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FK506 REVERSES ADRIAMYCIN RESISTANCE IN A MULTIDRUG-RESISTANT HUMAN LEUKEMIA CELL LINE

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INDEXING WORDS

FK506; cyclosporin A; multi-drug resistance; adriamycin; immunosuppressant

SYNOPSIS

We have examined the effect of FK506 on the Adriamycin sensitivity of the multidrug resistant human chronic myelocytic leukemia cell line (K562/ADM). In K562/ADM cells, 1.0 μg/ml FK506 reversed the resistance of Adriamycin, and increased the IC_{50} value for Adriamycin up to 17 fold. However, IC_{50} value for the parent cells (K562) increased only 1.5 fold. By cell cycle analysis, the accumulation in late S-G_2M phase was confirmed on K562/ADM cells, treated with 1.0 μg/ml FK506 and low-dose of Adriamycin. Cyclosporin A (CsA) could also restored the Adriamycin sensitivity in the K562/ADM cells, as previously reported. 1.0 μg/ml FK506 as well as CsA significantly increased radioactive Adriamycin accumulation in K562/ADM cells and blocked [^{3}H]azidopien photoaffinity labeling of P-glycoprotein. These results suggest that 1.0 μg/ml FK506 could reverse the Adriamycin resistance in a MDR human leukemia cells through the interaction with P-glycoprotein.

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INTRODUCTION

The overexpression of a 170-kDa transmembrane protein (P-glycoprotein) encoded by the MDR-1 gene causes multidrug resistance (MDR) in human and murine tumor cell lines (2). The homology between P-glycoprotein and a bacterial transport protein has been reported, and P-glycoprotein apparently acts to facilitate drug efflux from cells (5,13,14). A variety of drugs including calcium antagonists and calmodulin inhibitors has been demonstrated to interfere with drug efflux and thereby to enhance the chemosensitivity of MDR cells (40).

Cyclosporin A (CsA) and its analogues have also been reported to restore the sensitivity of a tumor cell line toward anthracyclines and *vinca alkaloids* (4,12,27,32,33,41). The property of CsA as a resistance modifier seems to be dissociated from the properties as an immunosuppressant (4,12,41).

FK506 as well as CsA, is a potent inhibitor of T cell activation, but these drugs are structurally unrelated immunosuppressants (18). FK506 is a macrolide and CsA is an acylic peptide. The immunosuppressive capacity of FK506 is approximately 10-100 times as potent as that of CsA both *in vivo* and *in vitro* (1). Recent studies have shown that FK506 binds a cytosolic protein which has the peptidyl-prolyl cis-trans isomerase activity. This is distinct from the CsA-binding protein, cyclophilin (15,34). Both FK506 and CsA can inhibit the transcriptional activation regulating interleukin-2 gene (10,26).

Therefore, we have examined the effect of FK506 on the anthracycline sensitization in the human MDR chronic myelocytic leukemia cell line, K562/ADM. FK506 could sensitize the MDR cells to anthracycline as well as CsA. We have also compared the mechanisms involved in the reversal of MDR induced by FK506 or CsA.

MATERIALS AND METHODS

Reagents.

FK506 and CsA were kindly provided by Fujisawa Pharmaceutical Co. (Osaka, Japan) and Sandoz Pharmaceuticals (Basel, Switzerland), respectively. Doxorubicin (Adriamycin) and its radiolabeled reagent, [14-^{14}C] doxorubicin hydrochloride (67 Ci/mmol) were obtained from Kyowahakko Co. (Tokyo, Japan) and Amersham (Buckinghamshire, UK), respectively. [^{3}H]azidopine (49 Ci/mmol) was obtained from Amersham (Buckinghamshire, UK). FK506 and CsA were dissolved in ethanol. The final concentration of ethanol added into medium was below 0.5%, which did not show any detectable effects on the cell viability.

Cell culture.

A human myelogenous leukemia-derived cell line, K562 (23) and its Adriamycin-resistant subline, K562/ADM (39) were kindly provided by Dr.Y. Imai of our laboratory and Dr. T. Tsuruo (the Institute of Applied Microbiology, the University of Tokyo). The cells were cultured in RPMI 1640 medium (M.A. Bioproducts, Rockville. MD.) containing 10% fetal bovine serum (M.A. Bioproducts) and kanamycin (100 μg/ml).

Growth inhibition studies.

Triplicate samples of $4x10^4$ cells per well were seeded in 96-well culture plates (Corning Glass Works, Corning, NY) with serial dilutions of Adriamycin in the presence or absence of 0.5-3.0 μg/ml FK506 and CsA, in a final volume of 200 μl each (41). After 72 h incubation at 37°C, the viability of 200 cells from each culture well were examined by using Trypan blue exclusion method (21). The cytotoxic activity of Adriamycin, in the presence or absence of FK506 and CsA, was determined by the concentration of drug required for 50% inhibition of the cell growth, IC_{50} (40).

Cell cycle analysis.

Each cell lines was treated by low-cytotoxic doses of Adriamycin with 1.0 μg/ml FK506 or 3.0 μg/ml CsA, then incubated in RPMI1640 medium containing 10% FCS at 37°C for 72h. The cells were stained by the standard propidium iodide method and analyzed by FACS analyzer (Beckton Dickinson,CA) (22).

[14-^{14}C]Adriamycin accumulation study.

Cells ($2x10^5$) were seeded in 96-well culture plates, and incubated for 2 h, 6 h and 10 h at 37°C with 2 μM [14-^{14}C] Adriamycin in the presence or absence of 1.0 μg/ml FK506 or 3.0 μg/ml CsA. The cells were collected on glass-fiber filters with a cell harvester unit, and washed by phosphate buffer solution as described previously (19,25). The radioactivities of the filters were counted by a Beckman LS7500 liquid scintillation system.

Photoaffinity labeling of [^{3}H]azidopine.

Crude cell membrane fraction from K562 and K562/ADM cells were prepared as described (31). The membrane protein (100 μg) were incubated with 0.75 μM [^{3}H]azidopine for 15 min at room temperature in the presence or absence of various drugs. After continuous irradiation at 366 nm for 20 min at 25 °C, the membrane proteins were solublized in a SDS sample buffer and subjected to a 7% polyacrylamide gel electrophoresis. The gel was soaked with autoradiography enhancer (EN^{3}HANCE,MEN,MA) and subjected to fluorography at -80 °C as described previously (31).

Immunoblot and northern blot analysis of DNA topoisomerase II

Whole cell lysates were mixed with SDS-PAGE loading buffer and SDS-PAGE were electrophoresed described detailed previously (6). Antibody to the 170-kDa form of topoisomerase II were kindly provided from Dr. Drake, Smithkline Beecham Pharmaceuticals (6). Total RNA isolated from cells incubated in the presence or absence of various drugs for 6 h was analyzed according to standard methods as described previously (38). rhTOP2/Z2, a 1.8 kb EcoR1 fragment of

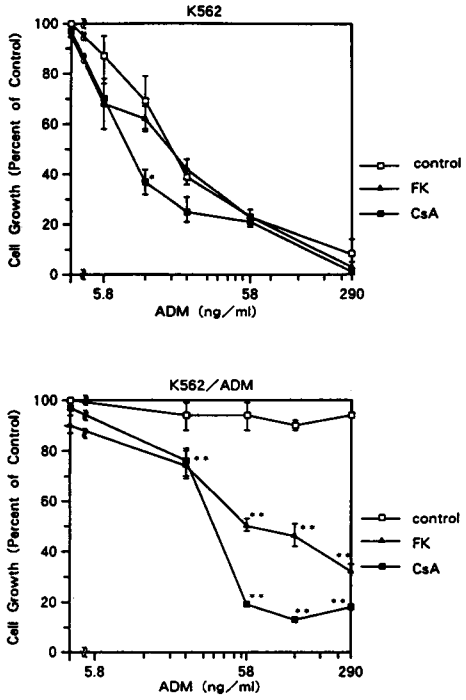

Figure 1. Effect of FK506 and CsA on Adriamycin in K562 and K562/ADM. Values are plotted as percentage of growth compared with (□) control in the absence of Adriamycin and in the absence of CsA or in the presence of (▲) 1 μg/ml FK506, (■) 3 μg/ml CsA. Each bar represents the mean ± SD of triplicate determinations. * significant at $P < 0.05$; ** significant at $P < 0.001$

a human topoisomerase II cDNA (kindly provided Dr. L.F. Lui, John Hopkins University School of Medicine, Baltimore, MD) was used as probe (37).

RESULTS

Effects of FK506 on Adriamycin sensitivity.

The effects of FK506 and CsA on the sensitivity of K562 and K562/ADM cells to Adriamycin were examined (Figure 1). Adriamycin inhibits the cell growth of K562 cells in a dose dependent manner (5.8-290 ng/ml). FK506 (1 μg/ml) did not enhance the cytotoxity of Adriamycin to K562 cells, although CsA show a little enhancement of

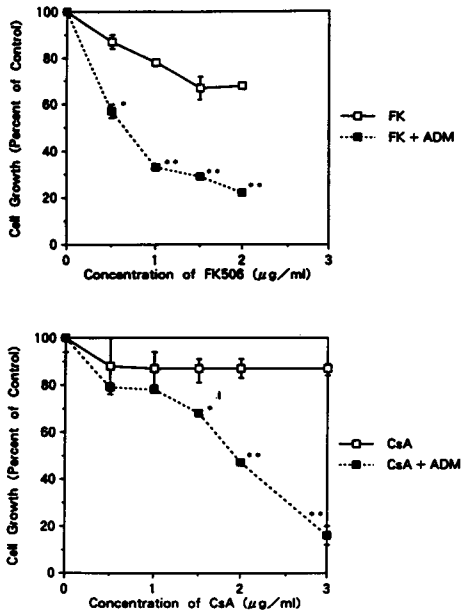

Figure 2. Dose-response relationship of FK506 and CsA in K562/ADM, that were cultured with Adriamycin at 290 ng/ml. Growth inhibition was measured as described in Material and Methods. Values are plotted as percentage of growth compared with control in the absence of Adriamycin in the absence of FK506 or CsA. Upper panel; (□) 1.0 μg/ml FK506, (■) FK506 + Adriamycin, lower panel; (□) 3 μg/ml CsA, (■) CsA + Adriamycin. Each bar represents the mean ± SD of triplicate determinations. * significant at $P< 0.05$; ** significant at $P< 0.001$

the Adriamycin cytotoxity. In contrast to the parent K562 cells, K562/ADM cell growth were not affected by the same doses of Adriamycin added to the parent cells. However, FK506 (1 μg/ml) remarkably sensitized the Adriamycin resistance of K562/ADM cells. Adriamycin in the presence of FK506 inhibited the growth of K562/ADM in a dose-dependent manner. CsA (3 μg/ml) also showed the remarkable sensitization of K562/ADM cells to Adriamycin. In K562/ADM cells, 50 % growth inhibition was observed at 58 ng/ml Adriamycin with 1 μg/ml FK506, although the growth of K562/ADM was not inhibited by 100 nM Adriamycin alon. The IC_{50} of Adriamycin for K562/ADM cells was increased 17 fold by the 1.0 μg/ml FK506 addition. However, little increase (1.5 fold) was observed in K562 cells by the same treatment. CsA (3.0 μg/ml) enhanced the cytotoxity of Adriamycin (IC_{50}; 37.1 fold) in K562/ADM cells, while CsA did not

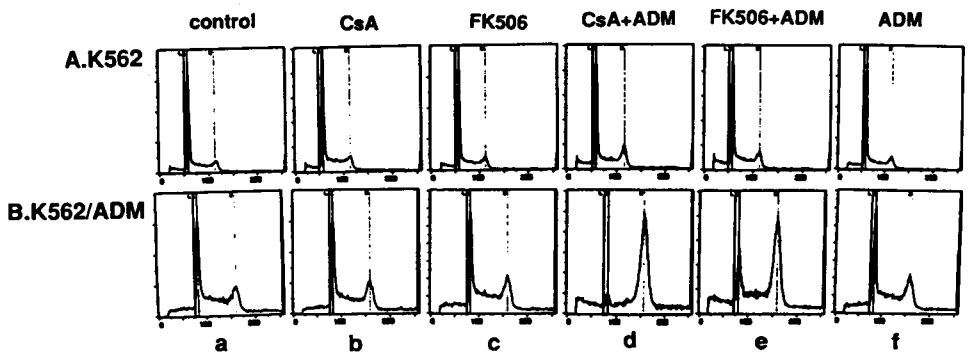

Figure 3. Flow cytometric analysis of the K562(A) and K562/ADM (B) cell cycles. (a) control, (b) 3 μg/ml CsA, (c) 1 μg/ml FK506, (d) CsA+Adriamycin (K562;11.6 ng/ml, K562/ADM;290 ng/ml), (e) FK506+Adriamycin, (f) Adriamycin. Cells were incubated for 72 h.

increase the IC_{50} so much (1.67 fold) in the parent K562 cells. The dose-response curves of FK506 or CsA on the Adriamycin sensitization of K562/ADM cells were shown in Figure 2. FK506 without Adriamycin showed slight growth inhibition in K562/ADM cells. FK506 significantly enhanced the cytotoxity of Adriamycin at 0.5 μg/ml, although, FK506 alone inhibited the growth of K562/ADM cells slightly. CsA alone showed little growth inhibition of K562/ADM cells but CsA remarkably enhanced the cytotoxity of Adriamycin.

Effect of FK506 on the cell cycle.

In order to confirm the sensitization to Adriamycin resistant cells by FK506, we employed a flow cytometric analysis of the cell cycle (Figure 3). The cell number of the K562/ADM cells at the S-phase was slightly increased than that of the parent cells. Neither FK506 or CsA alone affect the cell cycles in both cell lines. The combinations of FK506 or CsA with 290 ng/ml Adriamycin which did not inhibit the cell growth by itself, accumulated K562/ADM cells number

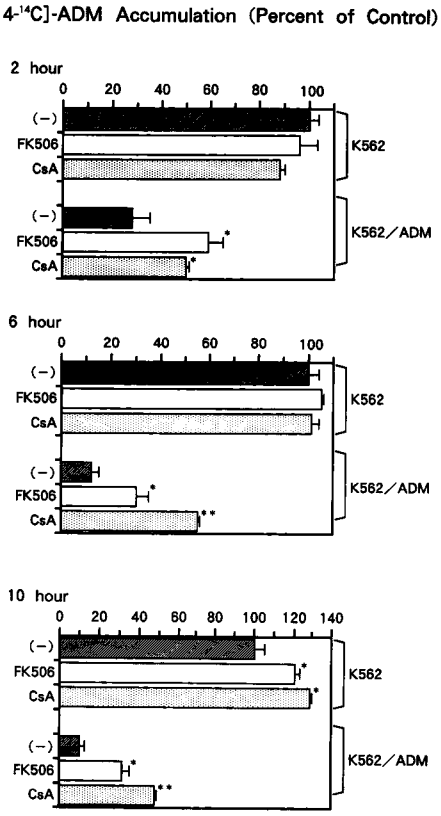

Figure 4. Effect of FK506 and CsA on intracellular accumulation of Adriamycin in K562 and K562/ADM. After the incubation of cells to 2 μM [14-^{14}C]-Adriamycin with in the absence(▨), or presence of 1.0 μg/ml FK506(□) and 3.0 μg/ml CsA (▩), [14-^{14}C]Adriamycin accumulation was measured as described in Material and Methods. The data were expressed as percent of the [14-^{14}C]Adriamycin accumulation in K562 cells without FK506 or CsA. Each bar represents the mean ± SD of triplicate determinations. * significant at P< 0.05; ** significant at P<0.001

in the late S-G_2M phase significantly. However, the same doses of FK506 or CsA with a low dose of Adriamycin (11.6 ng/ml) did not affects the cell cycle of the parent cells. High-dose of Adriamycin (290 ng/ml) accumulated the cells at the late S-G_2M phase in the parent cells (data not shown).

FK506 increased [14-^{14}C]Adriamycin accumulation in K562/ADM cells.

The accumulation of Adriamycin in K562 and K562/ADM cells in the presence of FK506 and CsA were measured (Figure 4). The

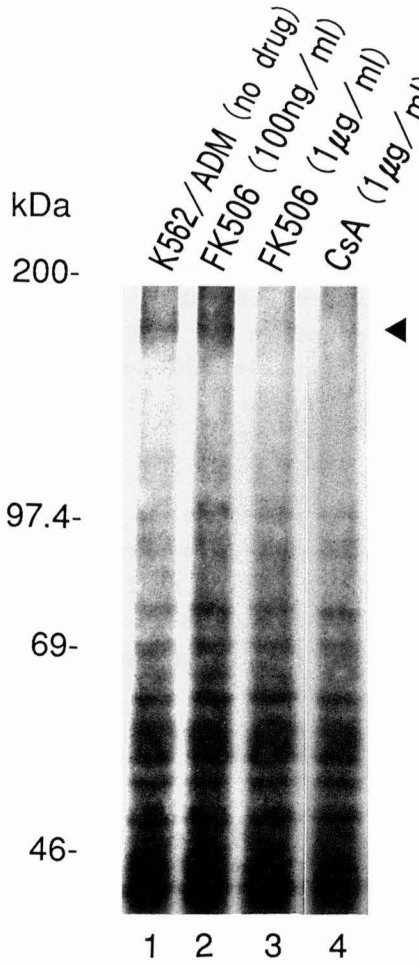

Figure 5. SDS-PAGE fluorography of [^{3}H]azidopine photoaffinity labelled plasma membranes (100 μg of protein/lane) of K562 and K562/ADM in the presence or absence of FK506 or CsA.
1, K562/ADM (no drug);2, K562/ADM +FK506(100ng/ml);3,K562/ADM+FK506 (1mg/ml);4,K562/ADM+CsA(1mg/ml). Arrowhead,170-kDa P-glycoprotein.

Adriamycin accumulation by 2 h in K562/ADM cells was 28 % of that in the control K562 cells. The Adriamycin accumulation in K562 cells was not affected by the FK506 or CsA treatment until 6 h incubation. After 10 h incubation, the Adriamycin accumulations were significantly increased by FK506 or CsA in the K562 cells. In the K562/ADM cells, 1.0 μg/ml FK506 or 3.0 μg/ml CsA remarkably

increased the accumulation at 2 h, 6 h and 10 h incubation.

FK506 blocked the photoaffinity labeling of [^{3}H] azidopine.

[^{3}H]azidopine photolabeling of P-glycoprotein on K562 and K562/ADM cells were analyzed by SDS-PAGE fluorograpy in the presence or absence of FK506 or CsA (Figure 5). The P-glycoprotein photolabelled by [^{3}H]azidopine was underdetectable in K562. [^{3}H]azidopine photolabeling of P-glycoprotein in K562/ADM was significantly reduced by 1.0 μg/ml FK506, while 100 μg/ml FK506 little. CsA, 1.0 μg/ml also blocked photolabeling of P-glycoprotein in K562/ADM .

Effect of FK506 on the expression of DNA topoisomerase II

The level of DNA topoisomerase II expression gene were examined by RNA blot and immunoblot analysis. FK506 or CsA on K562/ADM cells did not significantly affect the expression of the 6.5 kb transcript and 170-kDa form of topoisomerase II protein (data not shown).

DISCUSSION

In the present study, we have clearly demonstrated the ability of an immunosuppressive drug, FK506 to sensitize a MDR human leukemia cell line; K562/ADM to Adriamycin. The mechanisms of the reversal of MDR by FK506 have been analyzed. The modulation of MDR induced by CsA has been described in the recent reports (4,12,27,32,33,41). CsA and its synthetic nonimmunosuppressive analogues have been demonstrated to have sensitizing properties on both parent and MDR tumor cell liens (4,12,41). It is notable that CsA and its analogues sensitized MDR cell lines at clinically achievable concentrations (1~2.0 μg/ml) (4,12,41).

Recently, another potent immunosuppressant, FK506, has been isolated from the fermentation broth of a strain of *Streptomyces tsukubaensis* (18). It is a neutral macrolide structure unrelated to

CsA. The immunosuppressive potency of FK506 is approximately 10 to 100 times that of CsA (1). FK506 as well as CsA could inhibit the transcriptional activation of interleukin-2 gene in T lymphocyte (10,26).

CsA has been shown to arrest sensitive T-cell lines in the G_0 or G_1 phase of the cell cycle (20). However, the same effect was not found in epidermal keratinocytes (17). Our study has shown FK506 did not affect cell cycles of the chronic myelocytic leukemia cells, K562 at the dosage 1.0 μg/ml, as CsA at 3.0 μg/ml. The synergistic cytotoxity of FK506 in the presence of Adriamycin were confirmed by the accumulation of the MDR cells in the late S-G_2M phase. The accumulation of Adriamycin was modified by several sensitizers through the interaction with P-glycoprotein (40). In the parent cell line, the amount of Adriamycin accumulation was not significantly modified by CsA or FK506. However, in the MDR cell line, FK506 increased drug accumulation after 2 h and 6 h. This result suggested that FK506 enhanced the Adriamycin sensitivity by increasing the drug accumulation in MDR cells. A photoaffinity calcium channel blocker, [^{3}H]azidopine, is known to label P-glycoprotein. The photoaffinity was inhibited by MDR-modulators, vincristin and actinomycin D (31). Moreover, it significantly blocked the binding of photolabelled vincristin or [^{3}H]azidopine to P-glycoprotein (11,29,35). [^{3}H]azidopine photolabeling of P-glycoprotein was significantly reduced by FK506 up to 1.0 μg/ml in our experiments. These results suggests that FK506 reverse the drug resistance by the same mechanism through P-glycoprotein as CsA or calcium channel blockers.

CsA was also shown to be effective against parent cells lacking P-glycoprotein as a chemosensitizer (4,12,41). Nonimmunosuppressive analogues of CsA also sensitized MDR cells to anthracyclines (4,12,41). These results suggest that the other mechanisms than P-glycoprotein were involved in this sensitization (4). The relative molar potensity of FK506 as an immunosuppressant, is 10-100 times higher than that of CsA (1). However, FK506 as a chemosensitizer has

been shown to be effective as same as CsA. Therefore, the property of FK506 as a resistance modifier could be dissociated from the immunosuppressive properties.

Recently, other investigators reported that FK506 reversed drug resistance in both parent and resistant cells (9,28,30). However, these results showed that 3 μM to 10 μM FK506 modulated the chemosensitivity. In our system, 1 μg/ml (=1.2 μM) FK506 significantly enhanced the chemosensitivity to Adriamycin. The cell cycle study, the drug accumulation study and photoaffinity labelling confirmed this finding. Naito et al. showed that 10 μM FK506 was effective on K562/ADM, but not 1 μM FK506. This difference may be caused by using different cytotoxic assay method (Naito et al. employed MTT assay). Our results clearly demonstrated that FK506 reversed MDR at lower concentrations than CsA.

DNA topoisomerase II has been postulated to be another important cellular target for many anti-cancer drugs including anthracycline and epipodophyllotoxin derivatives (VP-16)(24). Alteration in the quantity or function of topoisomerase II has also been suggested as a possible mechanism for MDR (8). The expression of topoisomerase II mRNA is enhanced by G-CSF, and the sensitivity to daunorubicin is increased (36). Our hypothesis was that FK506 and CsA modulate the topoisomerase II mRNA expression, and enhance chemosensitivity in MDR cells. However, the expression of 170-kDa form of topoisomerase II transcript and protein were not affected by FK506 or CsA.

Recently, various type of FK506 binding protein have been revealed in eukaryotes (7,16). There is a possibility that one or more FK506 binding proteins are associated with MDR. Nonimmunosuppressive analogues of FK506 might qualify the precise mechanisms of FK506 as MDR modifier. It may be important to select the most potent MDR modifier among several FK506 analogues (3). Further to exploit the therapeutic window, the effect of FK506 as a chemosensitizer on MDR cells described in this paper should be explored *in vivo*.

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