Effects of a Water-Soluble Prodrug of Vitamin E on Doxorubicin-Induced Toxicity in Mice

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Effects of the administration of a water-soluble prodrug of vitamin E on doxorubicine (DXR)-induced lethal and oxidative toxicity in mice were studied. The prodrug used was d-α-tocopheryl N,N-dimethylaminooacetate hydrochloride (TDMA). It was intravenously administered to animals 2 h prior to an intraperitoneal administration of DXR (15 mg/kg). The single preadministration of the prodrug (10–50 mg/kg equivalent for d-α-tocopherol) delayed the DXR-induced death and the ameliorative effect was TDMA-dose dependent. The extent of total lipid peroxidation of the heart and liver was assessed by 2-thiobarbituric acid reactant substance levels. DXR significantly accelerated lipid peroxidation in the liver but not in the heart. The elevation of liver lipid peroxide was significantly suppressed to a normal range by a single preadministration of TDMA (50 mg/kg equivalent for d-α-tocopherol). TDMA did not significantly affect the antitumor activity of DXR in mice inoculated with L1210 leukemia cells.

Key words d-α-tocopherol N,N-dimethylaminooacetate; doxorubicin; lipid peroxidation; vitamin E; water soluble prodrug; oxidative stress

The toxicity of anticancer drugs to noncancerous cells is a major limitation in cancer chemotherapy. Doxorubicine (DXR) is one of the most effective and commonly used antineoplastic drugs in cancer chemotherapy.

However, its usefulness is limited by severe side-effects in numerous organs, including dose-related cardiotoxicity. The mechanism of this cardiotoxicity is not fully understood. Biochemical effects observed in experimental animals have afforded considerable information on the mechanism of the toxicity. Oxidative damage to membrane lipids and other cellular components is believed to be a major factor in the DXR-induced toxicity. However, the concept of a single biochemical mechanism to account for the toxic effects of DXR on several organs appears untenable, since Ito et al. reported that DXR selectively inhibits the expression of cardiac muscle genes in vivo and in vitro.

The presence of a quinone function in the DXR molecule offers the possibility of a conversion of DXR to a semiquinone free radical, which has been shown to be generated spontaneously under physiological conditions. Its formation is generally enhanced by an enzyme catalyzed one-electron reduction. The semiquinone reacts readily with molecular oxygen to produce active oxygen species, which induce undesired damage to the normal cells, producing a futile redox cycling. The net result of this redox cycling is oxidative stress, such as lipid peroxidation. A great deal of research has been directed toward reduction of this oxidative stress, such as the use of antioxidants for protection of the lipid peroxidation.

d-α-Tocopherol (vitamin E, Toc), a biological chain-breaking antioxidant, is currently receiving attention concerning its efficacy in preventing and reducing oxidative stress. Myers et al. reported that DXR administration lead to lipid peroxidation in cardiac tissues and that preadministration of Toc prevented this peroxidative process and ameliorated DXR-induced lethal toxicity without interfering with the effectiveness of the antitumor activity of DXR. Subsequent to their reports, DXR-induced lipid peroxidation and the protective effects of Toc have been confirmed by other workers both in vivo and in vitro. In all of the in vivo studies, a large amount of Toc or its acetate ester were administered by extravascular routes (e.g. intraperitoneally (i.p.), intramuscularly (i.m.), or subcutaneously (s.c.)). However, these dosage regimens of Toc are not applicable for clinical practice.

Toc is practically insoluble in water and is readily oxidized by atmospheric oxygen. Because of its high resistance to oxidation, the acetate ester of the vitamin is commonly supplied for clinical use. When a rapid onset of action is required via parenteral administration, however, significant problems arise from the fact that the acetate is also practically insoluble in water. In parenteral formulations, Toc and its acetate are solubilized by a large amount of a surfactant. It has been confirmed that hydrolysis of the acetate is the rate-limiting step in the course of the bioavailability of Toc. The use of surfactants in parenteral dosage forms generally induces toxicity such as the anaphylactoid reaction. The uncertain delivery of Toc seems to have forced the former researchers to use large doses of Toc or its acetate for protection against DXR-induced toxicity.

With the aim of overcoming the delivery problems of Toc, we synthesized d-α-tocopheryl N,N-dimethylaminooacetate hydrochloride (TDMA) and evaluated it as a water-soluble prodrug of Toc for parenteral use that can achieve the systemic liver-specific delivery of Toc. In the present study, the ameliorative effects of a single i.v. preadministration of TDMA on DXR-induced lethal toxicity and tissue lipid peroxidation in mice, as well as the effects on antitumor activity of DXR in L1210 inoculated mice, were investigated.

MATERIALS AND METHODS

Materials and Experimental Animals A doxorubicine hydrochloride (DXR) injection of clinical use grade (Adriacin) was obtained from Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan). TDMA was synthesized in our labora-

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tory by the method previously reported.24) 2-Thiobarbituric acid (TBA) was purchased from E. Merck (Darmstadt, Germany). The BCA protein assay kit was obtained from Pierce (IL, U.S.A.). dl-Tocol was kindly given by Eisai Co., Ltd. (Tokyo, Japan). All other chemicals were purchased from Wako Pure Chemical Ind., Ltd. (Osaka, Japan).

Male CDF1 mice, 20–24 g, were purchased from Charles River Japan (Yokohama, Japan) and were allowed free access to standard laboratory chow (CE-2, Clea Japan, Tokyo, Japan) and water from 1 week before to the end of the experiments. Leukemia L1210 tumor cells were a kind gift from Dr. Tazuko Tashiro, the Japanese Foundation for Cancer Research, Tokyo, and were maintained by i.p. transplantation in male CDF1 mice. Mice transplanted with leukemia L1210 cells were used for evaluation of the antitumor activity of DXR with and without the TDMA pretreatment.

**Alterations in DXR-Induced Lethality** The animals were randomly divided into four groups (10–14/group) and were intravenously (i.v.) given either saline (control group) or TDMA 2 h before DXR administration. Doses of TDMA were 0 (group A), 10 (group B), 25 (group C), or 50 (group D) mg equivalent for Toc per kilogram body weight (mg Toc equiv/kg). DXR (15 mg/kg) was administered i.p. to all groups of mice. The dose and route of DXR were comparable to those used by Myers et al.10) Numbers of surviving animals and their body weights were measured every day for 9 weeks. Statistical significances of survival numbers in the different experimental groups were estimated using the Cox-Mantel method.

**DXR-Induced Lipid Peroxidation** Sixty mice were divided into two groups (30/group) and treated according to the protocols described above for groups A and D. Five mice from each of the groups were sacrificed on day 0 to 5, consecutively. Blood was collected from the abdominal artery, and tissues were removed under light ether anesthesia. Sufficient volumes of 1.15% KCl solution were added to give 10% (w/v) homogenates of liver and kidney and 5% homogenates of heart and spleen. Homogenization was carried out using a Polytron homogenizer (Kinematica, Switzerland). Whole tissue homogenates were stored at −80°C until analysis and were submitted to lipid peroxide and Toc assays.

**Assay for Lipid Peroxides in Tissues** Lipid peroxides in tissues were evaluated by the amount of TBA-reactive substances (TBARS), and were expressed as nanomole of malondialdehyde (MDA) per milligram of protein. The method of the assay was essentially the same as that described by Ohkawa et al.26) The TBA reagent was used as a 0.6% aqueous solution because of its low aqueous solubility at room temperature (under 0.8%). The absorbance at 535 nm was measured relative to a reference at 520 nm and 1,1,3,3-tetraethoxycarbonyl was used as the MDA standard. The protein concentrations of the homogenates were determined by a BCA protein assay kit with bovine serum albumin as the standard.

**HPLC Analysis** The liver concentrations of Toc were determined by HPLC as described previously.29) To a 100 μl volume of the tissue homogenate was added 350 μl of ethanol containing 2 μg/ml dl-tocool. After vortex mixing for 2 min and centrifugation at 3000 rpm for 5 min, 50 μl of the supernatant was subjected to HPLC analysis.

**Effect of TDMA Pretreatment on the Antitumor Activ-

Fig. 1. Effect of TDMA i.v. Preadministration on DXR-Induced Lethal Toxicity in Mice
Each group of mice (10–14 mice/group) was administrated TDMA i.v. 2 h before DXR administration at doses of 0 (group A), 10 (group B), 25 (group C) and 50 (group D) mg Toc equiv/kg. DXR was administrated i.p. at a dose of 15 mg/kg. ◆, group A; ○, group B; ●, group C; △, group D.

ity of DXR** Four groups of mice (10/group) were inoculated with L1210 leukemia cells (10⁶ cells, i.p.). Two groups were administrated i.v. with TDMA (50 mg Toc equiv/kg) (TDMA alone group) or saline (control group) 22 h after the L1210 inoculation. To the other two groups, DXR (2 mg/kg) was administrated i.p. 24 h after the inoculation. One of the two groups received i.v. TDMA (50 mg Toc equiv/kg ) (TDMA+DXR group) and the other group saline (DXR alone group) 2 h before the DXR administration.

**RESULTS AND DISCUSSION**

**Effect of Preadministration of the Prodrug on DXR-Induced Lethal Toxicity** In order to determine the effective dose of TDMA against DXR induced lethal toxicity, TDMA was administrated (0 to 50 mg Toc equiv/kg, i.v.) 2 h prior to DXR administration (15 mg/kg, i.p.). The DXR dose was comparable to that employed by Myers et al.10) Figure 1 shows the survival curves after DXR administration with and without TDMA pretreatment. All of the animals in group A (0 mg/kg) died within 2 weeks, while at the end of the period the survival rates of groups B (10 mg/kg), C (25 mg/kg) and D (50 mg/kg) were 40, 70 and 90%, respectively. The survival rates of groups B, C and D at 60 d after the DXR administration were 10, 50, and 60%, respectively. The statistically significant differences in survival rates were confirmed between all TDMA pretreated groups and the control group (p<0.001, Cox-Mantel method). It is clear that a single i.v. pretreatment of TDMA delayed the DXR-induced death, and the ameliorative effect was dose dependent.

The effective doses of TDMA are equivalent to 10–50 mg/kg of Toc, which are less than one-third of those of Toc used by Myers10) and Minnaugh21) (4100 IU/kg, 3200 mg/kg), respectively. The results clearly indicate that i.v. administration of the prodrug can greatly reduce the effective dose of Toc for amelioration of DXR-induced lethal toxicity.

The loss in weight of the body and organs following DXR (15 mg/kg, i.p.) administration is shown in Table 1. The body weights of DXR administered mice decreased gradually; 22% of the initial weight was lost after 5 d. In contrast, body-
Table 1. Changes in Weights of Whole Body and Organs of Mice after DXR Administration with and without TDMA Pretreatment

<table>
<thead>
<tr>
<th>Dosage</th>
<th>Body weight (g)</th>
<th>Heart (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Liver (%)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Kidney (%)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Spleen (%)&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td>Control (Saline)&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>Initial day</td>
<td>22.0±0.6</td>
<td>0.57±0.07</td>
<td>5.62±0.48</td>
<td>1.47±0.61</td>
<td>0.29±0.01</td>
</tr>
<tr>
<td>Day 5</td>
<td>24.7±0.6&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.55±0.03</td>
<td>5.36±0.10</td>
<td>1.47±0.11</td>
<td>0.32±0.01</td>
</tr>
<tr>
<td>DXR without TDMA (Group A)&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>Initial day</td>
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<td>5.62±0.48</td>
<td>1.47±0.61</td>
<td>0.29±0.01</td>
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<tr>
<td>Day 5</td>
<td>17.2±0.3&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.45±0.02</td>
<td>4.38±0.43&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.45±0.03</td>
<td>0.12±0.02&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
<td>DXR with TDMA (Group D)&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>Initial day</td>
<td>21.7±0.5</td>
<td>0.56±0.06</td>
<td>5.11±0.66</td>
<td>1.53±0.15</td>
<td>0.35±0.07</td>
</tr>
<tr>
<td>Day 5</td>
<td>17.9±1.2&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.46±0.03</td>
<td>4.81±0.47</td>
<td>1.52±0.08</td>
<td>0.18±0.03&lt;sup&gt;e&lt;/sup&gt;</td>
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</table>

<sup>a</sup> Weights of organs are expressed as % of body weights.  
<sup>b</sup> Numbers of mice of control and groups A and D were 30 each and total numbers examined were 90.  
<sup>c</sup> DXR was administered 15 mg/kg i.p. on the initial day (day 0).  
<sup>d</sup> TDMA (50 mg/kg equiv. for Toc, i.v.) was given 2 h before the DXR administration.  
<sup>e</sup> The difference from the initial day was statistically significant (p<0.01 by Student’s t-test).

weight increased in the mice which received isotonic saline; the gain was 12% in the same observation period. The weights of heart, liver and spleen, expressed as percentages of body weight, in the DXR administered mice also decreased significantly after 5 d. For kidneys, no weight loss was observed. The preadministration of TDMA did not protect against the DXR-induced losses of body and organ weights.

The effects of DXR on lethality and on body and organ weights were in agreement with the results of previous reports, which used the same animals and DXR dosage regimens. Therefore, the acute toxic state of mice in the present study must be the same as those previously reported.

**Effect of TDMA on DXR-Induced Tissue Lipid Peroxidation**

To elucidate the mechanisms of the protection of TDMA against DXR-induced toxicity, it is important to consider the mechanisms whereby DXR exerts its toxicity, which are not fully understood. The mechanisms of the delayed lethality in mice have been investigated by several authors. It has been postulated that one of the major toxic effects induced by DXR is due to membrane lipid peroxidation. Therefore, the effect of the preadministration of TDMA against the DXR-induced tissue lipid peroxidation, assessed by TBARS, was studied in mice.

The time courses of lipid peroxidation of the tissues in response to the DXR administration (15 mg/kg, i.p.) are shown in Fig. 2. In liver, significant increases in lipid peroxide levels were observed 2 to 5 d after DXR administration (p<0.01, t-test) compared to those at day 0, while changes in the levels in the heart, kidneys and spleen were not significant. The preadministration of TDMA (50 mg Toc equiv./kg, i.v.) prevented an increase in liver lipid peroxide by DXR and kept it at the level of the control group.

Concentrations of Toc in liver homogenates of groups A and D were determined and the results are shown in Fig. 3. The Toc levels of group A did not change during 5 d, which indicated that DXR administration did not lower the liver Toc levels. In group D, the liver Toc level was 58-fold higher than that of the control mice at the initial day of the experiment. During the period of 5 d, the liver Toc levels of all of the TDMA-preadministered mice (group D) were significantly higher than those of the group A mice. These results may indicate that a single i.v. preadministration of TDMA maintained high concentration levels of Toc and prevented DXR-induced lipid peroxidation in the liver.

There have been conflicting reports in the literature concerning tissue lipid peroxidation after DXR administration. Myers et al. reported that TBARS concentration in the heart of mice increased significantly at day 4 and returned to a normal level at day 5 after DXR administration. Increased lipid peroxidation of the heart has also been reported in some in vivo studies. Tanizawa et al. indicated increased lipid peroxidation in both the heart and liver. Billingham et al. reported that the liver was relatively resistant to damage by DXR. In contrast to these reports, the results of the present...
study showed a significant increase in lipid peroxidation in the liver but not in the heart. Similar results have also been reported. Many in vitro studies showed a high susceptibility of liver components (hepatocytes, mitochondria and microsomes) to DXR-induced lipid peroxidation.

A decrease in relative organ weights was the largest in the spleen (Table 1) but TBARS levels in the spleen were not increased in this study. There has been no report of increased lipid peroxidation in the spleen. It seems likely that the toxicity of DXR towards the spleen is related to the suppressive effect on hematogenesis through the bone marrow. The increased lipid peroxidation in response to i.p. DXR and its reduction by TDMA may support the idea of an oxidative mechanism of DXR-induced toxicity. However, other biochemical mechanisms accounting for the toxic effects of DXR on several organs are probable, since the report of Ito et al. suggested that DXR selectively inhibits the expression of cardiac muscle genes in vivo.

Effect of Single i.v. Preadministration of TDMA on Antitumor Activity of DXR in Mice Inoculated with L1210 Leukemia Cells The effect of TDMA preadministration on the antitumor activity of DXR in mice transplanted with leukemia L1210 cells was examined. Two of the four groups of the transplanted mice received a single administration of DXR (2 mg/kg, i.p.), then the mice of one group each of the DXR- or non-DXR groups were administered TDMA (50 mg Toc equiv./kg, i.v.). The survival curves of the four groups are shown in Fig. 4. There is no significant difference between the curves of the groups which received saline alone or TDMA alone, indicating that the preadministration of TDMA has no effect on the life span of the animals. In contrast, the groups of mice with L1210 leukemia administered DXR with and without TDMA preadministration showed a prolonged life span. These results clearly indicate that the preadministration of TDMA does not affect the antitumor activity of DXR.

CONCLUSION

Our previous studies showed that TDMA was a potentially useful prodrg of Toc for i.v. administration. The effective delivery of Toc into the liver leads to an enhanced phar-
962 (1976).