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Chemosensitization to Adriamycin by Cyclosporin A and Verapamil in Human Retinoblastoma Cell Lines

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The chemosensitizing effects of cyclosporin A and verapamil on the cytotoxicity of adriamycin were investigated using MTT assay against two human retinoblastoma cell lines, Y79 and WERI-Rb-1. Y79 and WERI-Rb-1 were totally resistant to doses up to 5.0μg/ml of verapamil. Cyclosporin A inhibited the survival of Y79 and WERI-Rb-1 dose-dependently, however, the maximum inhibition at the highest concentration tested (5.0μg/ml) was less than 50% (% survival at 5.0μg/ml of cyclosporin A: 65.6% and 66.9% in Y79 and WERI-Rb-1, respectively). Combination of cyclosporin A and verapamil did not further inhibit the survival of Y79 and WERI-Rb-1 compared with cyclosporin A alone. Adriamycin inhibited the survival of Y79 and WERI-Rb-1 dose-dependently. The chemosensitizing effects of cyclosporin A and verapamil on the cytotoxicity of adriamycin were evaluated in terms of sensitizing index (SI: the ratio of IC_{50} to adriamycin alone to IC_{50} to adriamycin in the presence of cyclosporin A and/or verapamil). Cyclosporin A significantly enhanced SI and the addition of verapamil enhanced SI further: SI values at 5.0μg/ml of cyclosporin A, 5.0μg/ml of cyclosporin A plus 1.5μg/ml of cyclosporin A plus 1.5μg/ml of verapamil, 5.0 μg/ml of cyclosporin A plus 3.0μg/ml of verapamil were 2.0, 2.6 and 2.8 in Y79 and 2.6, 5.8 and 9.7 in WERI-Rb-1, respectively. These results suggest that cyclosporin A and verapamil are promising chemosensitizers to adriamycin in the treatment of retinoblastoma.

Key Words: *Chemosensitization, Adriamycin, Cyclosporin A, Verapamil, Retinoblastoma cell lines*

INTRODUCTION

Retinoblastoma, the most common pediatric ocular cancer, develops in one per 17,000 to 20,000 live births and accounts for 3.4% of the total annual cancer incidence in children less 15 years old in Korea (Pendergrass and Davis, 1980; Ministry of Health and Social Affairs, 1989). Although considerble improvement in survival, in recent years, has been achieved in patients in the early stage of retinoblastoma by the marked advancement of diagnosis and treatment, patients with advanced retinoblastoma, such as local invasion beyond surgical excision and distant metastasis, still have a poor prognosis.

Retinoblastoma confined in the eyeball has usually been treated with operation and radiation, 5-year survival being reported a 92-93% (Abramson and Ellsworth, 1980; Rubin et al., 1985). On the other hand, in patients in advanced stage, chemotherapy has frequently been tried but 5-year survival rate has been reported to be less than 30% (Kodilinye, 1967; Sinniah et al., 1980).

Despite numerous combination chemotherapies being tried to attempt to improve the response and survival, potent regimens have not yet been produced for retinoblastoma (Shields, 1983; Murphree and

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Rother, 1989). Adriamycin is now accepted as one of the most active and widely used agents for retinoblastoma. Response rate to adriamycin, however, is low, approximately 20% (White, 1983). Furthermore, even by adriamycin-based combination chemotherapy, the response rate still remains low and the duration of response is usually short, with the prolongation of survival often not being achieved by combination chemotherapy. To improve survival in patients with advanced retinoblastoma, it is of great importance to develop methods to enhance the responsiveness of cancer cells to chemotherapeutic agents.

Approaches directed at improving the response to chemotherapy have been focused primarily on the development of potent combination chemotherapy. However such potent regimens have not been reported for advanced retinoblastoma. An alternative approach for enhancing response is the chemosensitization of tumor cells. Numerous previous studies using cells acquiredly resistant to adriamycin have demonstrated that acquired resistance to adriamycin is usually associated with hyperexpression of P-glycoprotein and reduced intracellular drug accumulation and this type of resistance was reversed partially or completely by addition of cyclosporin A and/or verapamil by binding to P-glycoprotein (Kartner et al., 1983; Twentyman et al., 1987; Cornwell et al., 1987; Tsuruo et al., 1988; Kim et al., 1990).

Recently, some parent cell lines were reported to express low levels of P-glycoprotein (Kartner et al., 1985; Twentyman et al., 1990). If P-glycoprotein innately expressed affects the chemosensitizing effects of cyclosporin A and verapamil, cyclosporin A and verapamil may modify the intrinsic resistance to adriamycin. The present study was undertaken to investigate the chemosensitizing effects to adriamycin of cyclosporin A and verapamil on the sensitivity of two retinoblastoma cell lines.

MATERIALS AND METHODS

Cell lines

Two human retinoblastoma cell lines, Y79 and WERI-Rb-1, were maintained in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS) (Gibco), 100U/ml of penicillin and 100μg/ml of streptomycin, at 37°C in a highly humidified atmosphere of 5% CO_2 in air. These cell lines were kindly donated by Dr. M. Inomata, Japan National Cancer Research Institute.

Drugs

Adriamycin was kindly gifted from Dong-A Pharm. Co., Ltd., Korea). Verapamil and cyclosporin A were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Sandoz Korea Ltd. (Seoul, Korea), respectively. The stock solutions were made by dissolving the drugs in sterile distilled water and storing at −70°C. Immediately before the experiments, the stock solutions were diluted with RPMI-FCS to the required concentrations.

Chemosensitivity test

Cytotoxicity against two retinoblastoma cell lines was determined by MTT assay previously described in detail (Hong et al., 1992). Briefly, single cell suspension was made by gentle pipetting of exponentially growing cells followed by passing as gently as possible through 18-gauge injection needles. The seeding number of cells and incubation period were determined from the standard curve and growth curve of each cell line. In this study, 1 x10^4 cells/well and 6 days were used as a seeding number of cells and incubation period, respectively, in both cell lines. Y79 and WERI-Rb-1 were dispensed into each well of a 96-multiwell culture plate (1 x10^4 cells/150 μl) (Beckton Dickinson, Oxnard, CA, USA). After the plates were incubated for 4 hours, drugs diluted from stock solutions were added to each well at the volume of 15μl in single drug experiments and 30μl in combination experiments. And then the plates were incubated for 6 days, 15μl of MTT (3-(4,5-dimenthylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) (Sigma) solution diluted to 5mg/ml by phosphate buffered saline (Flow Laboratories, UK) were added to each well. After the plates were incubated for 4 hours, 150μl of acid isopropanol (0.04N) (Merck, Germany) were added to each well and the contents were subjected to repeated pipetting, about 20 times, to solubilize the intracellular formazan crystals formed. The absorbance was measured at 570 nm by using a spectrophotometer (Titertek Multiskan Plus MKII, Flow Laboratories, Finland). Each experiments was performed in triplicate and repeated three times.

Analysis of cytotoxicity

Cytotoxicity at various concentrations of drugs was evaluated using percent survival, which was determined by the formula: [(mean absorbance in three test wells-absorbance in background well)/(mean absorbance in control wells-absorbance in background well)] x100. Chemosensitization effect was analyzed using both IC_{50} and sensitization index (SI). IC_{50} was determined graphically from the dose-response curves. SI was determined as the ratio of IC_{50} to adriamycin

alone to IC_{50} to adriamycin in the presence of cyclosporin A and/or varapamil. Student's t-test was employed to testfor statistical differences between two sets of data.

RESULTS

Cytotoxicity of cyclosporin A and verapamil

The cytotoxicity of cyclosporin A and verapamil, either alone or in combination, was investigated in MTT assay against two human retinoblastoma cell lines, Y79 and WERI-Rb-1. Verapamil did not inhibit the survival of Y79 and WERI-Rb-1 (% survival at 5.0μg/ml of verapamil: 97.3% and 95.4% in Y79 and WERI-Rb-1, respectively) (Fig. 1). Cyclosporin A inhibit the survival of Y79 and WERI-Rb-1 dose-dependently, however, the maximum inhibition at the highest concentration tested (5.0 μg/ml) was less than 50% (% survival at 5.0% μg/ml of cyclosporin A: 65.6% and 66.9% in Y79 and WERI-Rb-1, respectively). Combination treatment with *cyclosporin A and verapamil did not further inhibit the* survival of Y79 and WERI-Rb-1 compared with cyclosporin A alone (data not shown).

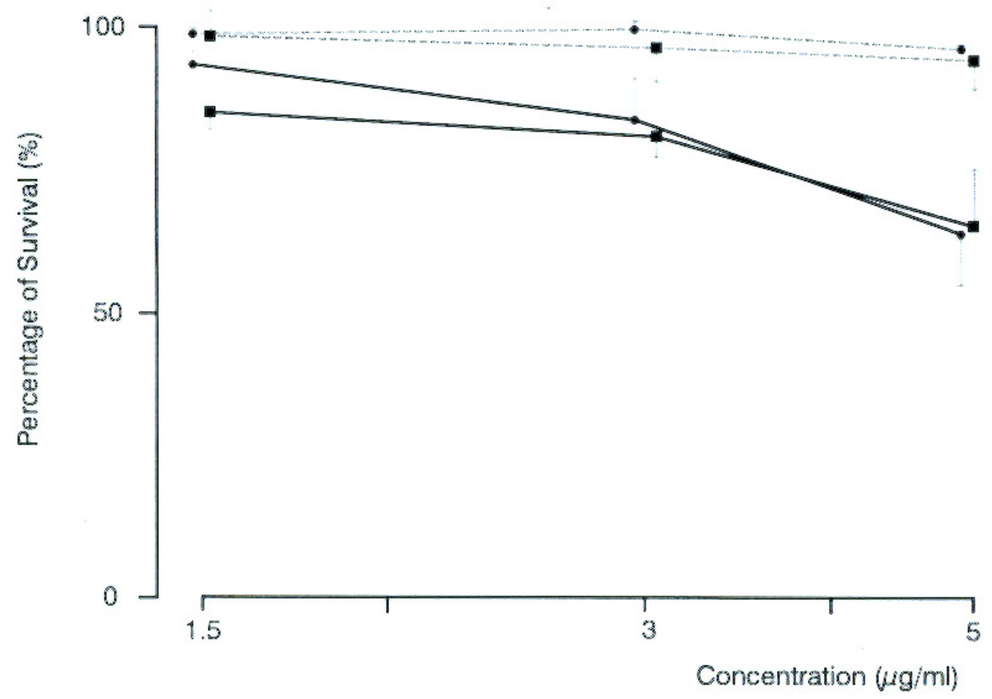

Fig. 1. Effects of cyclosporin A (—) and verapamil (---) on the survival of two human retinoblastoma cell lines, Y79 (•) and WERI-Rb-1 (■). Each point and bar represents the mean and SD three experiments.

Chemosensitizing effects of cyclosporin A and verapamil

The chemosensitizing effects of cyclosporin A and verapamil on the cytotoxicity of adriamycin were studied against Y79 and WERI-Rb-1. Adriamycin inhibited the survival of Y79 and WERI-Rb-1 dose-dependently and the addition of cyclosporin A and verapamil inhibited the survival to a greater extent (Fig. 2, 3). Chemosensitizing effects were determined using IC_{50} to adriamycin, demonstrating that IC_{50} was significantly decreased by the addition of 5.0μg/ml of cyclosporin A and further decreased by the addition of cyclosporin A and verapamil ($p<0.05$) (Table 1).

The chemosensitizing effects of cyclosporin A and verapamil were evaluated in terms of SI: SI values at 5.0 μg/ml cyclosporin A were 2.0 and 2.6 in Y79 and WERI-Rb-1, respectively; SI values at the combination of 5.0μg/ml of cyclosporin A and 1.5μg/ml of verapamil were 2.6 and 5.8 in Y79 and WERI-Rb-1, respectively; SI at the combination of 5.0μg/ml of cyclosporin A and 3.0μg/ml of verapamil were 2.8 and 9.8 in Y79 and WERI-Rb-1, respectively (Fig. 4). These results reveal that cyclosporin A or verapamil as a single agent does

Table 1. Combination effects of cyclosporin A and verapamil on IC_{50} adriamycin in human retinoblastoma cell lines, Y79 and WERI-Rb-1

	Y79	WERI-Rb-1
ADM alone	0.047±0.005*	0.029±0.008
ADM+VRP 1.5**	0.049±0.004	0.034±0.016
ADM+VRP 3.0	0.046±0.003	0.029±0.008
ADM+CsA 3.0	0.027±0.007	0.023±0.002
ADM+CsA 3.0+VRP 1.5	0.036±0.008	0.023±0.007
ADM+CsA 3.0+VRP 3.0	0.033±0.009	0.018±0.004
ADM+CsA 5.0	0.024±0.008[a]	0.011±0.003[a]
ADM+CsA 5.0+VRP 1.5	0.018±0.012[a]	0.005 ±0.004[a]
ADM+CsA 5.0+VRP 3.0	0.017±0.004[b]	0.003±0.002[b]

*, mean of IC_{50} (μg/ml) ± SD of three experiments
**, 1.5 μg/ml of verapamil
a) $p<0.05$, b) $p<0.01$
ADM; adriamycin, VRP; verapamil, CsA; cyclosporin A

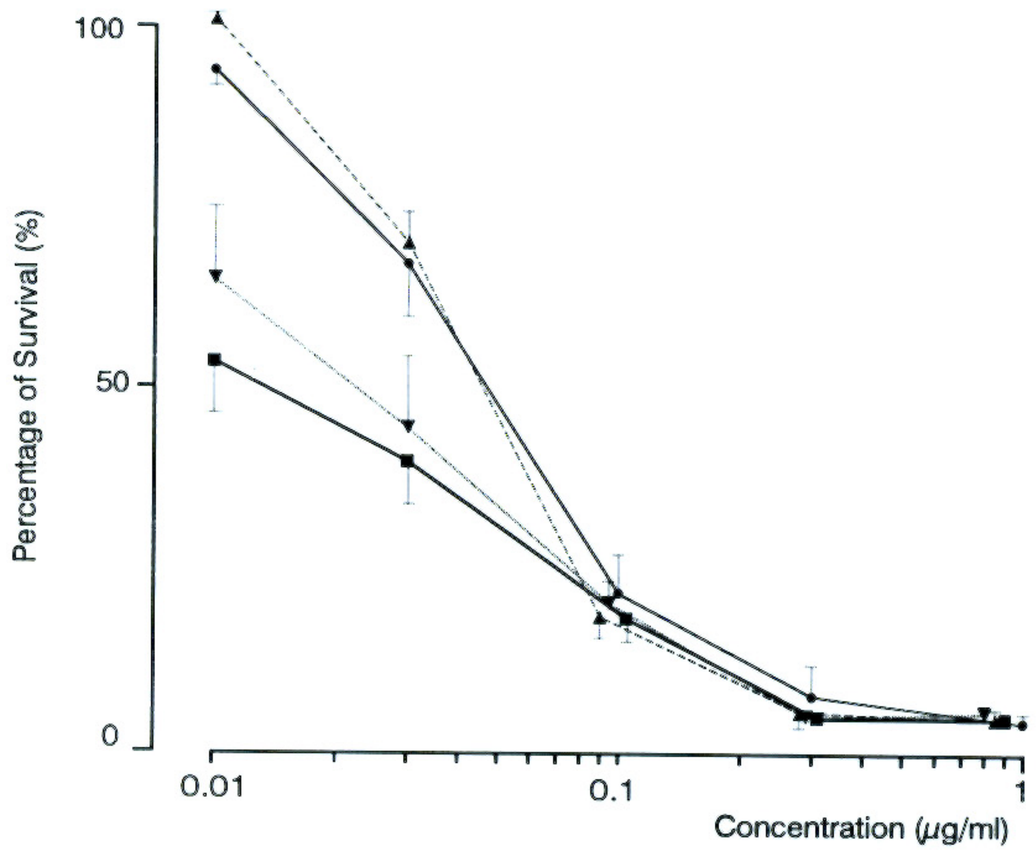

Fig. 2. Chemosensitization effects of cyclosporin A and verapamil on the cytotoxicity of adriamycin in Y79, a human retinoblastoma cell line: —●—, adriamycin alone; ···▼··· adriamycin + 3.0μml of cyclosporin A; -▲-, adriamycin + 3.0μg/ml of verapamil; —■—, adriamycin + 3.0μg/ml of cyclosporin A + 3.0μg/ml of verapamil. Each point and bar represents the mean and SD of three experiments.

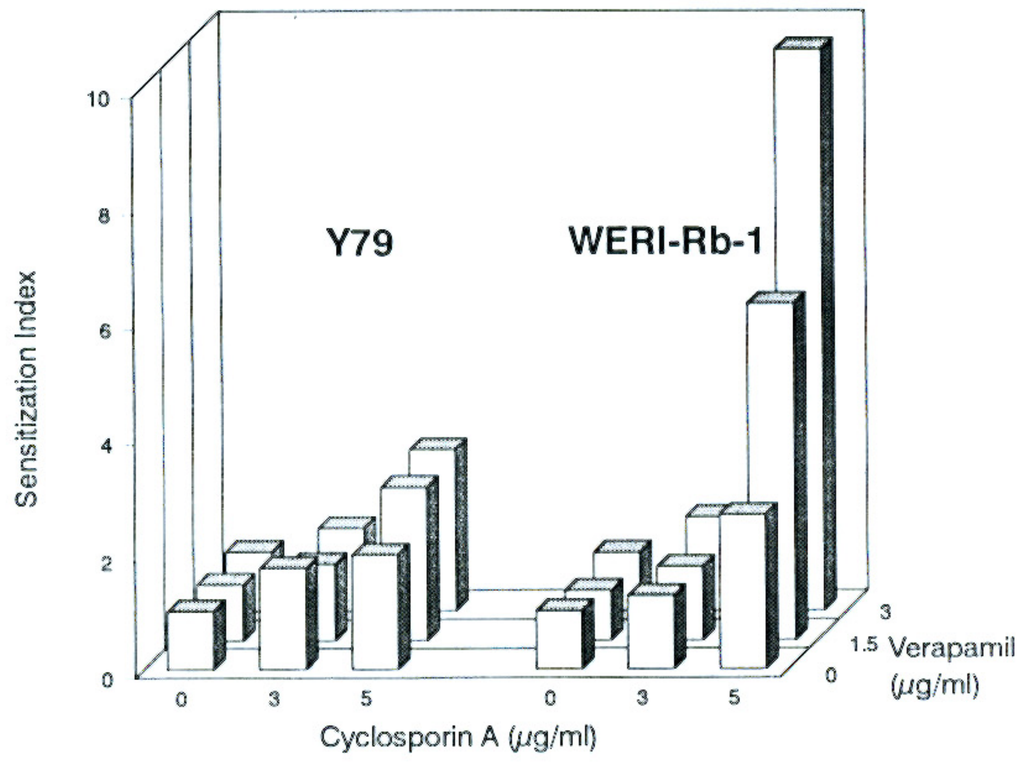

Fig. 4. Sensitization Index (SI) to adriamycin for Y79 and WERI-Rb-1, human retinoblastoma cell lines exposed to cyclosporin A and verampamil. SI was determined as the ratio of IC_{50} to adriamycin alone to IC_{50} to adriamycin in the presence of cyclosporin A and/or verapamil.

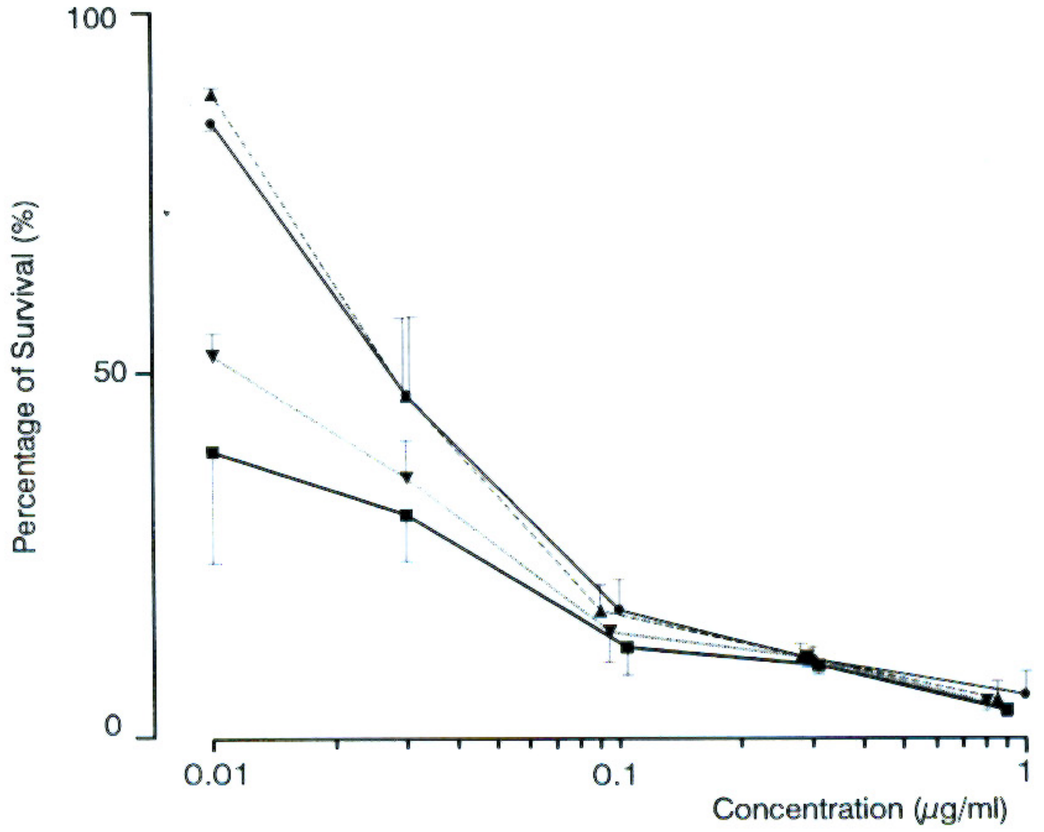

Fig. 3. Chemosensitization effects of cyclosporin A and verapamil on the cytotoxicity of adriamycin in WERI-Rb-1, a human retinoblastoma cell line: -●-, adriamycin alone: --▼-- adriamycin + 3.0μg/ml of cyclosporin A: ···▲···, adriamycin + 3.0μg/ml of verapamil: —■—, adriamycin + 3.0μg/ml of cyclosporin A + 3.0μg/ml of verapamil. Each point and bar respensents the mean and SD of three experiments.

not significantly inhibit the survival of Y79 and WERI-Rb-1 but the cytotoxicity of adriamycin is significantly enhanced by the combined use of cyclosporin A, being further enhanced by the addition of verapamil.

DISCUSSION

Despite much effort being paid to the development of potent chemotherapy regimens, the response rate still remains low and long-term prolongation of survival has not been achieved in patients with unresectable retinoblastoma (Shields, 1983; Murphree and Rother, 1989). To improve response and survival by chemotherapy, it is important to enhance the cytotoxicity of chemotherapeutic agents. A major aim of the present study was to investigate the chemosensitization effects of cyclosporin A and verapamil on the adriamycin-induced cytotoxicity against two human retinoblastoma cell lines, because adriamycin was known to be one of the most active agents for the treatment of retinoblastoma (White, 1983).

The resistance of cancer cells to chemotherapeutic agents is classified, in general, into two types, intrinsic resistance and acquired resistance. Intrinsic resistance means the innate insensitivity of cancer cells to chemotherapeutic agents, while acquired resistance is the resistance developed after continuing treatment despite initial efficacy. These two types of resistance are considered to be the major factors in chemotherapeutic failure.

The typical phenotype of acquired resistance to adriamycin is now established to be a multidrug resistance, which is associated with the hyperexpression of P-glycoprotein in cell membrane (Cornwell et al, 1987; Tsuruo et al., 1988). Hyperexpression of P-glycoprotein encoded by mdr-1 gene increases in drug efflux, *resulting in the decrease in intracellular accumula-*

tion of chemotherapeutic agents. There has been increasing evidence that multidrug resistance, such as adriamycin-induced acquired resistance, is partially or completely reversible by the addition of several drugs, such as cyclosporin A and verapamil, by binding P-glycoprotein hyperexpressed in cell membranes, although in some acquired resistant cell lines, the resistance develops by the expression of a low molecular weight, cytosolic, calcium binding protein, known as V19, CP22 or sorcin (Meyers and Biedler, 1981; Twentyman et al., 1990).

The mechanism of intrinsic resistance to adriamycin is not well understood at present. Several recent reports have shown that P-glycoprotein is constitutively *expressed at relatively low levels in some parent cell* lines that have never been exposed to chemotherapeutic agents (Kartner et al., 1985; Twentyman et al., 1990). These data suggest that P-glycoprotein express may also play a important role in the case of intrinsic resistance. If cyclosporin A and verapamil bind to P-glycoprotein innately expressed and inhibit the drug efflux, the sensitivity of cancer cells to chemotherapeutic agent may potentiated. On this supposition, this study was designed.

The enhancement of in vitro cytotoxicity of adriamycin was reported by cyclosporin A and verapamil in a human pulmonary adenocarcinoma cell line (Kim et al., 1990) and by recombinant human interferon-g and/or verapamil in a human stomach adenocarcinoma cell line (Hong et al., 1992). However, the chemosensitizing effects of cyclosporin A and verapamil on the cytotoxicity of adriamycin in retinoblastoma cells have not previously been reported. In the present study, we have demonstrated that the cytotoxicity of adriamycin was significantly enhanced by the addition of cyclosporin A and further enhanced by the combined use of cyclosporin A and verapamil.

To apply cyclosporin A and verapamil in the clinic, however, there are some problems. The clinical usefulness of these chemosensitizers mainly depends upon whether or not concentrations strong enough to modulate the sensitivity can be obtained in vivo without serious side effects, because cyclosporin A has an immunosuppressive activity and verapamil has an activity of lowering blood pressure. In this regard, the data presented here also gives useful information for developing analogues of cyclosporin A and verapamil, which have the same or more chemosensitizing activity without the adverse effects mentioned above.

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