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# Cyclosporin A and FK506 reverse anthracycline resistance by altering the cell cycle

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**We investigated the effect of cyclosporin A (CsA) or FK506 on the cytotoxicity of anthracyclines against a human laryngeal cancer cell line, KB cells, and a multidrug resistance cell line, VJ-300 cells. CsA and FK506 enhanced the cytotoxicity of anthracyclines, especially in the VJ-300 cells. The intracellular concentrations of epirubicin (EPIR), daunomycin (DM), adriamycin (ADM) and THP-adriamycin (THP) were increased by the addition of CsA or FK506 in VJ-300, but not in KB cells. The intracellular accumulation of EPIR was most increased when CsA or FK506 was concomitantly administered with the drug. We also asked whether CsA or FK506 might influence the cycle of KB or VJ-300 cells. The population of cells in each phase of the cell cycle was little changed in both KB and VJ-300 cells when 0.3 μM ADM was administered for 24 h. Both CsA and FK506 significantly increased the ADM-induced accumulation of VJ-300 cells in $G_2M$ phase, in comparison with findings with KB cells. Thus, the reversal of MDR by CsA or FK506 is related to increased intracellular concentrations of cytotoxic drugs and, as a result, the increased $G_2M$ accumulates in MDR cells. Among of antrhacyclines, EPIR was most effective when concomitantly combined with CsA or FK506 in VJ-300 cells.**

*Key words:* Cyclosporin A, FK506, multidrug resistance.

## Introduction

Multidrug resistance (MDR) is a major obstacle in clinical cancer chemotherapy. Tumor cells resistant to naturally occurring antitumor agents such as vinca alkaloids and anthracycline antibiotics are often associated with an increased expression of a 170 kDa transmembrane glycoprotein (P-glycoprotein, P-gp)[1,2] and *mdr* genes play an important role to reduce intracellular drug accumulation. By rapidly pumping anticancer drugs out of cells, P-gp decreases the intracellular anticancer drug concentration.

Some lipohilic compounds are able to reverse the MDR; *in vitro* these compounds include calcium channel blockers (e.g. verapamil, diltiazem), antimalaria drugs (e.g. quinacrine) and anti-arrhythmic agents (e.g. amiodarone and quinidine). The immunosuppressive agents [cyclosporin A (CsA), FK506], structurally unrelated immunosuppressive agents, are potent inhibitors of T cell activation. The immunosuppressive capacity of FK506 is 10- to 100-times as potent as that of CsA.[3] CsA and FK506 have been shown to reverse P-gp mediated MDR.[4–7] CsA, a cyclic indecapeptide with highly immunosuppressive effects, efficiently prevents binding of P-gp with anticancer drugs and overcomes the effects of MDR. The mechanism of action of CsA may be through binding to calmodulin[8,9] and calmodulin inhibitors can modify MDR.[10,11] A binding study using membrane vesicles of MDR cells indicated that CsA competitively interacts with a common drug-binding site of P-gp.[12] Recently it was reported that CsA or FK506 could serve as a substrate for P-gp to transport and P-gp reduced the accumulation of CsA or FK506 in cells.[13] In addition, FK506 seems to act through FK binding proteins (FKBPs) by interfering with the activity of protein kinase C (PKC).[14]

We investigated whether CsA or FK506 influences the cytotoxicity and drug uptake of anthracyclines, the cell cycle of the parent and resistant cell line, and which anthracycline was the most effective in resistance cell lines.

## Materials and methods

### Cell lines

The human laryngeal cancer cell line, KB cells, and its MDR cell line, VJ-300 cells, were obtained from Professor M Kuwano (Department of Biochemistry, Kyushu University).[15] Both cell lines were cultured in minimal essential medium (Nissui Seiyaku, Tokyo, Japan) containing 10% fetal bovine serum,

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L-glutamine (0.3 mg/ml), penicillin (80 units/ml), streptomycin (0.1 mg/ml) and gentamycin (0.3 mg/ml). VJ-300 cells were maintained in the presence of 3 ng/ml vincristine. In each experiment, VJ-300 cells were cultured out side of vincristine for at least 1 week.

## Drugs

CsA was supplied by Sandoz Pharmaceutical (Tokyo, Japan) and FK506 was supplied by Fujisawa Pharmaceutical (Osaka, Japan), adriamycin (ADM) and epirubicin (EPIR) were obtained from Kyowa Hakko (Tokyo, Japan), daunomycin (DM) and THP-ADM (THP) were obtained from Meiji (Tokyo, Japan).

## RNA isolated from monolayer cultures

Total cellular RNA was isolated from monolayer cultures by using the acid guanidium–phenol–chloroform technique.[16] The quality of RNA extracted was assumed on a 1% agarose gel stained with ethidium bromide and the quantity of RNA was determined by $A_{260/280}$ measurements.

## Reverse transcription of RNA and MDR expression by polymerase chain reaction (PCR)

RNA was immediately reverse transcribed to cDNA using Perkin-Elmer equipment. RNA was added to a master mixture containing 1 μl RT buffer (2.5 U/μl), 1 μl dATP, dCTP, dGTP and dTTP, 2 μl of 10 × PCR buffer [Tris–HCl (100 mM), pH 8.3, KCl (500 mM)], 4 μl $MgCl_2$ solution (25 mM), 1 μl RNase inhibitor (25 mM) and 1 μl Oligod(T)[16] (2.5 mM). The reaction was terminated by heated to 95°C for 5 min.

MDR expression was determined using a RT-PCR technique, details of which are described elsewhere.[17] This assay is based on the amplification of a target gene (MDR) and an endogenous gene (β-actin) which is used as an internal standard.[18,19]

| | | |
|---|---|---|
| β-actin | a: sense | 5′-ACCCCCACTGAAAAAGATGA-3′ |
| | b: antisense | 5′-ATCTTCAAACCTCCATGATG-3′ |
| MDR | c: sense | 5′-GGGACCGCAATGGAGGAG-3′ |
| | d: antisense | 5′-CAGATTCATGAAGAACCCTG-3′ |

PCR was a reaction with cDNA in a final volume of 100 μl of a reaction mixture [Tris–HCl (10 mM), pH 8.3, KCl (50 mM), 1.5 mM $MgCl_2$] containing 2.5 units of thermostable DNA polymerase (Amplitag, Parkin-Elmer). The PCR cycle consisted of 2 min of denaturation at 94°C, 2 min of primer annealing at 60°C and 2 min of extension/synthesis at 72°C. PCR primers were synthesized using DNA synthesizer (Applied Biosystem, model 394). PCR products were visualized by electrophoresis through a 1% agarose gel. PCR products using β-actin and MDR amplifiers yields were 102 and 308 bp, respectively.

## *In vitro* chemosensitivity assay

The assays were performed as described previously.[20,21] Briefly, immunosuppressive agents were added to a range of anthracycline agents in 96-well microplates, then, the cell suspension (100 μl), at a concentration of $2 \times 10^5$ cells/ml for the KB cells and VJ-300 cells, was dispensed into the plates. After 3 days of culture, cell viability was measured by the MTT calorimetric assay, a method which measures the content of viable cells by virtue of the activity in the mitochondria. Concentration of the anthracycline anticancer drugs affording 70% growth inhibition ($IC_{70}$) was determined from the dose–response curve by plotting cell viability versus concentration of the anthracycline anticancer drugs.

## Accumulation of anticancer drugs

KB and VJ-300 cells ($5 \times 10^5$) in monolayers were exposed to 1 μM of anthracycline and/or 2 μM of CsA, FK506 at 37°C for 60 min. After the incubation, the cells were washed twice with PBS, resuspended in PBS to a final concentration of $5 \times 10^5$/ml and accumulation of anticancer drugs was analyzed with by FACScan (Becton Dickinson). In each experiment, $1 \times 10^4$ cells were analyzed at a flow rate of 100–200 cells. Consort 30 software was used for data collection and analysis, the fluorescence signals were analyzed with excitation at 488 nm with emission integrated above 530 nm.[22] The related fluorescence intensity measured by flow cytometry is shown as fluorescence intensities of cells treated with 1 μM ADM for 60 min, in comparison with those of untreated cells. Each experiment was performed in triplicate.

### Cell cycle analysis

For cell cycle analysis, a range of 0–0.3 μM ADM, which has cytotoxicity in KB and VJ-300 cells, 0–0.7 μM CsA and 0–1 μM FK506 for 24 h was used. In the combination, drugs were concomitantly administered. Then cells were washed twice with PBS. Cells ($5 \times 10^5$/ml) were suspended in 0.2% (v/v) Triton X-100 detergent (Katayama Chemical, Osaka, Japan) and left at room temperature for 20 min. Then, 100 μg/ml propidium iodide (PI; Sigma, St Louis, MO) in sodium citrate buffer and 100 μl of 0.5 mg/ml ribonuclease A (RNase; Sigma) were added to $5 \times 10^5$ cells/ml cell suspension. The cell cycle distribution was analyzed by FACScan (Becton Dickinson). Cell-FIT software was used for data collection and analysis. Cells ($1 \times 10^4$) were analyzed at a flow rate of 100–200 cells. Gates were set on forward and side scattered light to eliminate debris and to select single cells. A total of $1 \times 10^4$ cells was used for cell cycle analysis of each sample.

### Statistics

Differences in data were analyzed using by Student's *t*-test. A *p* value of less than 0.05 was considered to be statistically significant.

## Results

### Expression of MDR using PCR

We analyzed the RT-PCR of MDR mRNA in KB cells and in a multidrug resistant cell line, VJ-300 cells. The specific MDR product was amplified by 35 cycles (Figure 1). This result suggested that expression of MDR is not recognized in KB cells, but in VJ-300.

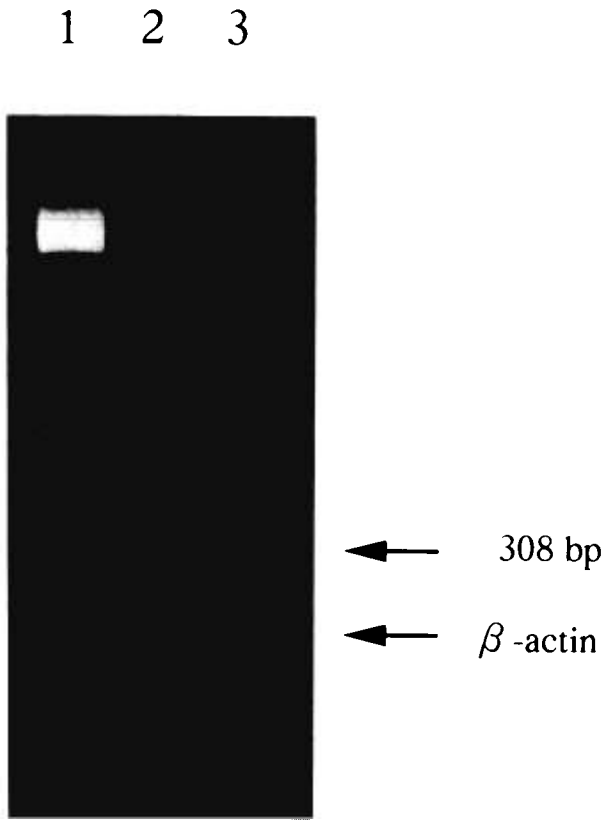


**Figure 1.** Expression of MDR using PCR. PCR studies on cDNA sample derived from cells. Lanes 2 and 3, amplification of cDNA using primers (a and b) and β-actin primers (c and d). ↑, indicates MDR (308 bp) and β-actin (120 bp). Lane 1, 100 bp ladder which is used as an internal standard. Lane 2, KB cells. Lane 3, VJ-300 cells.

### Sensitivity to anticancer drugs *in vitro*

We examined the effect of CsA or FK506 on the cytotoxic activity of ADM in both KB and VJ-300 cells, as shown in Table 1. The VJ-300 cells had 10.36-fold resistance to anthracycline compared with KB cells, when ADM $IC_{70}$ values against the VJ-300 cells were compared with those against KB cells. When 2.5 μg/ml of CsA or FK506 was added, the $IC_{70}$ values of ADM in VJ-300 cells were significantly decreased to the relative values by 0.33 or 0.93 times, compared with the $IC_{70}$ of each drug alone in KB cells.

For the other anthracyclines, VJ-300 cells had 4.86- to 25.38-fold resistance to DM, THP and EPIR (Table 2). When 2.5 μg/ml of CsA or FK506 was added to anthracyclines, the $IC_{70}$ values of anthracyclines in VJ-300 cells were significantly decreased to the relative values of 0.86 to 2.5 times.

When combined with 2.5 μg/ml of CsA or FK506, the $IC_{70}$ values for ADM/ADM plus CsA or FK506 were 0.032 or 0.089, for DM were 0.29 or 0.21, for THP were 0.125 or 0.135 and for EPIR were 0.036 or 0.042. CsA enhanced the sensitivity of VJ-300 cells to all the anthracyclines more effectively than did FK506.

### Intracellular accumulation of anthracycline

The intracellular accumulation of ADM in KB cells was little changed even in the presence of 2 μM CsA or FK506. However, in VJ-300 cells, this accumulation was greatly increased by CsA or FK506, as

**Table 1.** Effect of CsA or FK506 on cytotoxicity of ADM in KB and VJ-300 cells (numbers in parentheses indicate relative values of $IC_{70}$ doses of each drug, when $IC_{70}$ dose of ADM alone for KB cells was designated as 1)

| Cells | $IC_{70}$ (ng/ml) | | |
|---|---|---|---|
| | Alone | Combination | |
| | | +CsA (2.5 μ g/ml) | +FK506 (2.5 μ g/ml) |
| KB | 140 (1) | 110 (0.79) | 120 (0.86) |
| VJ-300 | 1450 (10.36) | 46 (0.33) | 130 (0.93) |

**Table 2.** Effect of CsA or FK506 on cytotoxicity of anthracyclines in KB and VJ-300 cells (numbers in parentheses indicate relative values of $IC_{70}$ doses of each drug, when $IC_{70}$ doses of each drug alone for KB cells were designated as 1)

| Cells | Drug | $IC_{70}$ (ng/ml) | | |
|---|---|---|---|---|
| | | Alone | Combination | |
| | | | +CsA (2.5 μg/ml) | +FK (2.5 μg/ml) |
| KB | DM | 140 (1) | 120 (0.86) | 130 (0.93) |
| | THP | 52 (1) | 51 (0.98) | 51 (0.93) |
| | EPIR | 130 (1) | 120 (0.92) | 120 (0.92) |
| VJ-300 | DM | 680 (4.86) | 140 (1) | 140 (1) |
| | THP | 960 (18.46) | 120 (2.31) | 130 (2.5) |
| | EPIR | 3300 (25.38) | 120 (0.92) | 140 (1.08) |

shown in Figure 2. When 2 μM CsA or FK506 was added to ADM, the intracellular accumulation of ADM was reversed by CsA, but 2 μM FK506 did not increase the intracellular accumulation of ADM over that seen with 2 μM CsA.

The intracellular accumulation of ADM in VJ-300 cells was examined with regard to the timing of administration of CsA (Figure 3A) or FK506 (Figure 3B), in relation to ADM. The level of intracellular accumulation was most increased when CsA was given concomitantly with ADM ($p < 0.01$). When CsA was given before ADM, the intracellular accumulation of the drug was slightly decreased, and when CsA was administered 1 h after ADM, it was significantly increased over that with ADM alone ($p < 0.05$).

Similarly, the level of intracellular accumulation of ADM was most increased when FK506 was given concomitantly with ADM ($p < 0.01$).

The intracellular accumulation of other anthracyclines was also increased. CsA and FK506 showed almost equal effects in increasing the intracellular accumulation of DM, THP and EPIR. For ADM, CsA was more effective than FK506. When CsA or FK506

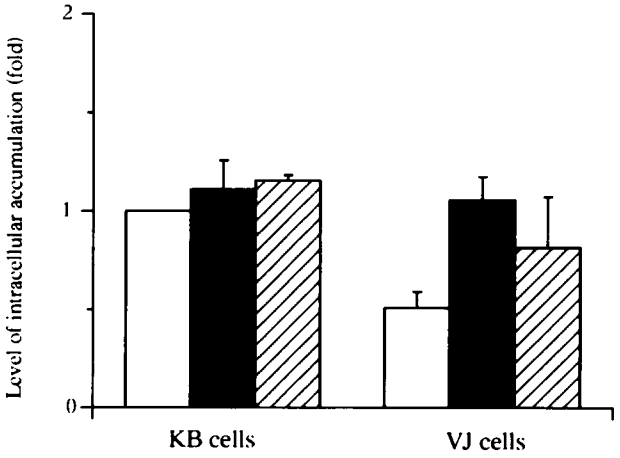


**Figure 2.** The intracellular accumulation of ADM, when CsA or FK506 was combined with ADM. The intracellular accumulation of ADM alone for KB cells was designated as 1. The cells were treated with ADM alone (□), ADM combined with CsA (■) or ADM combined with FK506 (▨). Data are from triplicate determinations. Means ± SD.

was combined with anthracyclines, the intracellular accumulation for DM alone/DM plus CsA or FK506 was 1.69 or 1.69, for THP it was 1.54 or 1.62 and for

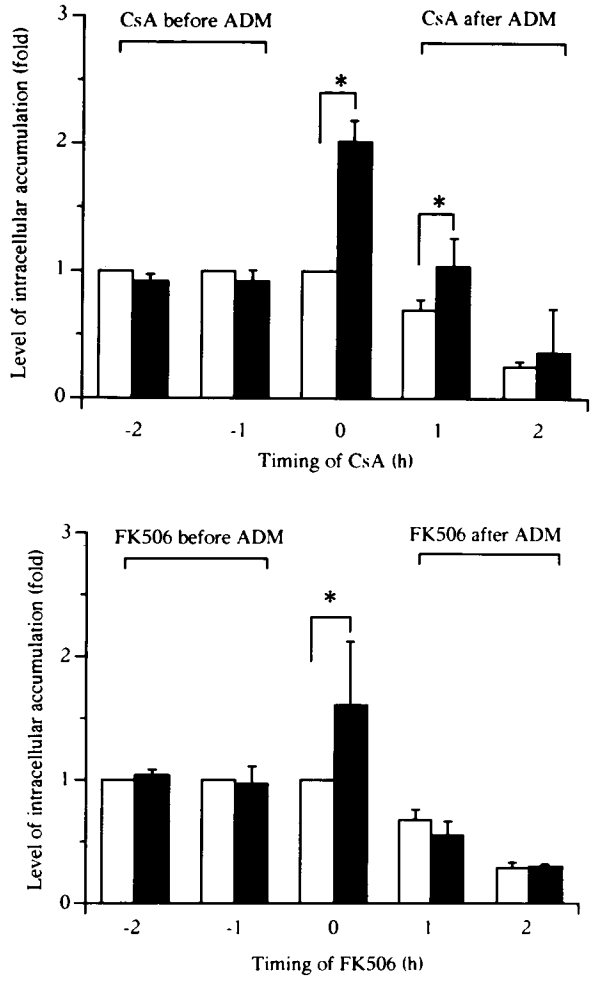


**Figure 3.** Timing of CsA or FK506. In VJ-300 cells, differences in the combined ADM and CsA (A) or FK506 (B) at various times. CsA or FK506 was added at −2, −1, 0, +1 and +2 h, using 0 h as the time of the action of ADM. When cells were immediately treated with 1 μM ADM for 60 min, intracellular accumulation was designated as 1. Cells were treated with ADM alone (□) or ADM combined with CsA or FK506 (■). Data are from triplicate determination. Means ± SD. *Significant difference ($p < 0.05$) from each group.

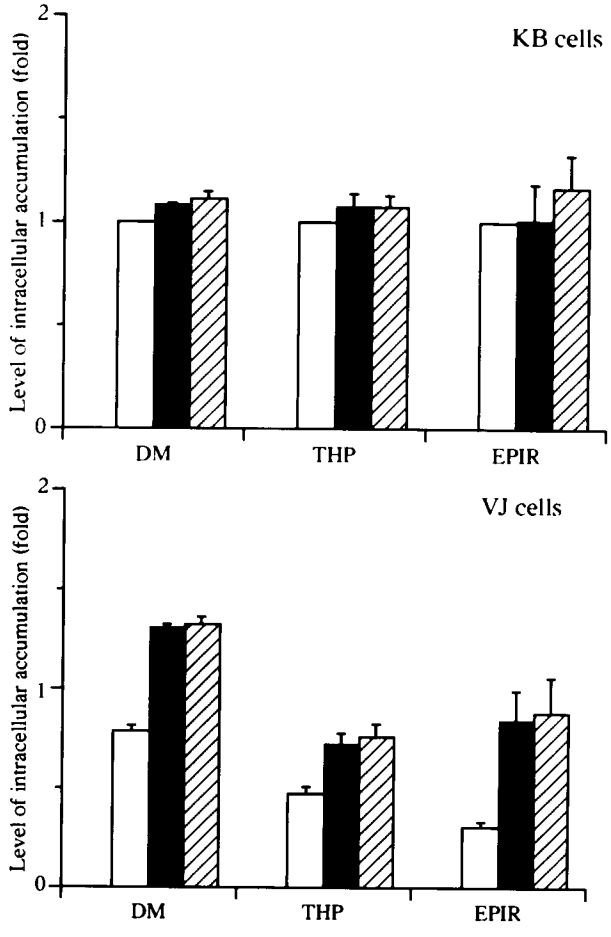


**Figure 4.** The intracellular accumulation of anthracyclines, when CsA or FK506 was combined with anthracyclines. Anthracyclines accumulating in KB cells (A) and VJ-300 cells (B) were determined as described in Materials and methods. The intracellular accumulation of anthracyclines alone for KB cells was designated as 1. The cells were treated with anthracycline alone (□) or anthracycline combined with CsA (■) or anthracycline combined with FK506 (▨). Data are from triplicate determinations. Means ± SD.

EPIR it was 2.8 or 2.93. The intracellular accumulation of EPIR was significantly enhanced by CsA or FK506 in VJ-300 cells, but not in KB cells (Figure 4A and B).

## Cell cycle analysis

Data on cell cycle analysis are given in Tables 3 and 4, and in histograms in Figure 5. The cell population in the $G_0/G_1$ phase in KB cells was unchanged by CsA or FK506 alone. In KB cells, the population in the S phase of the cell cycle was slightly increased with 0.3 μM ADM alone, but 0.7 μM CsA in combination with ADM slightly increased the cell population in the $S/G_2M$ phase. FK506 combined with ADM slightly increased the cell population in the S phase. However, in VJ-300 cells, 0.7 μM CsA or 1 μM FK506 combined with ADM significantly increased the cell population in the $G_2M$ phase, as compared with findings with ADM alone.

## Discussion

P-gp is a mediator of MDR and is an important therapeutic target to improve the results of clinical

**Table 3.** Changes in cell cycle of KB cells treated with ADM with or without CsA or FK506

| Dose ($\mu$M) | | Percentage of cells in | | |
|---|---|---|---|---|
| | | $G_1$ | S | $G_2M$ |
| ADM | CsA | | | |
| 0 | 0 (control) | 63.8 | 24.6 | 11.6 |
| 0 | 0.07 | 68.5 | 13.4 | 18.1 |
| 0 | 0.7 | 53.3 | 35.6 | 11.1 |
| 0.3 | 0 | 55.5 | 31.9 | 12.6 |
| 0.3 | 0.07 | 55.7 | 31.9 | 12.4 |
| 0.3 | 0.7 | 65.1 | 14.7 | 19.8 |
| ADM | FK506 | | | |
| 0 | 0.1 | 64.7 | 22.9 | 12.4 |
| 0 | 1 | 62.9 | 23.2 | 13.9 |
| 0.3 | 0 | 55.5 | 31.9 | 12.6 |
| 0.3 | 0.1 | 54.7 | 33.2 | 12.1 |
| 0.3 | 1 | 54.9 | 33.7 | 11.5 |

**Table 4.** Changes in cell cycle of VJ-300 cells treated with or without CsA or FK506

| Dose ($\mu$M) | | Percentage of cells in | | |
|---|---|---|---|---|
| | | $G_1$ | S | $G_2M$ |
| ADM | CsA | | | |
| 0 | 0 (control) | 51.3 | 31.4 | 17.1 |
| 0 | 0.07 | 58.4 | 23.5 | 18.1 |
| 0 | 0.7 | 65.2 | 13.3 | 21.5 |
| 0.3 | 0 | 45.2 | 39.4 | 15.4 |
| 0.3 | 0.07 | 47.8 | 29.4 | 22.9 |
| 0.3 | 0.7 | 28.9 | 15.4 | 55.7 |
| ADM | FK506 | | | |
| 0 | 0.1 | 55.6 | 28.9 | 14.6 |
| 0 | 1 | 77.1 | 10.1 | 12.8 |
| 0.3 | 0 | 45.2 | 39.4 | 15.4 |
| 0.3 | 0.1 | 59.5 | 28.5 | 11.9 |
| 0.3 | 1 | 11.3 | 55.8 | 32.9 |

chemotherapy. A variety of compounds inhibit the P-gp mediated transport of cytotoxic agents from tumor cells and thereby reverse the MDR phenotype. These MDR reversal agents include several calcium channel blockers, phenothiazines, progesterone, tamoxifen, CsA and some chemically modified derivatives.

We obtained evidence that the immunosuppressive agents, CsA and FK506, were capable of reversing the MDR phenotype to anthracyclines in VJ-300 cells. We examined whether the intracellular accumulation of anthracyclines would be increased by CsA or FK506 and we investigated the influence of these agents on the cell cycle of VJ-300 cells. In our study, when CsA or FK506 was combined with anthracyclines, the cytotoxicity of these agents, particular EPIR, was significantly enhanced in VJ-300 cells compared with other agents, with about 3-fold increase in EPIR. Cell cycle analysis indicated that CsA or FK506 combined with ADM significantly in-

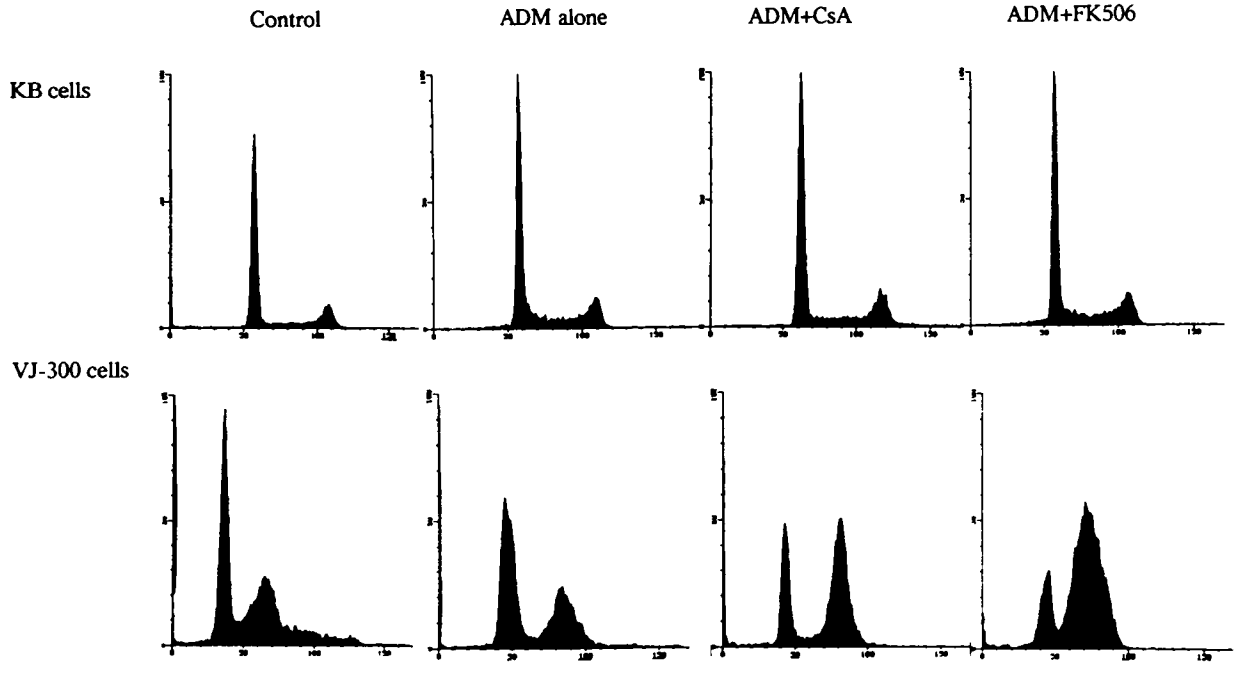


**Figure 5.** Cell cycle of CsA (0.7 $\mu$M) or FK506 (1 $\mu$M) combined with ADM (0.3 $\mu$M) in both KB and VJ-300 cells, and indicated on each of the histograms.

creased the cell population in the $G_2M$ phase when compared with findings with ADM alone in VJ-300 cells.

CsA was more effective than FK506 enhancing the cytotoxicity activity of antrhacyclines in VJ-300 cells. The intracellular drug accumulation in VJ-300 cells was greatly increased by CsA or FK506. These results suggests that the action of FK506 in overcoming MDR is similar to that of CsA. It was reported that the effect of each anthracycline was similar *in vitro*,[23,24] CsA or FK506 competitively bound to the drug binding site on P-gp and was transported from resistant cells by a mechanism similar to that seen for anthracyclines. The intracellular accumulation of drugs was most increased when ADM and CsA were concomitantly administered. Thus, simultaneous administration of CsA or FK506 and ADM caused a competitive inhibition of drug efflux. As the efflux of both CsA and FK506 is through P-gp, the intracellular concentration of ADM is increased. The level of accumulation of ADM was significantly increased when ADM was administered 1 h prior to CsA. This may be related to the molecular weight (MW) of CsA (MW = 1202) which exceeded that of FK506 (MW = 822) and this event may influence the kinetics of ADM efflux.

Barlogie *et al.*[25] reported that the number of cells in the $G_2M$ phase was increased by ADM alone. Although CsA was seen to arrest T cells in the $G_0/G_1$ phase,[26] this phenomenon was not noted with epidermal keratinocytes.[27] In our experiments, the number of KB and VJ-300 cells in the $G_0/G_1$ phase was slightly increased by CsA or FK506. On the other hand, CsA or FK506 combined with ADM greatly increased the number of cells n $S/G_2M$ phase compared with ADM alone in VJ-300 cells but not in KB cells. CsA or FK506 seem to influence little the cell cycle, CsA and FK506 were modulated of anticancer drugs, and cytotoxicity was increased in the MDR cells in combination with ADM. As the number of the cells in S $G_2M$ phase is targeted by anticancer agents, the cytotoxicity of the drugs may increase.

It was reported that P-gp was cell cycle dependent.[28] Newly synthesized P-gp had no apparent function before mitosis of the cells exposed to P-gp. As CsA or FK506 combined with ADM increased the number of cells in the $S/G_2M$ phase and P-gp was without apparent function before the mitosis, the increased cytotoxicity may be related to the increased intracellular drug accumulation, with increased $S/G_2M$.

The effect of each anthracycline on the kinetics of influxe or efflux of ADM, DM and THP was studied in the leukemic cell line, K562, and its MDR cell line. THP was rapidly taken up and excreted by the cells compared with findings with DM or ADM in these sensitive and resistant cells. The intracellular accummulation of DM was slightly increased compared with other agents, when CsA or FK506 was combined with ADM. In our experiment, the level of intracellular accumulation of EPIR was increased over that seen with anthracyclines in the presence of CsA or FK506 ($p < 0.05$) and this event is likely to be related to a delay in efflux, since CsA and FK506 were reported to be effluxed by P-gp.[13] If the efflux of anticancer agents was slower, this efflux would be relatively suppressed when combined with CsA or FK506.

CsA combined with anticancer agents (vincristine, vinblastine, etc.) was examined in clinical studies.[29] CsA is an immunosuppressive agent with potent side effects. Immunosuppresion combined with EPIR may be more effective and will have a good choice of series.

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