Therapeutic Index by Combination of Adriamycin and Docetaxel Depends on Dosing Time in Mice

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Abstract

Although the combination of adriamycin and docetaxel showed a better cure rate against metastatic breast cancer, severe myelosuppression and cardiotoxicity were dose-limiting factors. The purpose of this study was to establish a suitable dosing schedule, based on a chronopharmacologic approach, to relieve severe adverse effects. In experiment 1, adriamycin or docetaxel was injected i.p. at 2, 6, 10, 14, 18, or 22 hours after light onset (HALO) to estimate toxicities. In experiment 2, the dosing time dependency of toxicity and pharmacokinetics were assessed in the combination of adriamycin and docetaxel. In addition, G2-M phase in myelocyte cells was determined in nontreated mice. Adverse effects caused by adriamycin were shown to be the worst at 2 HALO and the best at 14 HALO. On the other hand, docetaxel-induced adverse effects were more severe at 14 HALO than at 2 HALO. In the combination study, the D(2)-A(14) group, in which docetaxel was administered at 2 HALO followed by adriamycin at 14 HALO, showed the most toxicity relief of all the treated groups. In the pharmacokinetic study, the dosing time dependency of toxicities was not related to the daily variation of pharmacokinetics of adriamycin and docetaxel. A significant 24-hour rhythm of G2-M phase distribution was found in myelocyte cells of nontreated mice. The daily variation of leukopenia caused by docetaxel corresponded to the 24-hour rhythm of G2-M phase distribution. These findings reveal that the therapeutic index of the combined chemotherapy can be improved by administering adriamycin and docetaxel at the time when the most adverse effects are relieved in each drug.

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Introduction

Metastatic breast cancer is a major public health problem for women and is almost always incurable (1). Whereas the median survival is ~18 to 24 months, survival ranges from a few weeks to several years (2). The management of metastatic breast cancer is a major clinical challenge for medical oncologists. To achieve prolongation of survival and improvement of quality of life, many drugs, such as hormonal, humanized antibody, and chemotherapy agents have been studied clinically in patients with metastatic breast cancer (3–5). Few (<10%) patients, however, remain disease-free beyond 5 years (6). There is, thus, no definite consensus on the effects of these treatments.

For metastatic breast cancer, anthracycline-containing regimens have proven superior to regimens that do not include anthracyclines in randomized clinical trials (7, 8). Therefore, over the last few decades, anthracycline therapy has been the backbone of palliative regimens for patients with metastatic breast cancer. In metastatic breast cancer, the polypharmacy is very effective and a new combination therapy with adriamycin and docetaxel has been clinically attractive. Due to the differences between their mechanisms, the combination is expected to enhance the antitumor effect (9, 10). In clinical studies, the combination therapy showed a better cure rate against metastatic breast cancer than the previous therapy. However, the combination of adriamycin and docetaxel induced severe myelosuppression and increased adriamycin-induced cardiotoxicity, limiting its clinical use in many patients with metastatic breast cancer (11, 12).

To relieve the adverse effects of the two-drug combination of adriamycin and docetaxel would be beneficial for safe chemotherapy. Many clinical studies with respect to the dosage and dosing sequence have examined this combination (13, 14). In these studies, docetaxel is almost always administered as a 1-hour infusion at 1 hour or immediately after a bolus infusion of adriamycin. The bolus and short-infusion regimens are associated with a high incidence of adverse effects. In our previous study in mice, we revealed that the dosing schedule in which adriamycin is administered 12 hours after docetaxel injection not only significantly reduced the leukopenia and toxic death but also significantly increased the inhibition rate of tumor growth compared with the dosing schedule without an interval between each injection used commonly in clinical practice (15).

Moreover, many attempts have been made to decrease the adverse effects induced by antitumor drugs, and one such approach has been the chronopharmacologic approach. Chronotherapy is defined as the administration of medications using biological rhythms to optimize the therapeutic outcomes and/or control adverse effects. To decrease adverse effects, many antitumor drugs have been particularly studied in humans and animals (16–19). The toxicities of adriamycin and docetaxel have also been found to depend on dosing time in animals and humans (17, 20–24).

The purpose of this study was to establish the most suitable dosing schedule to relieve severe adverse effects by adding a chronopharmacologic approach to the combination chemotherapy of adriamycin and docetaxel suggested by our previous study.

Materials and Methods

Animals and tumor cell line. Male ICR mice (6 weeks old) were purchased from Charles River Japan, Inc. (Kanagawa, Japan). Mice were housed 6 to 10 per cage under standardized light-dark cycle conditions (lights on and off at 07:00 a.m. and 7:00 p.m., respectively) at room temperature (24 ± 1°C) and at a humidity of 60 ± 10% with free access to food and water. Experiments were done after formal approval by the Institutional Ethical Committee for Research on Animals. Ehrlich cells,
which are murine mammary carcinomas, were obtained from the Cell Resource Center for Biomedical Research, Tohoku University.

**Preparation of dosing drug.** Adriamycin, supplied by Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan), was dissolved in saline. Docetaxel (Taxotere), supplied by Aventis Pharma, Ltd. (Tokyo, Japan), was dissolved in 95% ethanol and diluted with 5% glucose solution. Adriamycin (5.0 mg/kg) and docetaxel (12.5 mg/kg) were injected i.p.

**Dosing schedule.** In chronopharmacologic studies during which only one drug was administered, adriamycin (5.0 mg/kg) or docetaxel (12.5 mg/kg) was injected i.p. at 2, 6, 10, 14, 18, or 22 hours after the light was turned on (HALO) in mice. In the combination studies of adriamycin and docetaxel, mice were divided into four intermittent dosing groups, in which the second drug was administered 12 hours after the first drug injection: (a) A(2)-D(14) in which adriamycin was administered at 2 HALO followed by docetaxel at 14 HALO; (b) A(14)-D(2) in which adriamycin was administered at 14 HALO followed by docetaxel at 2 HALO; (c) D(2)-A(14) in which docetaxel was administered at 2 HALO followed by adriamycin at 14 HALO; and (d) D(14)-A(2) in which docetaxel was administered at 14 HALO followed by adriamycin at 2 HALO. There were also two simultaneous dosing groups in which both drugs were administered simultaneously: (e) A(2)/D(2) in which adriamycin and docetaxel were administered at 2 HALO and (f) A(14)/D(14) in which adriamycin and docetaxel were administered at 14 HALO and a saline-treated group (control group).

**Determination of tolerance (survival).** To study toxic death (tolerance), adriamycin alone was i.p. administered at 2, 6, 10, 14, 18, or 22 HALO every 7 days (total of 20 mg/kg of adriamycin; n = 11) in a chronopharmacologic study. The combination of adriamycin and docetaxel was i.p. administered every 7 days (a total of 20 mg/kg of adriamycin and 50 mg/kg of docetaxel; n = 14-15) according to an arbitrary dosing schedule. Survival time was recorded for 35 days in each mouse.

**Measurement of body weight change.** Docetaxel alone was i.p. administered at 2, 6, 10, 14, 18, or 22 HALO in a chronopharmacologic study (n = 6). In the combination study of adriamycin and docetaxel, the D(2)-A(14) group (n = 9) and the D(14)-A(2) group (n = 8) were i.p. administered. Body weight was monitored daily for 7 days in each mouse. Changes in body weights were calculated as the percent of weight change in each mouse from the initial value (day 0).

**Measurement of leukocyte counts.** In a chronopharmacologic study, after adriamycin (n = 8-12), docetaxel (n = 7-10), or saline was i.p. administered at 2, 6, 10, 14, 18, or 22 HALO, blood samples were drawn by orbital sinus collection on day 3 and then leukocyte counts were measured. The change rate in leukocyte counts was calculated as the percentage for each mouse from the mean value of the control group.

In a combination study of adriamycin and docetaxel, blood samples were drawn by orbital sinus collection on days 10 and 13 after twice weekly injections of drugs or saline in the three groups [D(2)-A(14), n = 9 or 10; D(14)-A(2), n = 10; and control, n = 15 or 16]. Leukocyte counts were measured immediately after blood drawing.

**Measurement of pharmacokinetics.** Blood samples were drawn from hearts under ether anesthesia at 0.5, 1, 2, and 4 hours after adriamycin and docetaxel were administered to the two pretreated docetaxel groups. All blood samples were immediately centrifuged at 12,000 × g for 15 minutes, after which the plasma was removed and frozen at −20°C until assay. Adriamycin (n = 4-7) and docetaxel (n = 4-6) concentrations in plasma were quantified by high-performance liquid chromatography with fluorescence or UV detection according to the previously published method (15).

**Determination of cell cycle (G2-M phase).** Bone marrow samples (two femora) were taken at different times (2, 6, 10, 14, 18, or 22 HALO; n = 7 or 8). The femora were flushed with 2 mL of PBS (−) per bone, and the suspension in myelocyte cells was centrifuged at 1,500 rpm for 4 minutes at 4°C. The pellets were washed with PBS (−), resuspended in PBS (−) to 1 × 10⁷ cells/mL, and 3 mL of ice-cold ethanol was slowly added dropwise. The cells were stored at 4°C until further processing for at least 12 hours. Fixed cells were centrifuged at 3,000 rpm for 4 minutes at 4°C. After centrifugation, the pellets were washed with PBS (−) twice, and the supernatant was removed. The residue (1 × 10⁷ cells) was added with 2.5 mL of ribonuclease [1 mg/mL in PBS (−)] and the sample was incubated at 37°C for 60 minutes. One hundred microliters of propidium iodide [1.25 mg/mL in PBS (−)] were added to the mixture. The cellular DNA content was then measured with a flow cytometer (BD FACSCalibur). The gated population was analyzed using the broadened rectangular model of ModFIT software. Change rates in G2-M phase were calculated as the ratio of G2-M phase in each mouse from the mean of G2-M phase in all mice.

**Determination of antitumor effect.** At 3 days after Ehrlich cells (5 × 10⁶ cells) were intracardially inoculated, combinations of adriamycin and docetaxel or saline were i.p. administered in the D(2)-A(14) group (n = 9), the D(14)-A(2) group (n = 8), and the control group (n = 9). After the initiation of drug injections, tumor weight was measured during 7 days according to the following 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 tumor volume from the initiation of adriamycin and docetaxel injection.

**Statistical analysis.** The survival days were plotted with the Kaplan-Meier method and compared by the log-rank test. The concentrations of drugs and leukocyte counts were shown as the mean ± SD and the other values were expressed as the mean ± SE. Statistical moment analysis was performed by a calculating pharmacokinetic parameter such as area under the plasma-time concentration curve (AUC). Groups were compared by one-way ANOVA and repeated ANOVA, and differences between groups were determined by Scheffé’s test. The statistical significance of circadian rhythmicity was documented by Cosinor analysis. P < 0.05 was considered to be significant.

**Results**

**Influence of dosing time on toxic death during adriamycin administration.** When adriamycin was administered every 7 days, the deceased mice showed marked accumulation of ascites. The mice treated at 2 HALO showed the worst survival rate among the adriamycin-treated groups (versus 6, 14, and 18 HALO: P < 0.01, respectively; versus 10 HALO: P < 0.05; Fig. 1). The mortality was significantly higher in the 22 HALO–treated group compared with the 6, 14, and 18 HALO–treated groups (versus 14 HALO: P < 0.01; versus 6 and 18 HALO, P < 0.05, respectively).

**Influence of dosing time on relative body weight change after docetaxel administration.** The body weight in the mice treated at 14 HALO decreased significantly more than those in the mice treated at 2 and 18 HALO (versus 2 HALO: P < 0.05; versus 18 HALO: P < 0.01; Fig. 2).

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**Figure 1.** Influence of dosing time on tolerance (survival) during adriamycin injection. Adriamycin (5 mg/kg, i.p.) was administered every 7 days (total of 20 mg/kg) at 2, 6, 10, 14, 18, or 22 HALO in mice. Survival time was recorded for 35 days in each mouse. The mortality in mice treated at 2 HALO showed the worst survival rate among the adriamycin-treated groups (versus 6, 14, and 18 HALO: P < 0.01, respectively; versus 10 HALO: P < 0.05; Fig. 1). The mortality was significantly higher in the 22 HALO–treated group compared with the 6, 14, and 18 HALO–treated groups (versus 14 HALO: P < 0.01; versus 6 and 18 HALO, P < 0.05, respectively).
Influence of dosing time on leukopenia after adriamycin or docetaxel administration. The decrease rates in leukocytes at 6 and 10 HALO were approximately four to six times as large as those at 14 and 18 HALO after adriamycin administration (Table 1). Leukopenia was severe in late light phase to mid dark phase (10, 14, and 18 HALO) and was mild in late dark phase to mid light phase (2, 6, and 22 HALO) after docetaxel administration (Table 1).

Influence of dosing time on toxic death during combination of adriamycin and docetaxel. When a combination of adriamycin and docetaxel was administered every 7 days, the D(2)-A(14) and D(14)-A(2) groups showed the best survival rate among all the groups except for the A(14)-D(2) group (P < 0.01, respectively) and showed lower mortality compared with the A(14)-D(2) group (P = 0.053 and 0.073; Fig. 3). There was no significant change in the survival rate between the D(2)-A(14) and D(14)-A(2) groups.

On day 35, the survival rates were 57.1% in the A(14)-D(2) group and 35.7% in the A(14)/D(14) group treated with adriamycin at 14 HALO but were 13.3% in the A(2)-D(14) group and 7.1% in the A(2)/D(2) group treated with adriamycin at 2 HALO. The mortality was significantly lower in the A(14)-D(2) group than in the A(2)-D(14) and A(2)/D(2) groups (P < 0.01, respectively). Survival on day 35 in the A(14)/D(14) group was 2.7 and 5 times as high as those in the A(2)-D(14) and A(2)/D(2) groups.

Influence of dosing time on relative body weight change after combination of adriamycin and docetaxel. We investigated the influence of dosing schedule on relative body weight change in the docetaxel-pretreated groups [D(2)-A(14) and D(14)-A(2)] that significantly improved the toxic death. The relative body weight decreased more in the D(14)-A(2) group than in the D(2)-A(14) group (P < 0.01; Fig. 4).

Table 1. Change rate in leukocyte counts after adriamycin or docetaxel injection

<table>
<thead>
<tr>
<th>Time of injection (HALO)</th>
<th>Drugs</th>
<th>Adriamycin (5 mg/kg)</th>
<th>Docetaxel (12.5 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td></td>
<td>-7.1 ± 6.8% (11)</td>
<td>1.7 ± 7.5% (10)</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>-23.5 ± 8.6% (8)</td>
<td>-1.1 ± 6.7% (10)</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>-20.8 ± 5.5% (8)</td>
<td>-9.5 ± 9.6% (10)</td>
</tr>
<tr>
<td>14</td>
<td></td>
<td>-5.3 ± 5% (12)</td>
<td>-7.1 ± 9.7% (7)</td>
</tr>
<tr>
<td>18</td>
<td></td>
<td>-4.2 ± 11.4% (10)</td>
<td>-12.1 ± 10.9% (8)</td>
</tr>
<tr>
<td>22</td>
<td></td>
<td>-12.0 ± 9.7% (9)</td>
<td>11.3 ± 10.4% (8)</td>
</tr>
</tbody>
</table>

NOTE: Data are shown as mean ± SE (n).

Figure 2. Influence of dosing time on relative body weight change after single administration of docetaxel. Docetaxel (12.5 mg/kg, i.p.) was administered at 2 (●), 6 (○), 10 (△), 14 (■), 18 (▲), and 22 (▲) HALO in mice. Points, mean; bars, SE (n = 6). *P < 0.05, **P < 0.01 compared with the 14 HALO-treated group using Scheffe’s test.

Figure 3. Influence of dosing time on tolerance (survival) during combined administration of adriamycin and docetaxel. Adriamycin (5 mg/kg, i.p.) and docetaxel (12.5 mg/kg, i.p.) were administered every 7 days simultaneously (A/D) or intermittently (A-D and D-A) at 2 or 14 HALO in mice (a total of 20 mg/kg of adriamycin and 50 mg/kg of docetaxel). The D(2)-A(14) and D(14)-A(2) groups showed the best survival rate among all the groups except for the A(14)-D(2) group (P < 0.01, respectively, log-rank test).

Figure 4. Influence of dosing time on relative body weight change after combined administration of adriamycin and docetaxel. Adriamycin (5 mg/kg, i.p.) and docetaxel (12.5 mg/kg, i.p.) were administered intermittently (●, D(2)-A(14); ▲, D(14)-A(2)) in mice. Points, mean; bars, SE (n = 8 or 9). The relative body weight in the D(14)-A(2) group decreased more than that in the D(2)-A(14) groups (P from repeated ANOVA = 3.12, P < 0.01).
markedly lower in the D(14)-A(2) group than in the control and D(2)-A(14) groups (P < 0.01 and P = 0.052). On day 13, the leukopenia was recovered in the D(2)-A(14) group, whereas severe leukopenia was observed in the D(14)-A(2) group compared with the control group (P < 0.05).

**Influence of dosing time on pharmacokinetics in plasma after combination of adriamycin and docetaxel.** When adriamycin and docetaxel were administered intermittently, there was no significant change in the plasma adriamycin concentrations between the D(2)-A(14) and D(14)-A(2) groups (Table 2).

The plasma docetaxel concentrations at 1 and 2 hours after docetaxel injection in the D(2)-A(14) group were higher than in the D(14)-A(2) group (P < 0.05; Table 2). The mean AUC_{0-4} (the pharmacokinetic parameter value calculated AUC between 0 and 4 hours) in the D(2)-A(14) group was about 1.75-fold higher than that in the D(14)-A(2) group (696.3 and 397.3 ng h/mL).

**Determination of change rate in the cell cycle (G2-M phase).** The G2-M phase of cell cycle in myelocyte cells in nontreated mice showed a significant 24-hour rhythm with a peak at 22 HALO and trough at 10 HALO (F from ANOVA = 5.51, P < 0.01; P from Cosinor < 0.01; Fig. 6).

**Table 2. Adriamycin and docetaxel concentrations after each drug administration**

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Groups</th>
<th>Time (h)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
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<tr>
<td></td>
<td></td>
<td>0.5</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Adriamycin (ng/mL)</td>
<td>D(2)-A(14)</td>
<td>103 ± 20 (5)</td>
<td>59 ± 13 (5)</td>
<td>36 ± 5 (4)</td>
<td>27 ± 13 (7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D(14)-A(2)</td>
<td>104 ± 25 (6)</td>
<td>62 ± 13 (5)</td>
<td>56 ± 19 (7)</td>
<td>26 ± 11 (4)</td>
<td></td>
</tr>
<tr>
<td>Docetaxel (ng/mL)</td>
<td>D(2)-A(14)</td>
<td>265 ± 48 (4)</td>
<td>327 ± 108 (5)</td>
<td>185 ± 45 (4)</td>
<td>41 ± 10 (6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D(14)-A(2)</td>
<td>173 ± 124 (5)</td>
<td>159 ± 29 (4)</td>
<td>105 ± 56 (6)</td>
<td>34 ± 11 (4)</td>
<td></td>
</tr>
</tbody>
</table>

Data are shown as mean ± SD (n).

*P < 0.05.

**Influence of dosing time on tumor growth after combination of adriamycin and docetaxel.** The D(2)-A(14) and D(14)-A(2) groups showed significantly inhibited tumor growth compared with the control group (P < 0.05, respectively; Fig. 7). No difference in inhibition of tumor growth was seen between the D(2)-A(14) and D(14)-A(2) groups.

**Discussion**

The biological functions of most living organisms are organized along an approximate 24-hour time cycle or circadian rhythm. Chronobiological investigations have shown that the therapeutic indices of drugs can be affected by varying circadian drug timing. Toxic death caused by adriamycin varied significantly depending on the dosing time despite the same dose being used in all groups. Survival on day 35 markedly decreased by 72.7% and 54.5% in the mice treated at 2 HALO compared with the mice treated at 14 and 18 HALO, respectively. The decrease rate in leukocyte counts after single administration of adriamycin showed a dosing time-dependent change with higher values in the rest phase and lower values in the active phase. A similar observation has been obtained for repeated administration in rats, and the mortality and leukopenia were severer in the group treated at the rest phase compared with the active phase (21). On the other hand, when docetaxel was administered, the body weight in the mice treated at 14 HALO decreased more than that in the mice treated at 2 and 18 HALO. Although the leukopenia did not show a significant difference, the mice treated with docetaxel during the active phase tended to show more severe leukopenia than those treated during the rest phase. A similar observation has been obtained for B6D2F1 mice, and both body weight loss and leukopenia were more toxic when docetaxel was administered in the active phase; dosing during the rest phase was better tolerated (20). Consequently, the time when adverse effects are relieved is separated by approximately 12 hours between adriamycin and docetaxel. In our previous study, the docetaxel-adriamycin group in which adriamycin was administered 12 hours after docetaxel injection showed marked improvement in toxic death and leukopenia compared with the other groups. Therefore, greater reduction of adverse effects after coadministration of adriamycin and docetaxel may be expected as a result of adding dosing time to the dosing schedule suggested by our previous study.

In the present study, toxic death was observed in the mice given 10 to 15 mg/kg adriamycin as the total dose. On day 35, the survival rate in the A(14)-D(2) group was four times as high as that in the
A(2)-D(14) group, and the survival rate in the A(14)/D(14) group was five times as large as that in the A(2)/D(2) group. These groups were different only in dosing time, which agreed well with the result that toxic death caused by adriamycin administration alone largely depended on dosing time in the present study and in our previous studies (21). It is clear that dosing time is an important factor to decrease the toxic death from concurrent administration of adriamycin and docetaxel. Quite strikingly, the pretreated docetaxel groups [the D(2)-A(14) and D(14)-A(2) groups] showed markedly improved toxic death compared with all of the adriamycin alone and combined groups. There was no change in survival rate between the D(2)-A(14) and D(14)-A(2) groups, regardless of the dosing time. This finding suggests that pretreatment of docetaxel rather than dosing time contributes greatly to decreasing toxic death in the concurrent administration of adriamycin and docetaxel. These findings may help increase the safety of continuous long-term use of this combination therapy.

The body weight loss after administration of adriamycin and docetaxel was more severe in the D(14)-A(2) group than the D(2)-A(14) group. The leukocyte counts after the repeated administration of adriamycin and docetaxel were more severe in the D(14)-A(2) group compared with the D(2)-A(14) group also. It was a common point with these results that administering the combination of adriamycin and docetaxel at the time when adverse effects were most decreased for the separate drugs also relieved more adverse effects in the combination. These findings, thus, reveal that a safer combination chemotherapy of adriamycin and docetaxel becomes possible by including a chronopharmacologic approach to the dosing schedule suggested by our previous study (15).

The mechanism underlying the dosing time–dependent leukopenia was investigated from the viewpoint of pharmacokinetics and pharmacodynamics. In a clinical study, the AUC of adriamycin or docetaxel was closely associated with the decrease in leukocyte counts in patients treated with adriamycin or docetaxel (25, 26). However, there was no difference in the AUC_{0–4h} of adriamycin between the D(2)-A(14) and D(14)-A(2) groups, although the leukopenia in the D(14)-A(2) group was more severe than that in the D(2)-A(14) group. On the other hand, lower AUC_{0–4h} of docetaxel was observed in the D(14)-A(2) group compared with the D(2)-A(14) group, although the leukopenia in the D(14)-A(2) group was more severe than that in the D(2)-A(14) group. In the preliminary study, there were no significant differences between the D(2)-A(14) and D(14)-A(2) groups in terms of adriamycin concentrations and docetaxel concentrations in the myelocyte cells after the drug injection, irrespective of the dosing time (data not shown). Therefore, the adriamycin and docetaxel concentration may not contribute directly to the dosing time dependency of leukopenia.

A significant 24-hour rhythm of G2-M phase distribution was found in mice myelocyte cells in the present study. The 24-hour rhythm in ICR mice myelocyte cells corresponded to that in B6D2F1 mice myelocyte cells (20). Leukopenia caused by docetaxel-alone administration was highest at the time when the proportion of G2-M phase cells increased and the lowest at the time when the proportion of G2-M phase cells decreased. In the combination study, the D(2)-A(14) group in which docetaxel was preadministered at 2 HALO markedly showed leukopenia compared with the D(14)-A(2) group in which docetaxel was preadministered at 14 HALO. Docetaxel induces phosphorylation of bcl-2 at the G2-M phase and leads to apoptotic death of cells (27). This is in good agreement with a report that cells in G2-M phase are the most sensitive to docetaxel (27). These results suggest that the difference of leukopenia in mice treated docetaxel alone or combination with adriamycin is related to the 24-hour rhythm of G2-M phase distribution in myelocyte cells.

In the present study, there were no significant changes between the D(2)-A(14) and D(14)-A(2) groups in mice inoculated with Ehrlich tumor cells, which are derived from the mammary gland. Antitumor effects, however, were better in the pretreated docetaxel groups [D(2)-A(14) and D(14)-A(2) groups] compared with the pretreated adriamycin groups [A(2)-D(14) and A(14)-D(2) groups] and the simultaneous groups [A(2)/D(14) and A(14)/D(2) groups; data not shown]. This result suggests that the antitumor effect in this combination depends not on the dosing time but on the dosing sequence, which supports our previous findings (15). It is

![Graph](image-url)
reported that the antitumor effect by the coadministration of adriamycin and docetaxel is highly dependent on the dosing schedule in vitro in human breast cells, and the exposure of tumor cells to adriamycin after docetaxel shows an inhibitory effect on tumor cell death with inhibition of the mitotic arrest and apoptosis, although the exposure of tumor cells to adriamycin simultaneously or before docetaxel could result in pronounced antagonism (28, 29). These results of an in vitro study corresponded to our results from in vivo study.

Experimental chronotherapy for the combination of adriamycin and docetaxel has already been reported in mice by Granda et al. (30), and there were two principal differences between this report and the present study. First, the recommended dosing interval between adriamycin and docetaxel was different. The dosing interval used by Granda et al. was adapted from simultaneous dosing in a study in which docetaxel was given at 3, 11, 15, or 23 HALO, then adriamycin 1 minute, 12 hours, or 24 hours later (31). In our previous study, we revealed that the mice pretreated with docetaxel significantly showed not only reduced adverse effects (leukopenia and toxic death) but also improved antitumor effect compared with the mice treated with adriamycin and docetaxel simultaneously (15). Moreover, the survival rates (tolerance) were 11.1% in the simultaneous dosing group and 94.1% in the pretreated docetaxel group in which adriamycin (2.5 mg/kg) and docetaxel (12.5 mg/kg) were administered every 7 days (a total of 20 mg/kg Adria- mycin and 100 mg/kg docetaxel), and 30.0% in the simultaneous dosing group and 90.9% in the pretreated docetaxel group after single administration of adriamycin (15 mg/kg) and docetaxel (12.5 mg/kg). Both results showed significantly high survival in the pretreated docetaxel group compared with the simultaneous dosing group (data not shown). Therefore, we adopted a dosing interval and dosing sequence based on these findings.

Second, the circadian time dependency of adriamycin tolerability was different between the study reported by Granda et al. and the present study, although that of docetaxel tolerability corresponded between the studies. In the present study, adverse effects were decreased by administration of adriamycin in the active phase. We have already revealed the same chronotolerance in rats (21). Moreover, it has been recommended that adriamycin be administered in the morning (active phase) based on clinical studies (17, 24). On the other hand, Granda et al. reported that the overall lethal toxicity was 81%, 19%, and 25% with 13.8, 8.3, and 5 mg/kg when mice received three weekly administrations of four adriamycin dose levels (13.8, 8.3, 5, or 3 mg/kg) at six different times (30). The lethal toxicity caused by adriamycin depends on the dosing schedule and total dose, and the cumulative dosage of 15 to 20 mg/kg produces high mortality and accumulation of ascites in rodents (15, 21, 32, 33). adriamycin dose of 13.8 mg/kg agrees with lethal dose on a single administration, and it seems that repeated administration of the dose has a severe impact on mice compared with other doses (8.3, 5, or 3 mg/kg). Thus, the difference in chronotolerance by adriamycin-alone administration between Granda et al. and our studies may be due to differences in study design. There is, however, an important point in common between Granda et al. and our studies: The therapeutic index of the combined chemotherapy can be improved by coadministering adriamycin and docetaxel at the times when the most adverse effects are relieved in each drug regardless of differences in study design and dosage. In fact, the availability of combined chronotherapy was reported in few clinical studies (16, 34). Thus, we consider that choosing the optimal dosing time for each drug is an essential to yield an excellent therapeutic index for the combination.

In conclusion, the findings of the present study suggest that the pretreated docetaxel group showed not only dramatically improved lethal toxicity but also inhibition of most tumor growth compared with the other groups. Moreover, the D(2)-A(14) group in which docetaxel was administered at 2 HALO followed by adriamycin at 14 HALO showed reduced body weight loss and leukopenia compared with the D(14)-A(2) group. The differences, especially that of leukopenia, may be caused by the 24-hour rhythm of G2-M phase distribution in myelocyte cells. Choosing the optimal dosing schedule, including chronopharmacology, would be expected to lead to safe and effective chemotherapy with combinations of adriamycin and docetaxel.

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Therapeutic Index by Combination of Adriamycin and Docetaxel Depends on Dosing Time in Mice

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