

Reversal of Resistance to Doxorubicin with Cepharanthine in Murine P388 Leukemia Cells

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Abstract—Cytotoxic effect in vitro and antitumor effect in vivo of doxorubicin (DOX) combined with cepharanthine were investigated on DOX-resistant murine P388 leukemia (P388/R) cells. Cepharanthine was minimally cytotoxic in the cell line, but reversed DOX-resistance in a dose-related manner in P388/R cells. The administration of cepharanthine to mice bearing the P388 leukemia enhanced the antitumor activity of DOX. These results indicate that cepharanthine is an effective agent to reverse DOX-resistant cells.

An anthracycline antitumor drug, doxorubicin (DOX), has been proven to be one of the most useful agents available in medical oncology. The emergence of acquired resistance to DOX represents a serious problem in the treatment of patients with cancer (1).

Cepharanthine, a biscochlorine alkaloid, has been known to affect cell membranes (2, 3) and alter the transmembrane movement of Ca^{2+} (4). Kato and Suzumura (5) have recently reported that cepharanthine overcomes vincristine resistance in a multidrug-resistance in murine leukemia cells in vitro.

In this study, we found that cepharanthine reversed resistance to DOX in a line of murine P388 leukemia cells selected for multidrug-resistance in vitro.

Cepharanthine and doxorubicin (DOX) were kindly provided by Kaken Shoyaku Co., Ltd., Tokyo, and Kyowa Hakko Kogyo., Ltd., Tokyo, respectively. Roswell Park Memorial Institute (RPMI) 1640 medium and its supplements were obtained from Nissui Co., Ltd., Japan; and fetal bovine serum (FBS) was purchased from GIBCO (Life Technologies, Inc., U.S.A.). DOX-sensitive (P388/S) cells were kindly supplied by Japanese Cancer Research Resources Bank, Tokyo, Japan.

P388 cells were maintained in suspension culture in RPMI 1640 medium supplemented with 10% FBS, 5×10^{-5} M 2-mercaptoethanol, 100 units per ml of penicillin, and 100 μg per ml of streptomycin. They were grown in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. DOX-resistant (P388/R) cells were selected by the method of Zijlstra et al. (6). The resistant cell line usually demonstrates a 30- to 40-fold resistance to DOX in soft agar. The effects of drugs on cellular proliferation were determined by seeding cells in culture at a concentration of 1×10^5 cells/ml. The drugs were freshly prepared in phosphate-buffered saline (PBS) and added to the cultures. The number of viable cells was determined by the trypan blue dye exclusion test in a hemacytometer. Each experiment was performed in triplicate. Intracellular DOX content was measured fluorometrically. The excitation wavelength was 470 nm and emission was collected at 585 nm (7). Female DBA/2 and C57BL/6 \times DBA/2 (BDF₁) mice were obtained from Japan SLC, Hamamatsu, Japan. They were housed in cages and supplied with a CE-2 pellet diet (Clea Japan, Inc., Japan) and water ad libitum. P388/R cells were passaged weekly through DBA/2 mice. Each dose of the drugs was evaluated in 6–10 mice. P388/R cells

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(10^6 cells in 0.1 ml PBS) were implanted i.p. into BDF₁ mice (18–22 g) on Day 0, and then DOX (0.5–2 mg/kg) and/or cepharanthine (5–10 mg/kg) was given i.p. on Days 3, 6, and 9 or Days 1–7. Antitumor activity was evaluated by the mean survival time for each group and also expressed by the T/C (mean survival time of the treated group divided by mean survival time of the control group) and T/D (mean survival time of the group treated with DOX and cepharanthine divided by the mean survival time of the group treated with DOX alone) values (percentage).

The cytotoxicity of cepharanthine on P388/S and P388/R cultured cells was examined. Cepharanthine inhibited the cell growth in a dose-dependent fashion. The 50% inhibitory concentration (IC₅₀) values of cepharanthine (1 hr-exposure) on the growth of P388/S and P388/R cells were 30 and 37 μ g/ml, respectively, whereas IC₅₀ values of continuous 48 hr-exposure of the same drug on the growth of P388/S and P388/R cells were 5.0 and 3.5 μ g/ml, respectively (data not shown). Figure 1A shows the IC₅₀ values of DOX with or without cepharanthine

against P388 cultured cells. Cepharanthine (0.1, 0.2, 0.5, or 1.0 μ g/ml) did not affect the cell growth, but these doses enhanced the cytotoxicity of DOX in a dose-dependent manner. The enhancement of DOX activity by cepharanthine was evident with all concentrations of DOX examined, and the IC₅₀ value of DOX decreased from 0.024 to 0.0016 μ g/ml on P388/S cells by 1 μ g/ml of cepharanthine. Against P388/R cells, the IC₅₀ value of DOX alone was 0.65 μ g/ml, but IC₅₀ values of DOX combined with cepharanthine doses of 0.1, 0.2, 0.5, and 1 μ g/ml were 0.34, 0.24, 0.14, and 0.12 μ g/ml, respectively. Thus, the enhancement of DOX activity depended on the dose of cepharanthine. The increase of DOX uptake was also found in P388/R cells treated with cepharanthine (0.1–1 μ g/ml, Fig. 1B). In a combination with cepharanthine (1 μ g/ml), the DOX uptake increased about 2.2-fold higher than that of DOX alone during the initial 1 hr after administration. In addition, we attempted to elucidate the effect of the combination of DOX and cepharanthine on the antitumor activity in tumor-bearing mice. As shown in Table 1, cepharanthine

Table 1. Effect of cepharanthine on the antitumor activity of DOX in P388/R-bearing mice

| Treatment (drugs and doses, mg/kg) | MST ^a (days) | T/C ^b (%) | T/D ^c (%) |
|---------------------------------------|----------------------------|-------------------------|-------------------------|
| (A) Saline (control) | 11.0±0.3 | 100 | |
| Cepharanthine, 10 | 11.0±0.5 | 100 | |
| DOX, 0.5 | 12.0±0.4 | 109* | 100 |
| DOX, 0.5 + Cepharanthine, 10 | 13.2±0.7 | 120** | 110 |
| DOX, 1 | 13.2±0.4 | 120** | 100 |
| DOX, 1 + Cepharanthine, 10 | 13.8±0.6 | 125** | 104 |
| (B) Saline (control) | 10.7±0.2 | 100 | |
| Cepharanthine, 5 | 11.0±0.2 | 103 | |
| DOX, 1 | 12.3±0.2 | 115** | 100 |
| DOX, 1 + Cepharanthine, 5 | 13.6±0.3 | 127** | 110# |
| (C) Saline (control) | 11.1±0.1 | 100 | |
| Cepharanthine, 10 | 11.5±0.2 | 104 | |
| DOX, 2 | 13.6±0.5 | 123** | 100 |
| DOX, 2 + Cepharanthine, 10 | 15.6±0.2 | 141** | 115## |

Groups of 6–10 mice were used. (A): Each group of BDF₁ mice was inoculated i.p. with 10^6 cells of P388/R cells on Day 0, and drugs were given i.p. on Days 3, 6, and 9. (B) and (C): P388/R (10^6 cells) were inoculated i.p. into BDF₁ mice on Day 0, and drugs were given i.p. daily from Days 1–7. Cepharanthine was administered 10 min before DOX administration. ^a: Mean survival time. Each value represents the mean and S.E. of 6–10 mice. ^b: Values calculated on the basis of the MST of the treated vs. control. ^c: Values calculated on the basis of the MST of the cepharanthine- and DOX-treated group vs. DOX alone group. Significantly different from the control group by Student's *t*-test, **P*<0.05, ***P*<0.01. Significantly different from the DOX alone group by Student's *t*-test, #*P*<0.05, ##*P*<0.01.

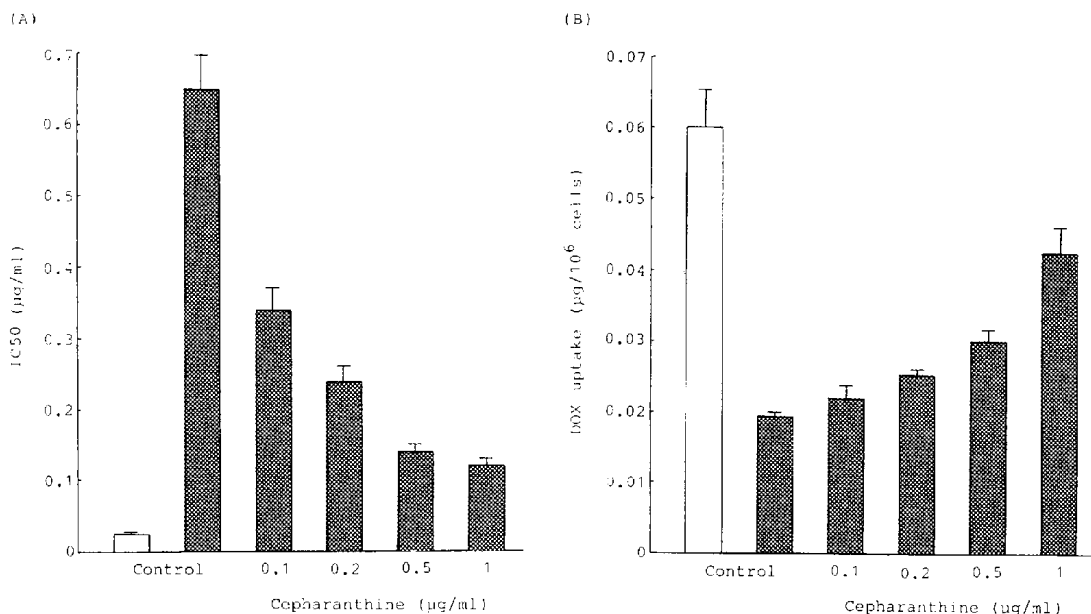


Fig. 1. Cytotoxic effects and intracellular accumulation of doxorubicin (DOX) on DOX-resistant P388 leukemia (P388/R) cells in the presence or absence of cepharanthine. (A): Cytotoxicity was measured using the dye exclusion test. DOX-sensitive P388 leukemia (P388/S, \square) cells were exposed to DOX (0.001–1 μ g/ml) for 48 hr in the absence of cepharanthine. P388/R (▨) cells were exposed to DOX (0.001–1 μ g/ml) for 48 hr in the presence or absence of cepharanthine. IC₅₀ values were determined from dose-response curves obtained from each concentration of compound. Each value represents the mean \pm S.E. of three experiments. (B): Intracellular DOX content was determined fluorometrically. P388/S (\square) and P388/R (▨) cells were exposed to DOX (1 μ g/ml) for 1 hr in the presence or absence of cepharanthine (0.1–1 μ g/ml). Each bar in the figure represents the means \pm S.E. of three experiments.

alone did not have antitumor activity in P388/R-bearing mice. However, the antitumor effect of DOX was promoted by the combination with cepharanthine. Namely, when cepharanthine (5 mg/kg, i.p.) was given in combination in a daily dosage for 7 days, DOX (1 mg/kg, i.p.) significantly increased the life span of the P388/R-bearing mice ($P < 0.05$, Table 1B). In this case, the mean survival time was prolonged from 12.3 to 13.6 days, T/C values rose from 115 to 127%, and the T/D value was 110%. Furthermore, the effect of the combination of cepharanthine (10 mg/kg, i.p.) and DOX (2 mg/kg, i.p.) on the life span of P388/R-bearing mice was examined in the same injection schedule (Table 1C). The mean survival time of the mice administered with DOX (2 mg/kg, i.p.) was significantly prolonged from 13.6 to 15.6 days (T/D value 115%, $P < 0.01$) by the combination with cepharanthine (10 mg/kg, i.p.). The synergistic effect of cepharanthine to DOX on

the prolongation of survival time observed in this study may result in part from enhancement of intracellular DOX accumulation in P388/R cells. On the other hand, the combined effect of cepharanthine and DOX in three times administration of drugs on Days 3, 5 and 9 after tumor inoculation was not observed (Table 1A). For the P388/S-bearing mice, the mean survival time of the animals treated with 1 mg/kg of DOX alone in the same drug injection schedule after tumor transplantation (10^6 cells) was prolonged from 11.2 to 15.8 days and the T/C value was 141%. From the results of the in vivo experiments, a long-term contact of drug to DOX-resistant cells in the coadministration schedule of DOX and cepharanthine is important for the potentiating action of cepharanthine. The optimum schedule of drug administration should be further investigated.

Recent pharmacological studies have reported the ability of several calcium channel

blockers, including verapamil and calmodulin inhibitors such as trifluoperazine, to reverse the resistance of chemotherapeutic drugs (8–10).

The drug-resistant cell line is known to have reduced intracellular drug accumulation associated with the overexpression of P-glycoprotein when compared to the sensitive parent cell line. The exact function of P-glycoprotein is unknown; however, recent studies have demonstrated drug binding to this protein, suggesting that it is involved in drug transport (11, 12). Shiraishi et al. (13) have demonstrated that the *in vitro* intracellular concentrations and retention of daunomycin in resistant cells can be restored by cepharanthine. Several other studies have indicated that resistance to DOX is related to decreased DNA damage and increased rapid DNA repair (8, 14).

In conclusion, this study demonstrated that cepharanthine, a membrane-active agent without antitumor activity, enhanced the cytotoxic effects of DOX in DOX-resistant P388 cells *in vitro* and *in vivo*. Hence, the authors think that potentiation of DOX activity by cepharanthine may be due to circumvention of multidrug resistance by inhibiting drug efflux and increasing intracellular accumulation of the drug.

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