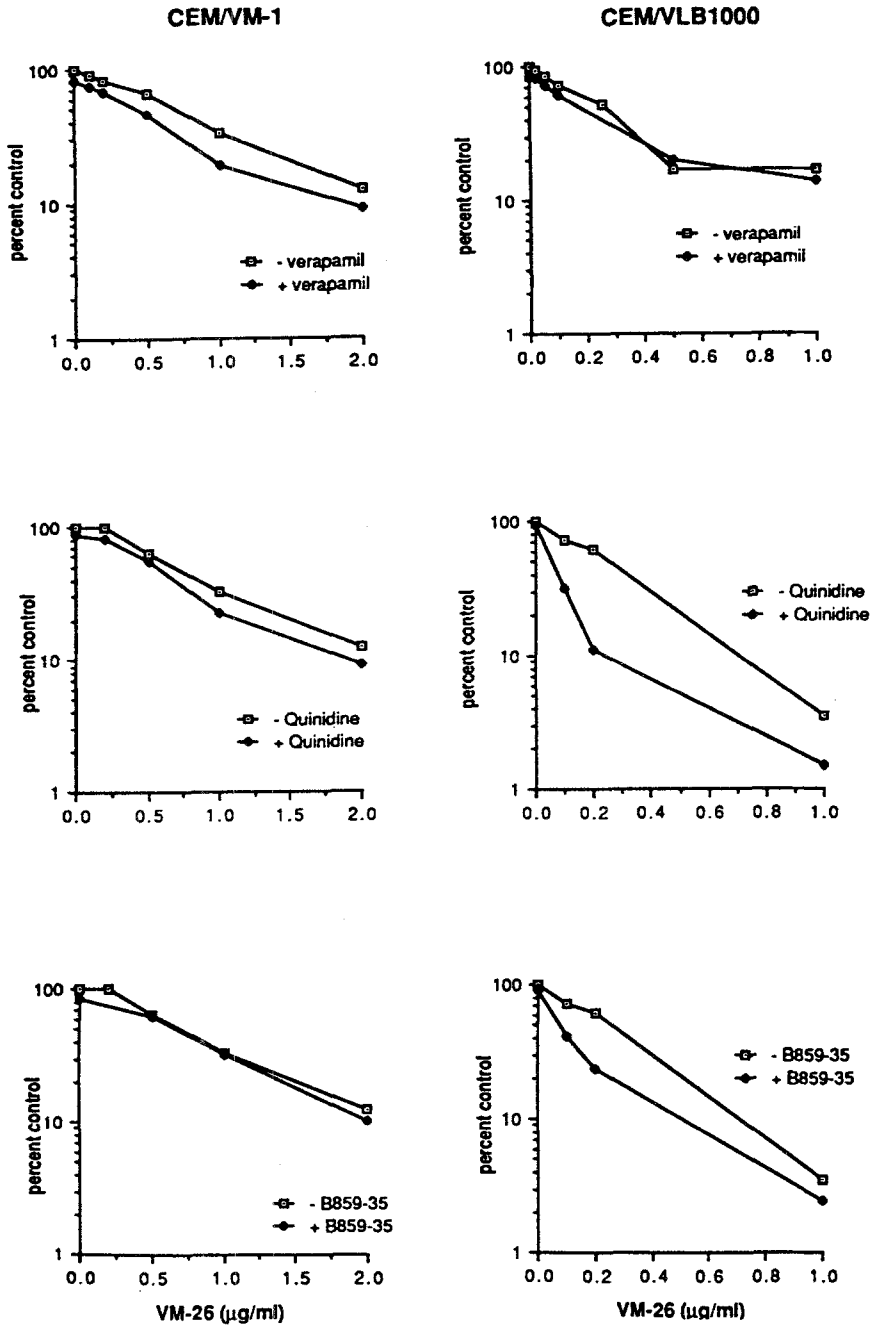


**Fig. 2.** Comparative growth-inhibitory effects of teniposide ± modulator (verapamil, quinidine or B859-35) on the CEM/VM-1 and CEM/VBL1000 drug-resistant cell lines. Tumor cells were exposed continuously for 5 days to a range of drug concentrations in the presence or absence of each modulator

[5] also reported that 10 µ*M* verapamil enhanced the cytotoxicity of vinblastine in these parental CEM cells, although Hu et al. [24] stated that verapamil addition had no significant effect on the Adriamycin $IC_{50}$ value (50% growth-inhibitory concentration) in these cells. Indeed, our data on vinblastine and teniposide also imply that the antitumor drug itself may be important in determining whether or not activity is modulated in drug-sensitive cells. Although this is a relatively modest modulatory effect in these parental cells as compared with the extent of enhancement noted in the highly vinblastine-resistant VBL1000 cells, it highlights the need to carry out these comparative types of studies in the laboratory when new agents are being evaluated. Studying various MDR sublines of the EMT6 murine tumor system, Twentyman and colleagues [2, 53] also reported examples where sensitization of the MDR lines was no greater than that of the parent line. Indeed, as emphasized previously [20], many of these potential modulators can exert significant cytotoxicity in their own right, and positive in vitro modulation is often highly concentration-dependent or even tumor cell-line-dependent [25, 30, 34, 53]. Such observations obviously need to be considered in the selection of newer agents for clinical evaluation. In this respect it was noticeable that for B859-35 and cyclosporin A at 2 and 0.5 µ*M*, respectively, no enhancement of vinblastine cytotoxicity in the parental sensitive cells was observed when they were used either as single agents or in combination.

All of the compounds tested exerted some modulatory effects on vinblastine cytotoxicity in the classic MDR

VBL1000 cells, with dSIs varying from 2.4 to 123, although only quinidine, B859-35 and the combinations of B859-35 with either cyclosporin A or with verapamil proved significantly more effective at sensitization than verapamil itself.

The relatively ineffective sensitization observed with cyclosporin A in this series was unexpected, although it was similar to that reported recently in a vincristine-resistant CEM MDR subline [27], since several authors had reported that it proved superior to verapamil in reversing vinca alkaloid [25, 35] and Adriamycin resistance [33]. In general, though, these groups had used considerably higher concentrations (1–8 μ*M*) and, in view of the strong dose-dependent cytotoxicity reported [2, 24, 25, 34, 53] and confirmed in our present study, this may at least in part explain our differing results. Increasing the cyclosporin A concentration to 2 μ*M* in the present study resulted in considerable cytotoxicity (i.e. growth inhibition of ≥ 90%).

The marginal superiority of S9788 over verapamil confirms our earlier study using a range of human tumor cell lines [20], although Pierré et al. [37] described it as being 2–5 times more active, depending on the tumor cell line tested. The comparable modulation of vinblastine produced by flunarizine and verapamil was also identified in relation to vincristine or Adriamycin cytotoxicity in three Pgp-expressing human neuroblastoma cell lines and one classic MDR vincristine-resistant MCF-7 human breast-carcinoma subline (Hill et al., unpublished data).

Our data showing increased sensitization of vinblastine cytotoxicity with B859-35 over verapamil is consistent with the earlier report that it proved superior in reversing doxorubicin resistance [21]. The marked superiority of quinidine in this study, however, was encouraging and consistent with the results of a previous study using the MCF-7/ADR resistant subline [46], although our concentration of 30 μ*M* was clearly in excess of their 6.6 μ*M*, a concentration shown to be achievable in vivo [58]. In an earlier study, however, comparable modulation of daunomycin was reported using equimolar (10 μ*M*) quinidine or verapamil with MDR human KB cells [57].

In contrast to the data obtained with vinblastine, modulation of teniposide cytotoxicity by the three agents tested was relatively modest with quinidine and B859-35 (dSIs of 6 and 3, respectively) and absent with verapamil. The relative ineffectiveness of resistance modulators in influencing etoposide cytotoxicity has indeed been reported in a number of experimental model systems in tests of verapamil [5, 7, 34, 42] or cyclosporin A [34, 53]. Indeed, verapamil itself clearly exerts differential sensitization of the various drugs implicated in the MDR phenotype, proving generally most effective with the vinca alkaloids, then the anthracyclines, followed by colchicine and the epipodophyllotoxins [5, 7, 11, 42]. The highest degree of sensitization noted with B859-35 confirms an earlier report of synergism between etoposide and this modulator against an MDR subline of the Walker carcinoma [21].

This apparent drug-dependent modulation, if confirmed, needs to be taken into account in designing clinical studies where drug combinations are used and also argues against a generalized effect of these modulators in influencing Pgp-mediated drug efflux. This point also serves to emphasize the observation that in general, the precise mechanism(s) of action of these various compounds in modulating resistance remain to be defined. However, these data also indicate that none of the modulators tested in this study appears to influence either vinblastine or teniposide cytotoxicity in the "at-MDR" VM-1 cell line. Beck et al. [4] reported that neither verapamil (10 μ*M*) nor chloroquine (50 μ*M*) sensitized these VM-1 cells to vinblastine. This finding would be consistent with the proposal that these various modulators exert their effects by Pgp-mediated pathways, none of which operates in these VM-1 cells with their altered topoisomerase II-mediated resistance [12]. Indeed, data from a number of in vitro studies indicate that these various resistance modulators appear ineffective in enhancing chemosensitivity selectively in those MDR tumor cells that do not express Pgp [2, 10, 31, 44].

The experimental model system used in these investigations provides a rapid, readily reproducible means of screening new modulators and of determining whether all drugs implicated in the MDR phenotype and likely to be used clinically in combination protocols are similarly modulated. Ideally, new modulators should not significantly influence drug cytotoxicity in the parental cells and, we suggest, should provide a dSI in excess of 100, indicating a significant differential and definite superiority over the "gold-standard" compound verapamil. In addition, we need to identify modulators of non-Pgp-mediated MDR. Furthermore, our data add weight to the proposal that optimal use of these newer agents may result from their application in combination [24, 25, 30, 35, 40], and in this respect the in vitro model system described herein may serve as a useful initial in vitro screen.

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