Influence of Dosing Schedule on Toxicity and Antitumor Effects of a Combination of Adriamycin and Docetaxel in Mice

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ABSTRACT

Purpose: Although the combination of Adriamycin (ADR) and docetaxel (DOC) showed a better cure rate against metastatic breast cancer in a clinical study, severe myelosuppression and cardiotoxicity were dose-limiting factors. The purpose of this study was to establish the most suitable dosing schedule to relieve severe adverse effects and improve the antitumor effects.

Experimental Design: Both ADR and DOC were administered simultaneously in the simultaneous-dosing group (ADR/DOC), whereas in the intermittent-dosing groups (ADR-DOC and DOC-ADR), the second drug was administered 12 h after the first drug. Leukocyte counts and survival were measured to estimate adverse effects. After administration, ADR and DOC concentrations in blood, myelocyte cells, and heart were determined. To clarify the antitumor effect, tumor growth was measured in Ehrlich-cell-bearing mice after the initiation of drug injections.

Results: The simultaneous-dosing group showed severe leukopenia compared with the saline-treated group. However, the toxicity was reduced in the intermittent-dosing groups. The DOC-ADR group showed the best survival rate in the dosing groups. In the pharmacokinetic study, ADR and DOC concentrations in plasma, myelocyte cells, and the heart were markedly higher in the simultaneous-dosing group than the intermittent-dosing groups. These results indicate that pharmacokinetic interactions may contribute to the change in leukopenia induced by concurrent administration of ADR and DOC. The antitumor effect in the DOC-ADR group was the highest in the dosing groups.

Conclusions: In the present study, the findings suggest that ADR administered 12 h after DOC injection (DOC-ADR group) not only inhibits tumor growth more strongly but also significantly reduces leukopenia compared with results for the simultaneous-dosing (ADR/DOC) group and significantly reduced the number of toxic deaths compared with the other groups.

INTRODUCTION

Docetaxel (DOC), a semisynthetic taxane prepared from a noncytotoxic precursor extracted from the needles of the European yew tree Taxus baccata, is one of the most active chemotherapeutic agents. The cytotoxic effect of DOC is primarily attributable to its ability to promote tubulin assembly and inhibit microtubule depolymerization (1). DOC acts as a mitotic spindle poison, inducing a mitotic block in proliferative cells (2), and also induces apoptosis through Bcl-2 phosphorylation (3).

Anthracyclines have been used in clinical practice since the 1960s and represent one of the most commonly used classes of antitumor drugs. Adriamycin (ADR) was one of the first anthracyclines in clinical use, has a broad antitumor spectrum, and has been used against hematopoietic malignancies such as lymphoma, myeloma, and leukemia and solid tumors such as breast cancer, ovarian cancer, and sarcomas (4, 5). ADR was suggested to be the most effective agent for the management of breast cancer, and the inclusion of ADR in a combination regimen increases the response rate and the duration of survival (6).

In metastatic breast cancer, the polypharmacy is very effective, and a new combination therapy with ADR and DOC has been clinically attractive. Because of the differences between their mechanisms, the combination therapy of ADR and DOC is expected to enhance the antitumor effect (7, 8). In a clinical study, the combination therapy showed a better cure rate against metastatic breast cancer than the previous therapy. However, the combination of ADR and DOC induces severe myelosuppression (9). DOC is almost always administered as a 1-h infusion at 1 h or immediately after a bolus infusion of ADR. With this dosing schedule, myelosuppression is the dose-limiting factor. Approximately 90% of patients experienced severe (grade 3 or 4) neutropenia (10, 11). Therefore, despite its strong antitumor effect, combination therapy with DOC is often interrupted because of adverse effects, particularly myelosuppression. In addition, ADR-induced cardiotoxicity limits its clinical use and threatens the cardiac function of many patients with cancer (12).

To relieve the adverse effects of the two-drug combination of ADR and DOC would be beneficial to safe chemotherapy. Although many studies have been conducted, dosing schedules with respect to the dosing interval and the dosing sequence between ADR and DOC injections have not been studied in
The purpose of this study was to establish the most suitable dosing schedule (dosing interval and dosing sequence) to relieve the severe adverse effects. We investigated the influence of dosing schedule on the adverse effects induced by ADR and DOC in mice and the influence of dosing schedule on the pharmacokinetics of ADR and DOC to clarify the mechanisms underlying the dosing-schedule-dependent pharmacological actions. In addition, we investigated the influence of dosing schedule on the antitumor effects.

MATERIALS AND METHODS
Animals and Tumor Cell Line. Male ICR mice (6 weeks of age) were purchased from Charles River Japan, Inc. (Kanagawa, Japan). Mice were housed 6–10/cage under standardized light–dark cycle conditions (lights on and off at 7:00 a.m. and 7:00 p.m., respectively) at a room temperature of 24°C ± 1°C and humidity of 60 ± 10% with free access to food and water. Experiments were performed after formal approval by the Institutional Ethical Committee for Research on Animals. Ehrlich cells, which are a murine mammary carcinoma, were obtained from Cell Resource Center for Biomedical Research, Tohoku University.

Drugs and Dosing Schedule. ADR, supplied by Kyowa Hakko Kogyo Co. Ltd. (Tokyo, Japan), was dissolved in saline. DOC (Taxotere), supplied by Chugai Pharmaceutical Co. Ltd. (Tokyo, Japan) and Aventis Pharma Ltd. (Tokyo, Japan), was dissolved in 95% ethanol and diluted with 5% glucose solution. ADR (5.0 mg/kg) and DOC (12.5 mg/kg) were injected i.p. Mice were divided into a simultaneous-dosing group (ADR/DOC), in which both drugs were administered simultaneously; intermittent-dosing groups (ADR-DOC and DOC-ADR), in which the second drug was administered 12 h after the first drug injection; and a saline-treated group (control group).

Measurement of Leukocyte Counts. After the drugs or saline were administered to the four groups (n = 10–15), blood samples were drawn by orbital sinus collection on days 3 and 6, and leukocyte counts were measured.

Determination of Tolerance (Survival). To study toxic death, a combination of ADR and DOC or ADR alone was administered i.p. every 7 days (total of 20 mg/kg ADR and 50 mg/kg DOC; n = 22–29). Survival time was recorded for 35 days in each mouse.

Measurement of Pharmacokinetics. Blood samples were drawn from the hearts of mice anesthetized with ether at 0.5, 1, 2, 4, and 6 h after ADR was administered to the three ADR-treated groups (n = 4–12) and at 0.25, 0.5, 1, 2, 4, and 8 h after DOC was administered to the three DOC-treated groups (n = 5–8). All blood samples were centrifuged immediately at 16,000 × g for 15 min, after which the plasma was removed and frozen at −20°C until assayed. Mice were killed at 2 h after ADR or DOC injection in each dosing, and femura (n = 4 or 5) and hearts (n = 10–12) were removed. The femora were flushed with 2 ml of PBS(−) per bone, and the suspension in myelocyte cells was centrifuged at 800 rpm for 5 min at 4°C. The pellets were washed with PBS(−), resuspended in PBS(−) to 1 × 107 cells/ml, and frozen at −20°C until assayed. The hearts were frozen at −80°C until assayed.

ADR concentrations were quantified by a high-performance liquid chromatography system according to a previously published method (13). Plasma (100 μl) or myelocyte cells (1 × 107 cells) were mixed with 1 ml of Colihoff buffer, 0.1 ml of solution buffer [0.01 M phosphate buffer (pH 3.0):methanol (1:1, v/v)], 0.1 ml of the internal standard (epirubicin), and 4 ml of extraction solvent ethyl acetate:2-nbutanol (4:1, v/v). The hearts (0.12 g) were homogenized with the solution described above. These samples were centrifuged at 3000 rpm for 10 min, and the organic phase was dried with Speed Vac Plus SC110A (Savant Instruments, Inc., New York, NY). The residue was resuspended in 300 μl of solution buffer. The solution (150 μl) was injected into the high-performance liquid chromatography system.

DOC concentrations were measured by high-performance liquid chromatography according to the previously published method (14). The mixture of 0.4 ml of plasma and 50 μl of internal standard (paclitaxel) was applied to a C2 (1.0 ml) Bond Elute microcolumn (Varian, Harbor City, CA) that was pre-treated with 1.0 ml of methanol and 1.5 ml of water. The plasma containing was eluted with 1.0 ml of water and 1.0 ml of 50% methanol, and DOC was eluted in 0.3 ml of 90% methanol. The mixture of myelocyte cells (1 × 107 cells), internal standard, and 3 ml of chloroform was mixed for 2 min and centrifuged at 3000 rpm for 5 min. The top aqueous layer was aspirated, and the bottom organic layer was transferred to a new tube and dried in a Speed Vac Plus SC110A. The residue was resuspended in methanol:water (1:1, v/v). The drug was extracted from the suspension by the solid-phase extraction described above. The isolate (150 μl) was injected into the high-performance liquid chromatography system.

Determination of Antitumor Effect. ADR and DOC were injected i.p. in the ADR/DOC (n = 18), ADR-DOC (n = 17), DOC-ADR (n = 17), and saline (n = 9) groups 3 days after mice received s.c. inoculations containing 5 × 106 Ehrlich cells. After the initiation of drug injections, the tumor weight was measured for 7 days according to the after equation: tumor weight = A × B2/2, where A is the longer and B is the shorter diameter (mm). Relative tumor growth rate was expressed as the change in the tumor volume after the initiation of ADR and DOC injections. The antitumor activity of the treatments was also evaluated in terms of inhibition rate (IR), which was calculated as IR (%) = (1 – Rt/Rc) × 100, where Rt is the relative tumor growth rate in each drug-treated mouse on day 7, and Rc is the mean relative tumor growth rate in the control group on day 7.

Statistical Analysis. The survival days were plotted with the Kaplan–Meier method and compared by the log-rank test. Inhibition of tumor growth is expressed as the mean ± SD, and other values are shown as the mean ± SE. The ADR and DOC concentrations in plasma were collected using the naive averaging of data method, and the pharmacokinetic parameters were calculated with moment analysis. Groups were compared by one-way ANOVA, and differences between groups were determined by Scheffe’s test. P < 0.05 was considered to be significant.

RESULTS
Influence of Dosing Schedule on Leukocyte Counts. The leukocyte counts were measured on days 3 and 6 after ADR (5 mg/kg i.p.) and DOC (12.5 mg/kg i.p.) were administered
simultaneously (ADR/DOC) or intermittently (ADR-DOC and DOC-ADR; Fig. 1). On day 3, the leukocyte counts were significantly decreased in the three dosing groups (ADR/DOC, ADR-DOC, and DOC-ADR) compared with the control group ($P < 0.01$, $P < 0.01$, and $P < 0.05$, respectively). In addition, the leukopenia was more severe in the ADR/DOC group than in the ADR-DOC and DOC-ADR groups ($P = 0.056$ and $P < 0.05$, respectively). On day 6, the leukocyte counts were significantly decreased in the ADR/DOC group compared with the control group ($P < 0.05$). However, there were no significant decreases in the ADR-DOC and DOC-ADR groups compared with the control group.

**Influence of Dosing Schedule on Toxic Death.** When the combination of ADR and DOC or ADR alone was administered every 7 days, the deceased mice showed marked accumulation of ascites. The DOC-ADR group showed a significantly higher survival rate compared with the ADR/DOC, ADR-DOC, and ADR-alone groups ($P < 0.01$ for all), and the survival rates on day 35 were 86.2% in the DOC-ADR group, 40.9% in the ADR-alone group, 34.5% in the ADR-DOC group, and 22.2% in the ADR/DOC group (Fig. 2). However, there was no significant change in the survival rate among the ADR-alone, ADR/DOC, and ADR-DOC groups.

**Influence of Dosing Schedule on Pharmacokinetics in Plasma.** When ADR and DOC were administered simultaneously or intermittently, the plasma ADR concentrations at 2 and 4 h after ADR injection showed a significant increase in the ADR/DOC group compared with the ADR-DOC group ($P < 0.05$ and $P < 0.01$, respectively; Fig. 3). The plasma ADR concentrations in the ADR/DOC group showed a significant increase at 4 h compared with that in the DOC-ADR group ($P < 0.01$); however, there was no significant change in the ADR concentrations between the ADR-DOC and DOC-ADR groups. The area under the plasma–time concentration curve (AUC) for ADR increased by $\sim 30\%$ in the ADR/DOC group compared with the ADR-DOC and DOC-ADR groups.

Although there were no significant changes in the DOC concentrations in the ADR/DOC and ADR-DOC groups compared with the DOC-ADR group, plasma DOC concentrations after DOC injection tended to increase in combination with
ADR irrespective of the dosing schedule (simultaneous or intermittent; Fig. 3). The AUC for DOC was 39 and 49% higher in the ADR/DOC and ADR-DOC groups than in the DOC-ADR group. These results corresponded to the results for the ADR concentrations in plasma. The DOC concentrations increased by 61% in the ADR-DOC group compared with the DOC-ADR group, although the difference was not significant.

**Influence of Dosing Schedule on ADR Concentrations in Myelocyte Cells.** When ADR and DOC were administered simultaneously or intermittently, the ADR concentrations in myelocyte cells after ADR injection were 69 and 64% higher in the ADR/DOC group than in the ADR-DOC and DOC-ADR groups, respectively (Fig. 4). ADR concentrations in the DOC-ADR group were the same as those in the ADR-DOC group. These results corresponded to the results for the ADR concentrations in plasma. DOC concentrations in myelocyte cells in the ADR/DOC group after the DOC injection were twice as high as those in the DOC-ADR group \((P < 0.05)\). These results also corresponded to the results for the DOC concentrations in plasma. The DOC concentrations increased by 61% in the ADR-DOC group compared with the DOC-ADR group, although the difference was not significant.

**Influence of Dosing Schedule on ADR Concentrations in the Heart.** When ADR and DOC were administered simultaneously or intermittently, the ADR concentrations in the heart 2 h after ADR injection were significantly higher (28 and 33%) in the ADR/DOC group than in the ADR-DOC and DOC-ADR groups \((P < 0.05\) and \(P < 0.01\), respectively; Fig. 5); however, there was no significant difference between the ADR-DOC and DOC-ADR groups.

**Influence of Dosing Schedule on Tumor Growth.** When ADR and DOC were administered simultaneously or intermittently, tumor growth was significantly inhibited in the DOC-ADR group compared with the control group \((P < 0.05;\) Fig. 6), and the other dosing schedules tended to inhibit tumor growth compared with the control group. On day 7, inhibition of tumor growth in the DOC-ADR group was twice as high as that in the ADR/DOC group \((P < 0.05)\).

**DISCUSSION**

Leukocyte counts after the co-administration of ADR and DOC showed a significant dosing-schedule-dependent change. When the two drugs were injected simultaneously, the leukocyte counts on day 3 decreased to 65% of values for the saline group. However, when the two drugs were injected intermittently, the leukocyte counts on day 3 were maintained at ~80% of the values for the saline group. The simultaneous-dosing group showed markedly severe leukopenia compared with the intermittent-dosing groups. On day 6, the recovery rate in leukocyte counts was 1.57-fold higher in the simultaneous-dosing group compared with the intermittent-dosing groups, and there were no differences in leukocyte counts among the drug-treated groups. However, the simultaneous-dosing group had significantly lower leukocyte counts than the control group. Therefore, a longer time was required for leukocyte counts to return to normal when the two drugs were injected simultaneously. These results suggest that the dosing schedule with a 12-h interval between ADR and DOC injections reduces leukopenia compared with the dosing schedule without an interval between each injection, which is used commonly in clinical practice.

Survival on day 35 was markedly increased (51.7 and 64.0%) in the DOC-ADR group compared with the ADR-DOC and ADR/DOC groups, respectively. Moreover, survival rate for the DOC-ADR group was 45.3% higher than in the ADR-alone group, although mortality was higher in the combination groups.
except for the DOC-ADR group, than the ADR-alone group. These findings reveal that ADR-induced death is decreased markedly by a slight change in dosing sequence and dosing interval. In the present study, because the dosing interval was investigated only with a 12-h interval, it is uncertain whether this interval is necessary. However, previous clinical studies used protocols where DOC was almost always administered as a 1-h infusion at 1 h or immediately after a bolus infusion of ADR, and none of the authors of these studies reported that making the dosing interval longer would be useful for the concurrent administration of ADR and DOC (15, 16). Thus, clinical studies should be undertaken to examine the adverse effects and antitumor effects in protocols using intervals of 6–24 h based on the 12-h conditions.

ADR-induced myocardial damage is irreversible and lethally toxic, although the toxicity depends on the dosing schedule and total dose, and the cumulative dosage of 15–20 mg/kg produces high mortality and accumulation of ascites, decreases body weight gain, and depresses cardiac function in rats (17, 18). Although the exact mechanisms underlying ADR-induced cardiac damage are not fully understood, composite factors may contribute to the cardiotoxicity (19, 20). At present, the use of an antioxidant significantly prevents the ADR-induced cardiotoxicity (21). Oxygen radical-induced injury of membrane lipids was suggested to be the most important factor responsible for the development of ADR-induced cardiotoxicity. We also reported that the concentration of lipid peroxide (LPO), which is a factor in ADR-induced congestive heart failure, differs with dosing time and that the daily variation in LPO coincides with that in ADR-induced toxic death (13). In the preliminary study, an increase in LPO was observed in mice treated repeatedly with both ADR and DOC at the same time compared with mice treated repeatedly with ADR alone. There was no significant difference in LPO between the DOC-ADR and ADR-alone
groups; however, no increase in LPO was observed in the ADR-DOC group, which showed an equally high mortality similar to the mortality for the ADR/DOC group compared with the DOC-ADR and ADR-alone groups (data not shown). From these findings, a clear mechanism for the toxic death caused by the combination of ADR and DOC has not been found. However, it is clear from the present study that the dosing interval and dosing sequence were important factors in the decrease in adverse effects compared with the concurrent administration of ADR and DOC. These findings may help in improving the safety of continuous long-term use of this combination therapy.

The mechanism(s) underlying the dosing-schedule-dependent leukopenia and survival was investigated from a pharmacokinetic viewpoint. In a clinical study, the AUC of DOC increased in the combination of ADR and DOC (22). Moreover, increases in the AUC of DOC or ADR were closely associated with a decrease in leukocyte counts in patients treated with DOC or ADR (23, 24). In the present study, the plasma AUC and concentration in myelocyte cells of both ADR and DOC increased 1.3–2-fold in the simultaneous-dosing group compared with the intermittent-dosing groups, and the leukopenia in the simultaneous-dosing group was more severe than that in intermittent-dosing groups. Therefore, increases in the AUC of both ADR and DOC appear to be among the mechanisms underlying the severe leukopenia after simultaneous injection of both drugs. On the other hand, severe leukopenia was not observed in the ADR-DOC group compared with the DOC-ADR group despite the increase in the AUC and the concentration in myelocyte cells of DOC. However, leukopenia in rats was more severe in the ADR-DOC group than the DOC-ADR group on day 27 after the combination of ADR and DOC was administered every 7 days (the fourth administration) in the preliminary study (data not shown). Thus the sequence of ADR and DOC injections also appears to be important for relieving myelosuppression during the repeated treatment, although it was not suggested by the results obtained with a single treatment of ADR and DOC in the present study.

Higher concentrations of ADR in the heart were observed in mice treated with ADR and DOC simultaneously, which supported previous findings (25). ADR stimulates mitochondrial superoxide formation in a dose-dependent manner in vitro (26). The high concentration of ADR in the heart induced by the simultaneous injection of ADR and DOC appeared to cause high cardiotoxicity and high mortality; however, there was no significant difference in ADR concentrations between the intermittent-dosing groups despite the ~50% difference in mortality.

Fig. 6 Influence of dosing schedule on tumor growth after a combination injection of Adriamycin (ADR) and docetaxel (DOC) in mice inoculated with Ehrlich tumor cells. ADR (5 mg/kg i.p.) and DOC (12.5 mg/kg i.p.) were administered simultaneously (ADR/DOC; ■), or intermittently [ADR-DOC (△) and DOC-ADR (▲)]. Saline was administered in the control group (●). Relative tumor weight (mean; above) and inhibition rate [mean ± SE (bars; below)] were calculated as described in "Materials and Methods." *P < 0.05, Scheffe’s test. The DOC-ADR group showed significant inhibition of tumor growth compared with the control group (P < 0.05). On day 7, the DOC-ADR group showed the highest inhibition in the dosing groups, and the inhibition rate was increased ~1.4–2-fold.
between the two groups. Therefore, the ADR level in the heart may not contribute directly to toxic death.

In the present study, the antitumor effects in mice inoculated with Ehrlich tumor cells, which are derived from mammary gland, were best in the DOC-ADR group and worst in the ADR/DOC group. The antitumor effects did not depend on increases in the plasma concentration produced by co-administration of ADR and DOC. It has been reported that the antitumor effect of co-administered ADR and DOC is highly dependent on the dosing schedule in vitro in human breast cells and that the exposure of tumor cells to ADR after DOC shows an inhibitory effect on tumor cell death with inhibition of mitotic arrest and apoptosis, although the exposure of tumor cells to ADR simultaneously with or before DOC could lead to pronounced antag-

In conclusion, the present results suggest that the leukopenia induced by the two-drug combination of ADR and DOC varies according to dosing interval and that changes in this toxicity correspond to dosing-schedule-dependent changes in the pharmacokinetics of both drugs. Although no clear mechanism is known for the toxic death, the mortality revealed the marked dosing-sequence-dependent difference underlying the 12-h dosing interval. The DOC-ADR group showed the highest inhibition of tumor growth among the dosing groups. These results indicate that different mechanisms probably participate in the leukopenia, toxic death, and antitumor effects produced by co-administration of ADR and DOC. The findings of the present study suggest that the DOC-ADR group, in which ADR was administered 12 h after DOC injection, had significantly reduced leukopenia compared with the ADR/DOC group, significantly lower toxic death compared with the ADR/DOC and ADR-DOC groups, and the highest antitumor effects among the dosing groups. Therefore, choosing the optimum dosing schedule (dosing interval and dosing sequence) may lead to safe and effective chemotherapy with combinations of ADR and DOC.

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