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Effect of Cyclosporin and Verapamil on the Cellular Kinetics of Daunorubicin

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Both cyclosporin and verapamil modulate the multidrug-resistant (MDR) phenotype in the classical MDR cell lines, CEM/VLB100 and CEM/VLB1000. Initial studies demonstrated a significant reduction in daunorubicin accumulation in the two resistant lines compared with the drug-sensitive parent line CEM/CCRF. Both cyclosporin and verapamil increased drug accumulation in the resistant lines. This effect was dose-dependent although a plateau occurred in CEM/VLB100 cells at concentrations of cyclosporin exceeding 4.2 μmol/l. Cyclosporin 4.2 μmol/l and verapamil 10 μmol/l significantly increased daunorubicin uptake and reduced drug efflux in the CEM/VLB100 and CEM/VLB1000 lines. At low clinical concentrations of cyclosporin (0.8–1.6 μmol/l and verapamil (1–2 μmol/l), there was a synergistic increase in drug accumulation in the two resistant cell lines ($P < 0.007$). These data suggest that cyclosporin modulates the classical MDR phenotype by altering the cellular kinetics of daunorubicin. The *in vitro* synergistic action of cyclosporin and verapamil could be interesting clinically.
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INTRODUCTION

THE mutlidrug-resistant (MDR) phenotype has been described in several tumour types [1] and cell lines [2,3]. The classical MDR phenotype shows cross-resistance to several structurally unrelated cytotoxic drugs [2] and may be defined by the presence of P-glycoprotein [2,4], reduced cellular accumulation of drugs in drug-resistant variants [3] and modulation of resistance by drugs such as calcium-channel blockers [5] and calmodulin antagonists [5,6], which may increase drug accumulation. This accumulation is independent of changes in calcium metabolism

[7] but may relate to binding of these compounds to P-glycoprotein [8]. Cyclosporin also modulates the expression of the MDR phenotype [9,10]. This action may be independent of changes in cellular daunorubicin levels [9] although cyclosporin increased drug accumulation in daunorubicin-resistant P388 murine leukaemia cells [11], mouse mammary tumour cells and a human small-cell lung cancer cell line [12]. We have examined the effect of cyclosporin and verapamil which we have found act synergistically, on the cellular kinetics of daunorubicin in classical MDR cell lines.

MATERIALS AND METHODS

Cell lines

Two resistant variant lines of the drug-sensitive line CEM-CCRF, originally from a patient with a T-cell lymphoblastic leukaemia, were used. The moderately resistant CEM/VLB100 and the highly resistant CEM/VLB1000 were grown in sub-lethal concentrations of vinblastine, 10 and 100 ng/ml, respectively. Lines were maintained in RPMI 1640 supplemented with 10% foetal calf serum (Flow at 37°C in a humidified chamber containing 5% CO_2). The lines were regularly screened for mycoplasma contamination and discarded accordingly. All three lines were provided by Dr D. Bell. The two resistant variants are typical MDR lines with cross-resistance to doxorubicin and enhanced expression of P-glycoprotein proportional to the degree of resistance [13].

Materials

Doxorubicin, cyclosporin (pure analytical) and verapamil were bought from Farmitalia, Sandoz and Schering, respectively. RPMI 1640 was purchased from Gibco and supplemented with gentamicin (80 μg/ml), minocycline (1 μg/ml), hepes (20 mmol/l), sodium bicarbonate (0.21%) and L-glutamine (0.8 mmol/l). ^{3}H-Daunorubicin (specific activity 18.1 × 10^4 MBq/mmol, 97–98% pure) was bought from New England Nuclear.

Drug accumulation

Cells were adjusted to 5 × 10^6/ml and viability assessed with trypan blue. The cells were pipetted into 96-well plates (Flow) to give a final number of 7.5 × 10^5 per well and incubated at 37°C in 5% CO_2 with varying concentrations of cyclosporin or verapamil and tracer amounts of [^{3}H]daunorubicin (final concentration 1.85 × 10^4 Bq/ml, 0.05 μg/ml). The cells were harvested onto glass-fibre filters at designated times with an automated harvester (Titertek). The filter papers were dried and dissolved in 10 ml Beckman's solution and radioactivity was measured. All assays were in triplicate.

Cyclosporin was initially dissolved in analytical grade alcohol and diluted in RPMI 1640 to give a stock solution of 0.42 μmol/l. We used cyclosporin at 0.8–8.3 μmol/l. In control experiments, cells were exposed to equivalent concentrations of alcohol (0.07–0.7%), which had no effect on drug uptake, efflux or cell viability.

Uptake was studied at frequent times during the first 60 min of exposure of cells to tracer. Drug accumulation was the total cellular content of [^{3}H]daunorubicin over 1–4 h.

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Table 1. Effect of cyclosporin and verapamil on [^{3}H]daunorubicin accumulation over 150 min in two resistant variants of CEM/CCRF

Cell lines	Control	Cyclosporin (8.3 μmol/l)	Verapamil (40 μmol/l)
CEM/CCRF	62.3 (4.76)*	58.2 (1.26)	60.3 (0.97)
CEM/VLB100	25.3 (1.93)†	57.2 (1.03)	53.4 (2.19)§
CEM/VLB1000	15.7 (1.24)†	42.6 (2.80)§	38.5 (1.91)§

*10^3 (cpm), mean (S.D.).
†$P < 0.001$ compared with CEM/CCRF; $P < 0.001$ compared with CEM/VLB100; §$P < 0.001$ compared with same cell line in controls.

Drug efflux

Drug efflux was measured after incubating cells with trace quantities of [^{3}H]daunorubicin in 96-well plates for 60 min at 37°C. Plates were centrifuged at 4°C and washed three times in cold RPMI 1640. The cells were then reincubated at 37°C in 5% CO_2 in 4.2 μmol/l cyclosporin or 10 μmol/l verapamil in an excess of unlabelled doxorubicin (25–50 μg/ml). At designated times, cells were harvested. All assays were in triplicate.

Statistics

We used paired *t* tests for drug accumulation studies and regression analysis to compare uptake and efflux curves and to evaluate the interaction between various combinations of cyclosporin and verapamil. The uptake of daunorubicin above the predicted additive effect of these drugs was analysed with analysis of variance and the significance of this interaction was tested with the Sign test.

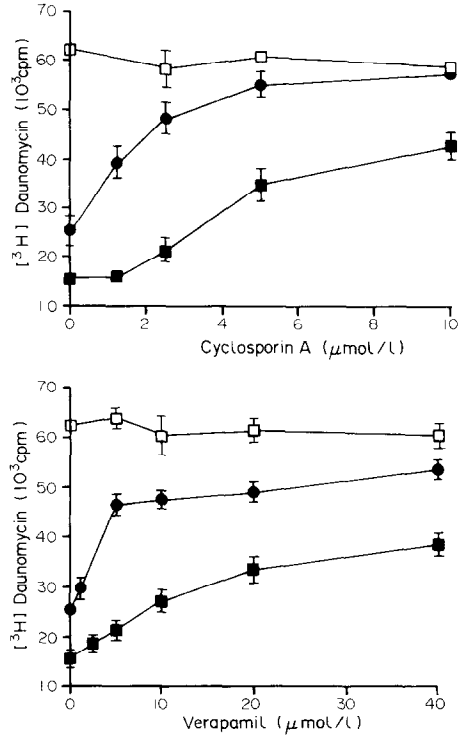

Fig. 1. Effect of cyclosporin (upper) or verapamil (lower) on the accumulation of [^{3}H]-daunorubicin in the CEM/CCRF (□–□), CEM/VLB100 (●–●), and CEM/VLB1000 (■–■) cell lines. Mean (S.D.).

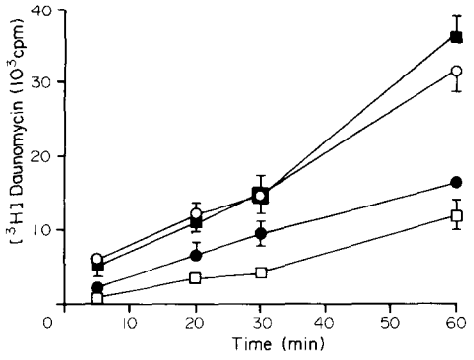

Fig. 2. Uptake of [³H]daunorubicin in the absence (○–○) or presence (■–■) of cyclosporin 4.2 μmol/l in the CEM/CCRF and CEM/VLB100 (□–□, •–•) cell lines.

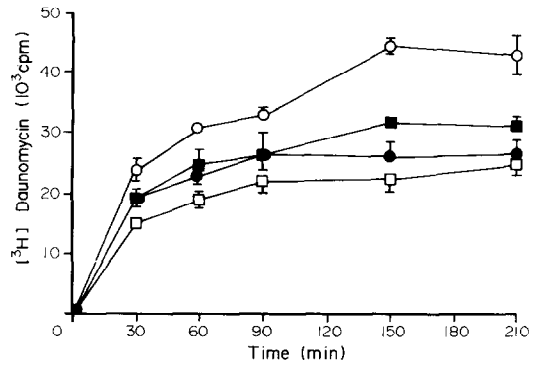

Fig. 4. Accumulation of [³H]daunorubicin in the absence of drugs (□–□) or in the presence of verapamil 1 μmol/l (•–•), cyclosporin A 1.6 μmol/l (■–■), or both (○–○).

RESULTS

In the absence of cyclosporin or verapamil, the accumulation of daunorubicin varied considerably in each cell line at 150 min (Table 1). There was an increased amount of [³H]daunorubicin in the sensitive line with significantly lesser amounts in the moderately resistant and highly resistant lines respectively ($P < 0.001$). In addition, drug accumulation was significantly reduced in the highly resistant line compared to the moderately resistant line ($P < 0.01$). Thus with increasing levels of resistance, there was significant decrease in drug accumulation. With cyclosporin or verapamil present, there was no significant change in drug accumulation in the sensitive line. However, total drug accumulation was increased significantly in both the moderately resistant and highly resistant lines.

The effect of cyclosporin or verapamil on drug accumulation was dependent on concentration (Fig. 1). As the concentration of cyclosporin was increased, the amount of radioactivity taken up into CEM/VLB100 cells plateaued with total accumulation approaching that in the sensitive line. This plateau was not as obvious in CEM/VLB1000. Increasing concentrations of cyclosporin had no effect on drug accumulation in CEM/CCRF. Similarly, increasing concentrations of verapamil had no effect on drug accumulation in the sensitive cell line but were associated with a progressive increase in the moderately and highly resistant cell lines. Even in high concentrations of verapamil, drug accumulation did not reach the levels in the sensitive line.

Drug accumulation at any one time represents the net result of initial drug uptake, drug efflux and re-uptake by the same cells. In each cell line, daunorubicin uptake was measured during the first 60 min of exposure of cells to tracer quantities of [³H]daunorubicin. At 1 min, 5–10% of the maximum level of tracer eventually taken up into cells had entered all three cell lines. This result was thought to represent non-specific binding to cells and/or the glass filters. There was a significant ($P < 0.05$) increase in the uptake of [³H]daunorubicin in the CEM/VLB100 cells in the presence of cyclosporin compared with controls (Fig. 2). Similar results were seen with the CEM/VLB1000 cell line (data not shown). Cyclosporin had no significant effect on the rate of uptake of [³H]daunorubicin in the sensitive cell line.

Drug efflux for CEM/VLB100 is shown in Fig. 3. In the absence of cyclosporin or verapamil, the cellular content of [³H]daunorubicin fell rapidly with almost a 50% decrease in radioactivity occurring by 30 min and a 75% decrease by 60 min. However, there was a significant reduction ($P < 0.05$) in the relative rate of drug efflux in the presence of cyclosporin or verapamil compared with controls. Neither modulator had any effect on drug efflux in the sensitive cell line (data not shown).

The combined effects of clinical concentrations [14,15] of cyclosporin or verapamil on drug accumulation are shown in Fig. 4 and Table 2. In the CEM/VLB100 line, drug uptake was increased by the addition of either verapamil or cyclosporin. There was a significant increase in drug uptake in the presence of both compounds compared with the effect of either agent alone ($P < 0.01$). In addition, the effect of both drugs was significantly greater than expected if both agents had an additive effect on drug uptake ($P < 0.007$). Thus this combination has a synergistic effect on drug accumulation.

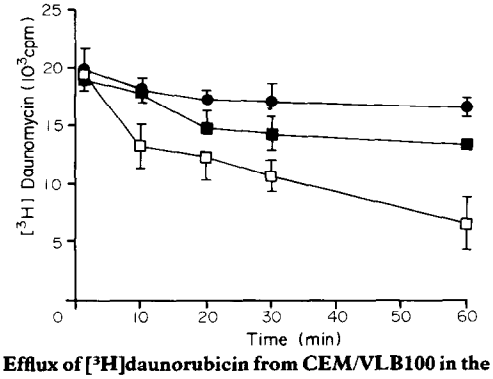

Fig. 3. Efflux of [³H]daunorubicin from CEM/VLB100 in the absence of drugs (□–□) or in the presence of cyclosporin 4.2 μmol/l (•–•), or verapamil 10 μmol/l (■–■).

Table 2. Effect of combinations of cyclosporin and verapamil on [³H]daunorubicin accumulation in CEM/VLB100 over 90 min

Cyclosporin (μmol/l)	Verapamil (μmol/l)	10^3 cpm
0	0	19.6 (1.64)
0.8	0	19.7 (1.25)
1.6	0	23.1 (0.96)
0	1	22.9 (1.08)
0	2	27.0 (1.6)
0.8	1	28.3 (1.22)*
0.8	2	31.1 (0.93)*
1.6	1	30.8 (0.74)*
1.6	2	35.9 (2.21)*

Mean (S.D.).
*All combinations led to superior drug accumulation ($P < 0.007$ to < 0.0008) compared with either modulator alone.

The relation between different concentrations of verapamil and cyclosporin on drug accumulation at a fixed time point (90 min) is shown in Table 2. There was a significant difference in total drug accumulation in the presence of both compounds compared with the contribution of either modulator used alone ($P < 0.003$). Furthermore, this difference was significantly greater ($P < 0.007$) than the predicted additive effect of both agents on drug accumulation. Similar drug accumulation data were seen at 150 min (data not shown).

DISCUSSION

Cyclosporin enhanced drug accumulation and drug uptake and inhibited drug efflux in MDR cell lines. In this sense, cyclosporin behaved like calcium-channel blockers and calmodulin antagonists (e.g. trifluoperazine) in modulating drug resistance by altering the cellular kinetics of an anthracycline. Cyclosporin's effect on drug accumulation was dose-dependent, as were the results with for verapamil. However, drug accumulation is the net effect of uptake and efflux. During the first 60 min of exposure of cells to tracer quantities of [^{3}H]daunorubicin, the rate of uptake was directly related to the sensitivity of the three lines tested (data not shown). This association between the degree of resistance and rate of drug uptake has been reported in other MDR cell lines [3] and, in conjunction with the change in drug accumulation associated with exposure to verapamil or cyclosporin characterizes the classical MDR phenotype.

Our data differ from results reported by Slater *et al.* [9], who did not observe a significant effect on daunorubicin transport in a drug-resistant human T-cell acute lymphatic leukaemia cell line. However, using laser flow-cytometry and high-performance liquid chromatography, Nooter *et al.* [11] demonstrated that cyclosporin increased the intracellular accumulation and uptake of daunorubicin in resistant P388 cells, although any effects of cyclosporin on drug efflux were not reported. Similar effects on drug accumulation were demonstrated in a resistant mouse mammary line and human small cell lung cancer cell line [12].

We found that the efflux of [^{3}H]daunorubicin was inhibited by the cyclosporin. Slater *et al.* [9] found no such effects. However, these experiments were done in the absence of unlabelled daunorubicin, which might have allowed the rapid reuptake of [^{3}H]daunorubicin to confuse interpretation of the efflux data. Similar efflux data have been reported with verapamil [15, 16].

Our data extend the observations of Nooter *et al.* [11] to indicate that cyclosporin promotes cellular accumulation of daunorubicin in MDR cell lines by both increasing drug uptake and decreasing drug efflux. Cyclosporin may modulate the MDR phenotype by interfering with the transport of anthracyclines, similar to the mechanisms proposed for calcium-channel blockers. It is unclear how cyclosporin might alter drug transport. Several structurally unrelated agents alter drug accumulation in MDR cell lines [5,6]. Furthermore, these modulatory effects are unrelated to the mechanism of action of the class of drugs being tested. For example, verapamil is not thought to act via blockade of voltage-dependent calcium channels but rather via competitive binding to membrane glycoproteins responsible for drug transport [7,8]. Many of the reported modulators are lipophilic, which may underlie their ability to alter the transport of some cytotoxic drugs across cell membranes. Cyclosporin may act in a similar manner in classical drug-resistant lines. Cyclophilin [17], the putative cyclosporin receptor, calmodulin or other targets of cyclosporin interaction may also be important in determining the overall impact of the drug on resistant cells. Cyclosporin and verapamil synergistically modulate the MDR phenotype in resistant variants of the CEM/CCRF cell line. Our data show that the two compounds also have a synergistic effect on drug accumulation, presumably due to the combined impact on drug uptake and efflux. This effect occured at clinical concentrations [14] of cyclosporin and verapamil. There may be a clinical advantage in using combinations of modulators that have been shown to increase drug uptake and/or decrease drug efflux *in vitro*.

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