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Cremophor EL modifies adriamycin-induced action on human leukemia K562 cells

Hiroko Matsui[1], Yoko Sakanashi[1], Yumiko Nishimura[1], Kanna Horimoto[1], Kaori Kanemaru[1], Shiro Ishida[2], Yoshiro Okano[2], Yasuo Oyama[1],*

[1] *Laboratory of Cell Signaling, Faculty of Integrated Arts and Sciences, The University of Tokushima, Tokushima 770-8502, Japan and* [2] *Department of Pharmaceutical Care and Clinical Pharmacy, Faculty of Pharmaceutical Sciences, Tokushima Bunri University, Tokushima 770-8512, Japan*

* *Correspondence: Yasuo Oyama, Ph.D.*

ABSTRACT

Adriamycin and paclitaxel are simultaneously used for cancer treatment in some cases. The formula of paclitaxel contains cremophor EL as a solvent. Since this solvent exerts diverse biological actions, the modification of adriamycin action by cremophor EL has been studied on human leukemia K562 cells. Cremophor EL did not significantly affect the concentration-response relation for antiproliferative action of adriamycin and the cell cycle changed by adriamycin. However, the induction of morphological change by adriamycin was significantly augmented by cremophor EL. The simultaneous application of cremophor EL increased the intensity of fluorescence from adriamycin trapped inside the cells in a concentration-dependent manner, suggesting an increase in intracellular concentration of adriamycin by cremophor EL. Adriamycin alone at concentrations higher than those to completely inhibit the growth induced morphological change in K562 cells. Therefore, cremophor EL may potentiate some of actions induced by adriamycin when adriamycin and paclitaxel are simultaneously applied.

Keywords: adriamycin; cremophor EL; cytotoxicity; human leukemia K562 cells

1. INTRODUCTION

Cremophor EL, a surfactant for pharmaceutical products, augments cytotoxicity of hydrogen peroxide (H_2O_2) in rat thymocytes (Iwase *et al.*, 2004). In brief, cremophor EL decreased cellular content of glutathione. Simultaneous incubation with cremophor EL and H_2O_2 significantly decreased the cell viability of rat thymocytes. Furthermore, the surfactant accelerated the process of cell death induced by H_2O_2. Results suggest that cremophor EL increases cell susceptibility to oxidative stress. In addition, cremophor EL increased population of dead cells in rat thymocytes simultaneously treated with A23187, a calcium ionophore increasing intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) (Yamaguchi *et al.*, 2005). Removal of Ca^{2+} from external solution diminished the cremophor EL-induced increase in dead cell population. Results show that Ca^{2+}-dependent process is involved in the cremophor EL-induced decrease in the cell viability under the simultaneous presence of A23187. Therefore, cremophor EL may affect the process of cell death induced by oxidative stress and excessive Ca^{2+} overloading (Iwase *et al.*, 2004; Yamaguchi *et al.*, 2005).

Cremophor EL is used as a vehicle or a stabilizer for formulation of antineoplastics, photosensitizers, immunosuppressives, sedatives, and anesthetics (for a review, Gelderblom *et al.*, 2001). Furthermore, it is also used for preparing colloidal systems such as microcapsules, microspheres, nanoparticles, or micromolecule complexes. Although this surfactant exerts a range of clinical adverse effects (Michaud, 1997; Szebeni *et al.*, 1998; Gelderblom *et al.*, 2001), it is expected that the agent may potentiate the cytotoxicity of adriamycin because of following reasons. First, adriamycin is capable of inducing oxidative stress and this capacity has been considered critical for its antiproliferative action (for a review, Keizer *et al.*, 1990). It is reasonable to suggest that cremophor EL enhances the adriamycin-induced cytotoxicity because it augments the cytotoxicity of H_2O_2 (Iwase *et al.*, 2004). Second, adriamycin induces apoptosis in normal and neoplastic cells (Skladanowski and Konopa, 1993; Anand *et al.*, 1995; Yu *et al.*, 1996; Azmi *et al.*, 1997; Bilim *et al.*, 1997; Gruber *et al.*, 2001). The membranes with exposed phosphatidylserine, proposed to occur during an early stage of apoptosis (Koopman *et al.*, 1994;

Vermes *et al.*, 1995), are vulnerable to the surfactants (Iwase *et al.*, 2004; Tatsuishi *et al.*, 2005; Yamaguchi *et al.*, 2005, 2006). Thus, there is a possibility that cremophor EL may promote the process of cell death induced by adriamycin. In the present study, therefore, we have examined how cremophor EL modifies the action of adriamycin by using human leukemia K562 cells. We think that such a study is important because in some cases adriamycin is used for cancer treatment with paclitaxel containing cremophor EL as a solvent (Millward *et al.*, 1998).

2. MATERIALS AND METHODS

2.1. Cell Preparation

Human leukemia K562 cells were cultured in 24-well Falcon tissue culture plates placed in a CO_2 incubator (Sanyo, Tokyo) at a temperature of 37°C. Each well contained 2 ml of RPMI 1640 medium with glutamine (300 mg/l) and 10% f*et al.* bovine serum (Sigma Chemical, St. Louis, MO, USA).

2.2. Cell Viability

Cytograms (forward-scatter intensity versus side-scatter intensity) were obtained from a programmed number (2000 or 2500 cells) of K562 cells with a flow cytometer (Cyto-ACE150, JASCO, Tokyo). Cell viability was estimated using the propidium iodide (Molecular Probes Inc., Eugene, OR, USA) staining method. Since propidium iodide is unable to stain living cells, the cells exhibiting propidium fluorescence are dead. The fluorescence was measured at 2 min after adding propidium iodide to medium to achieve a final concentration of 5 μM. Excitation wavelength for propidium was 488 nm. Emission was monitored at wavelength of 600 ± 20 nm. The cell viability in this study indicates the percentage population of cells not stained with propidium to total cells.

2.3. Estimation of Growth

The growth and viability of K562 cells were estimated by the use of erythrocytometer with trypan blue staining. In some experiments, the number of cells counted during a programmed time (30 s) with a flow cytometer was correlated with those estimated by erythrocytometer with a coefficient of 0.98 (Chikahisa *et al.*, 1998). Thus, the flow cytometric technique with propidium iodide also allowed estimation on the effects of the drugs on the growth and viability of cells in the medium.

2.4. Analysis of Cell Cycle

To determine the distribution of DNA content in K562 cells, DNA was stained with propidium iodide. DNA staining solution contained 0.1% Triton X-100 (Sigma Chemical), EDTA 3 mM (Katayama Chemical, Osaka, Japan), RNase A 0.05 mg/ml (Sigma Chemical) and propidium iodide 100 μM in phosphate buffer solution. The cells were suspended in DNA staining solution for at least 1 h at room temperature (Umebayashi *et al.*, 2003). Propidium fluorescence was measured in the cells using a flow cytometer.

2.5. Data Presentation and Statistics

Numerical values of experimental data are presented as the mean ± standard deviation (SD) in this study. Statistical analysis was performed using Tukey multivariate test. A P value of < 0.05 was considered significant.

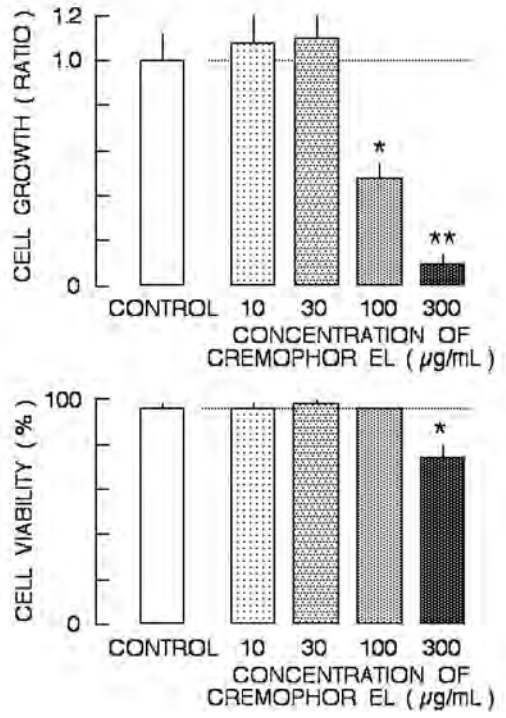

Figure 1 *Effects of cremophor EL on cell growth (upper panel) and viability (lower panel) of K562 cells. The cells were incubated with cremophor EL at concentrations ranging from 10 μg/mL to 300 μg/mL for 72 hr. Column and bar respectively indicate mean value and its standard deviation of 4-6 experiments. Symbols (* and **) show significant difference between control group and respective test groups. * P<0.05 and ** P<0.01.*

3. RESULTS

3.1. Effects of cremophor EL alone on viability and growth of K562 cells

To examine the effects of cremophor EL on viability and growth of K562 cells, the cells were incubated with the agent for 72 h. The concentrations of cremophor EL used ranged from 1 μg/mL to 300 μg/mL because the maximum blood concentrations were reported to be greater than 1000

μg/mL when patients received infusion of paclitaxel (Rischin *et al.*, 1996). The population of cells stained with trypan blue, indicating dead cells, was not significantly increased by the incubation with cremophor EL at 100 μg/mL or less for 72 h (Fig. 1). The threshold concentration of cremophor EL to increase the population of dead cells was 300 μg/mL under present experimental condition. However, the agent at 300 μg/mL significantly increased the population of dead cells to 95% or more of total cells (Fig. 1). Results suggest that cremophor EL at 100 μg/ml or less does not decrease the viability of K562 cells. As to the effect on the growth of K562 cells, the incubation with cremophor EL at 100 μg/mL or more for 72 h inhibited the growth in a manner dependent on concentration (Fig. 1). Thus, cremophor EL at 100 μg/mL seems to inhibit the growth without decreasing the viability.

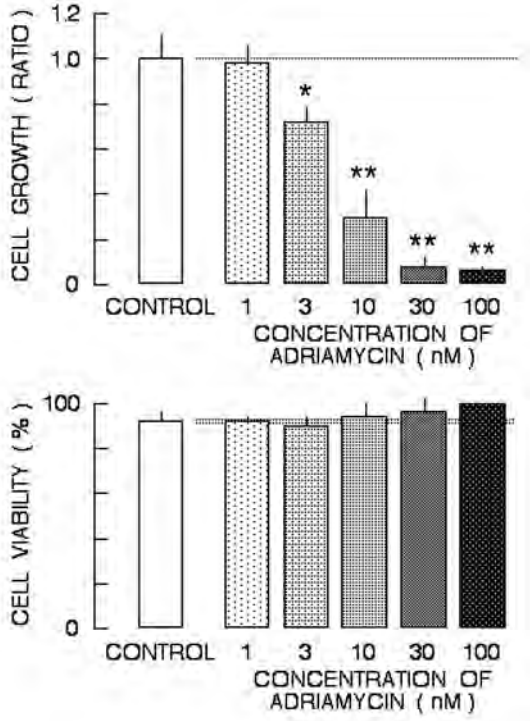

Figure 2 *Effects of adriamycin on cell growth (upper panel), and viability (lower panel) of K562 cells. The cells were incubated with adriamycin at concentrations ranging from 1 nM to 100 nM for 72 hr. Column and bar respectively indicate mean value and its standard deviation of 4-6 experiments. Symbols (* and **) show significant difference between control group and respective test groups. * P<0.05 and ** P<0.01.*

3.2. Effects of adriamycin alone on viability and growth of K562 cells

As shown in Fig. 2, adriamycin at concentrations ranging from 1 nM to 100 nM did not affect the viability. Adriamycin at 3 nM or more inhibited the growth in a concentration-dependent manner (Fig. 2). The agent at 30-100 nM almost-completely inhibited the growth. Therefore, adriamycin inhibited the growth of K562 cells without affecting the viability. Large cells appeared as the concentration of adriamycin increased (Fig. 3). The percentage population of cells with cell size larger than maximal size under control condition was significantly increased when the concentration was 30-100 nM.

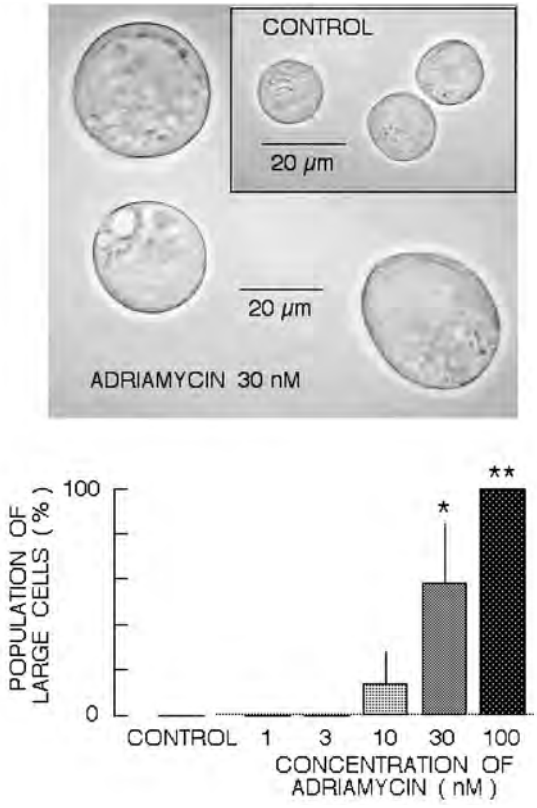

Figure 3 *Increase in population of large cells in K562 cells by adriamycin. The cells were incubated with adriamycin at concentrations ranging from 1 nM to 100 nM for 72 hr. Column and bar respectively indicate mean value and its standard deviation of 4-6 experiments. Symbols (* and **) show significant difference between control group and respective test groups. * P<0.05 and ** P<0.01.*

3.3. Effect of cremophor EL on antiproliferative action of adriamycin

As described above (Fig. 1), cremophor EL exerted antiproliferative action on K562 cells without affecting the viability when the concentration was 100 μg/mL. Further increase in the concentration (up to 300 μg/mL) decreased the cell viability. Therefore, the concentrations of 30 μg/mL (non-toxic) and 100 μg/mL were used to examine the effect of cremophor EL on the action of adriamycin. As shown in Fig. 4, although the dose-response relation for adriamycin-induced inhibition of cell growth was

not significantly affected, the tendency (as shown with # in Fig. 4) to augment the action of 10 nM adriamycin by 100 μg/ml cremophor EL was observed.

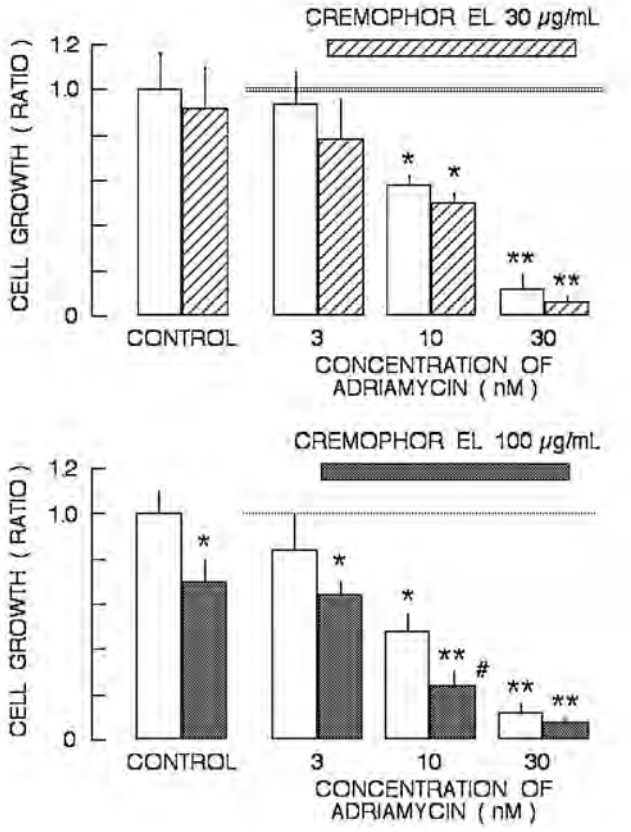

Figure 4 *Effect of adriamycin on cell growth of K562 cells in respective presence of 30 μg/mL (upper panel) or 100 μg/mL (lower panel) cremophor EL. The cells were incubated with adriamycin at concentrations ranging from 3 nM to 30 nM for 72 hr in the presence of cremophor EL. Column and bar respectively indicate mean value and its standard deviation of 4-6 experiments. Symbols (* and **) show significant difference between control group and respective test groups. * P<0.05 and ** P<0.01.*

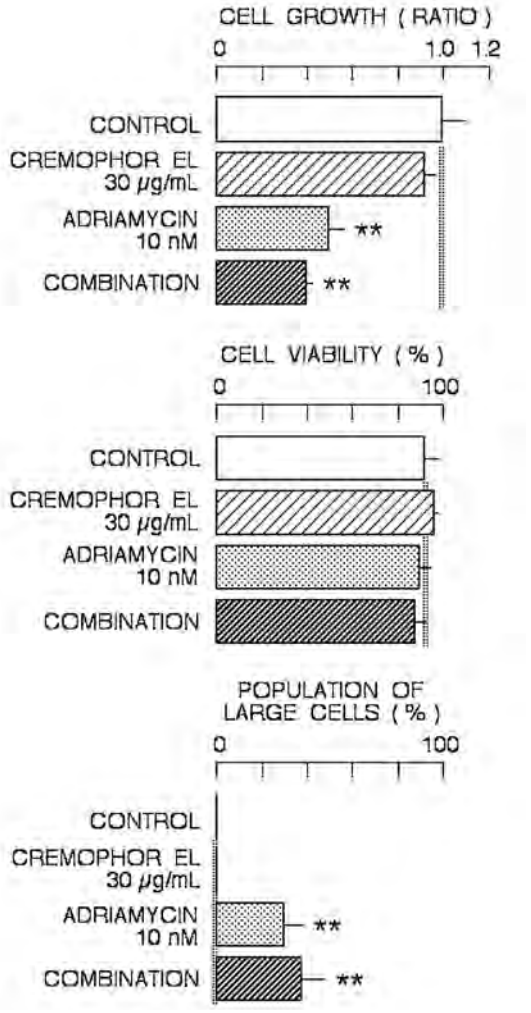

Figure 5 *Effects of 30 μg/mL cremophor EL alone, 10 nM adriamycin alone, and their combination on cell growth (upper panel), viability (middle panel), and population of large cells (lower panel) of K562 cells. The cells were incubated with agent(s) for 72 hr. Column and bar respectively indicate mean value and its standard deviation of 4 experiments. Symbol (**) shows significant difference between control group and respective test group. ** P<0.01.*

To confirm the tendency, the effect of 10 nM adriamycin on K562 cells in the presence of 30 μg/mL cremophor EL was further studied. As shown in Fig. 5, the cell viability was not affected by the simultaneous application of adriamycin and cremophor EL. Even the growth was further suppressed by the simultaneous application, it was not statistically significant, but cremophor EL seemed to have the tendency to augment the antiproliferative action of adriamycin. The percentage population of large cells was further, but slightly, increased in the simultaneous presence of two agents in comparison with that in the presence of adriamycin alone.

As to the effect on the cell cycle of K562 cells, 10 nM adriamycin induced G2M arrest while 30 μg/mL cremophor EL exerted no effect (Fig. 6). The profile of cell cycle in the simultaneous application of adriamycin and cremophor EL was similar to that in the application of adriamycin alone. From the results shown in Figs. 4, 5, and 6, it is unlikely that the simultaneous application of cremophor EL greatly modifies the antiproliferative action of adriamycin on K562 cells. Therefore, in our further experiments, we focused the effect of cremophor EL on morphological change in K562 cells induced by adriamycin.

3.4. Effect of cremophor EL on the adriamycin-induced increase in population of large cells

As shown in Fig. 7, under the microscopic observation, cremophor EL at concentrations ranging from 10 μg/mL to 300 μg/mL concentration-dependently increased the percentage population of large cells in the simultaneous application of 30 nM adriamycin. The increase was statistically significant in the case of 100-300 μg/mL (Fig. 7). To confirm this observation, the intensity of forward scatter monitored from the cells was compared in the groups of cells treated with adriamycin, cremophor EL, and their combination. The histogram of forward scatter intensity in the presence of 100 μg/mL cremophor EL alone was largely overlapped with the control histogram (Fig. 8). In the case of 30 nM adriamycin alone, the histogram was obviously shifted to a direction of higher intensity (Fig. 8), indicating an increase in cell size by adriamycin. The simultaneous application of 100 μg/mL cremophor EL with 10-30 nM adriamycin further shifted the histogram. Fig. 9 shows the mean intensity of forward scatter monitored from the cells treated with 100 μg/mL cremophor EL alone, 30 nM adriamycin, and their combination. The simultaneous application of cremophor EL at concentrations ranging from 1 μg/mL to 100 μg/mL increased the mean intensity of forward scatter monitored from the cells treated with 30 nM adriamycin in a concentration-dependent manner (Fig. 8). It is likely that cremophor EL augments the adriamycin-induced change in cell morphology.

3.5. Change in red fluorescence intensity measured from the cells incubated with cremophor EL, adriamycin, and their combination

As shown in Fig. 3, adriamycin increased the percentage population of large cells in a concentration-dependent manner. Therefore, the simultaneous application of cremophor EL may increase intracellular concentration of adriamycin. To test the possibility, the fluorescence of adriamycin trapped by the cells was compared in the absence and presence of cremophor EL. The mean intensity of fluorescence monitored from the cells increased as the concentration of adriamycin increased (Fig. 9). The mean intensity in the simultaneous presence of 10 nM or 30 nM adriamycin and 100 μg/mL cremophor EL was significantly higher than that in the presence of 10 nM or 30 nM adriamycin alone, respectively. Results may suggest that the intracellular concentration of adriamycin in the simultaneous application of adriamycin and cremophor EL is higher than that in the presence of adriamycin alone.

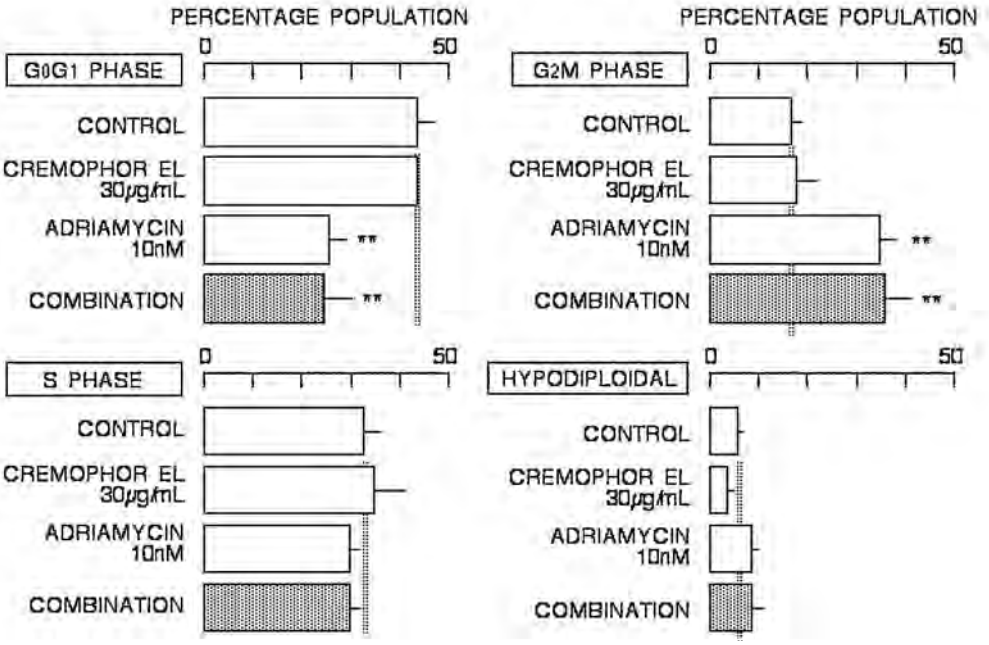

Figure 6 *Effects of 30 μg/mL cremophor EL alone, 10 nM adriamycin alone, and their combination on distribution of cellular DNA content of K562 cells. The cells were incubated with agent(s) for 72 hr. Column and bar respectively indicate mean value and its standard deviation of 4 experiments. Symbol (*) shows significant difference between control group and respective test groups. * P<0.05.*

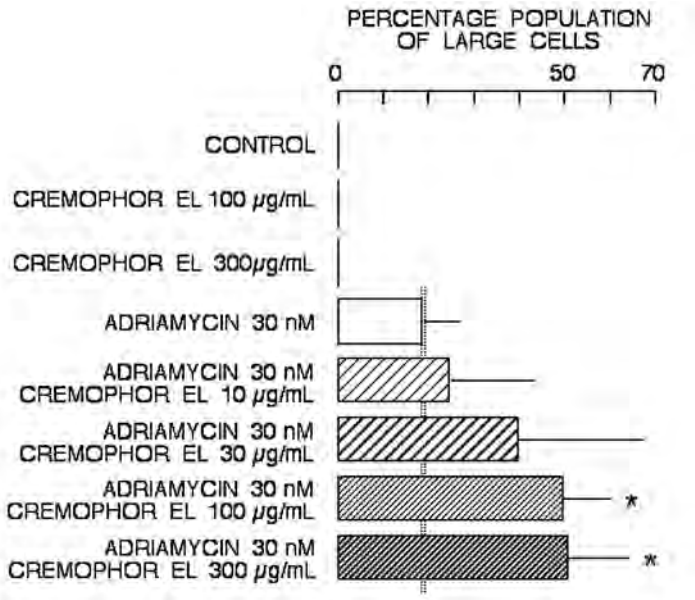

Figure 7 *Concentration-dependent change induced by cremophor EL in percentage population of large cells. K562 cells were respectively incubated with cremophor EL alone, adriamycin alone, or their combination for 72 hr. Column and bar respectively indicate mean value and its standard deviation of 4 experiments. Symbol (*) shows significant difference between control group and test groups. * P<0.05.*

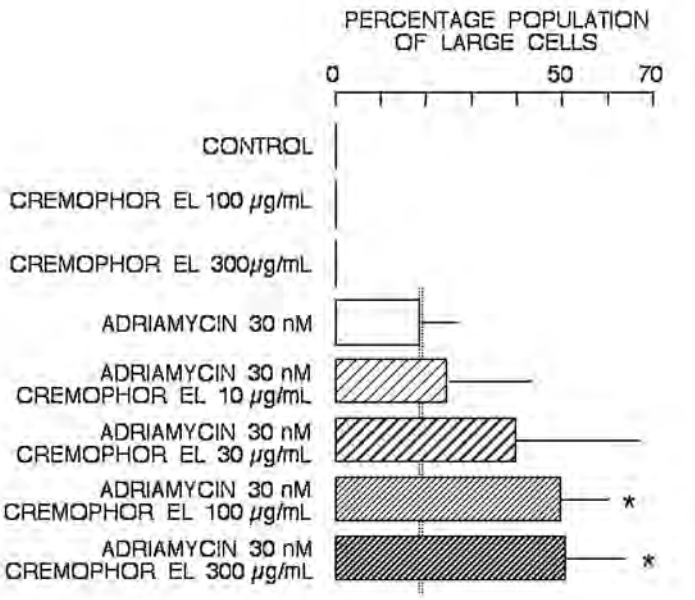

Figure 8 *Change in intensity of forward scatter (cell size) induced by cremophor EL alone, adriamycin alone, or their combination. K562 cells were treated with the agent(s) for 48 hr. Column and bar respectively indicate mean value and its standard deviation of 4 experiments. Symbols (*, **, and #) show significant difference respectively between control group and test group (* and **) and between the group treated with adriamycin alone and the group treated simultaneously with adriamycin and cremophor EL (#). * and # P<0.05, ** P<0.01.*

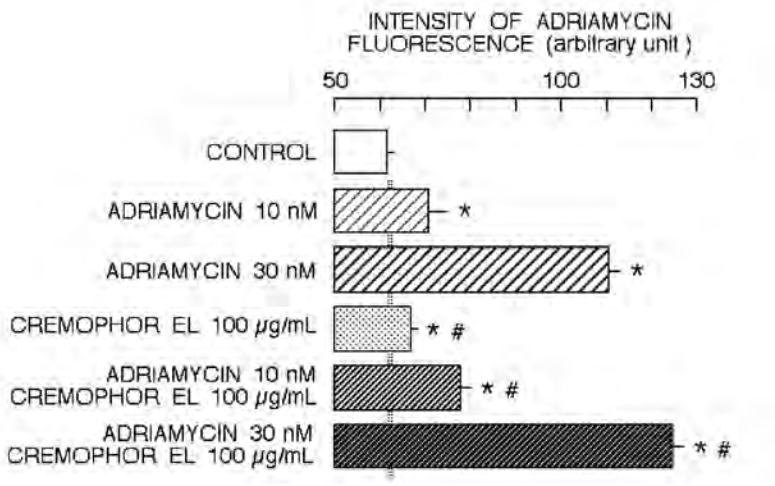

Figure 9 *Change in intensity of fluorescence monitored from the cells treated with adriamycin alone, cremophor EL alone, or their combination. Column and bar respectively indicate mean value and its standard deviation of 4 experiments. Symbols (* and #) show significant difference respectively between control group and test group and between the group treated with adriamycin alone and the group treated simultaneously with adriamycin and cremophor EL. * and # P<0.05.*

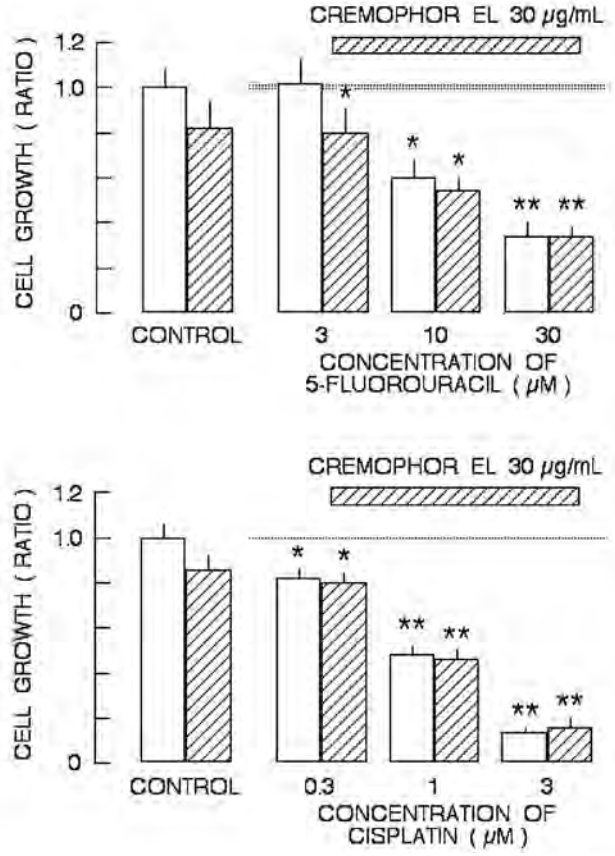

Figure 10 *Effects of 5-fluorouracil (upper panel) and cisplatin (lower panel) on cell growth of K562 cells in presence of 30 μg/mL cremophor EL. The cells were incubated with respective agent(s) for 72 hr. Column and bar respectively indicate mean value and its standard deviation of 4-6 experiments. Symbols (* and **) show significant difference between control group and respective test groups. * P<0.05 and ** P<0.01.*

3.6. Effects of 5-fluorouracil and cisplatin on K562 cells in the presence of cremophor EL

The effects of 30 μg/mL cremophor EL on antiproliferative action of 3-30 μM 5-fluorouracil and 0.3-3 μM cisplatin were also tested. As shown in Fig. 10, the simultaneous application of cremophor EL induced no significant change in the concentration- response relation for antiproliferative action of 5-fluorouracil and cisplatin.

4. DISCUSSION

Paclitaxel is used in a combination of adriamycin for some cancer treatments (Eng *et al.*, 2001; Hess *et al.*, 2003; Dreicer *et al.*, 2004; Briasoulis *et al.*, 2004). The formula of paclitaxel includes cremophor EL as a solvent. Cremophor EL exerts diverse biological actions (Michaud, 1997; Szebeni *et al.*, 1998; Gelderblom *et al.*, 2001). Therefore, it is reasonable to suggest the possibility that cremophor EL modifies some of adriamycin-induced actions on cancer cells.

Adriamycin induces oxidative stress and this is considered critical for its antiproliferative action (for a review, Keizer *et al.*, 1990). Cremophor EL accelerates the process of cell death induced by H_2O_2, an oxidative stress (Iwase *et al.*, 2004). Thus, cremophor EL was suggested to augment the antiproliferative action of adriamycin. However, in this study, cremophor EL did not significantly affect the concentration-response relation for antiproliferative action of adriamycin (Figs. 4 and 5) and the cell cycle changed by adriamycin (Fig. 6). Therefore, the suggestion is unlikely. The type of oxidative stress induced by adriamycin may be different from that induced by H_2O_2.

The induction of morphological change induced by adriamycin was significantly augmented by cremophor EL in a concentration-dependent manner (Figs. 7 and 8). This augmentation of adriamycin-induced change in morphology by cremophor EL is plausibly explained as follows. Adriamycin alone at concentrations higher than those to completely inhibit the growth induced morphological change in K562 cells (Fig. 3). The study on the fluorescence monitored from the cells treated with adriamycin alone, cremophor EL alone, or their combination revealed that the simultaneous application of cremophor EL and adriamycin increased the intracellular concentration of adriamycin (Fig. 9). Cremophor EL has been reported to reverse the multidrug resistance phenotype in vitro (Friche *et al.*, 1990; Woodcock *et al.*, 1990; Ross *et al.*, 1994) and to inhibit the elimination of etoposide in rat liver (Ellis *et al.*, 1996). Thus, cremophor EL probably exerts an inhibitory action on membrane pump that can eliminate adriamycin from the cells, resulting in an increased concentration of adriamycin inside the cells. Furthermore, cremophor EL at concentrations ranging from 3 μg/mL to 300 μg/mL concentration-dependently decreases the cellular content of glutathione in rat thymocytes (Iwase *et al.*, 2004). Glutathione has a role in cellular detoxification of xenotoxins. Therefore, the simultaneous application of cremophor EL and adriamycin can increase the population of large cells.

One may argue that the simultaneous application of cremophor EL and adriamycin should also augment the antiproliferative action of adriamycin if cremophor EL increases the intracellular concentration of adriamycin (Fig. 9). In fact, as shown in Figs. 4 and 5, the antiproliferative action by the simultaneous application of cremophor EL and adriamycin seemed to be more profound than that by adriamycin alone. It was obvious in the case of adriamycin with 100 μg/mL cremophor EL (Fig. 4). However, cremophor EL at 100 μg/mL itself exerted significant inhibitory action on the growth of K562 cells. Therefore, the augmentation of antiproliferative action by the simultaneous application cannot be simply explained by the increase in intracellular concentration of adriamycin by cremophor EL. The blood concentration of cremophor EL in cancer patients receiving infusion of anticancer agent was reported to be much higher than 100 μg/mL (Rischin *et al.*, 1996; Millward *et al.*, 1998). It can be suggested that cremophor EL may modify the cytotoxic action of adriamycin under clinical condition.

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