DOXORUBICIN (DOX) is an extremely effective antitumor anthracycline antibiotic. Unfortunately, DOX administration can induce a wide variety of acute cardiotoxic effects, including transient cardiac arrhythmia, nonspecific electrocardiographic abnormalities, pericarditis, and acute heart failure (4, 5). More frequently, DOX causes cardiomyopathy, which manifests as congestive heart failure months or even years after treatment.

The mechanism of DOX-induced cardiac injury has been an active area of investigation in the past three decades. Several hypotheses have been suggested to explain the acute and chronic cardiotoxicity of DOX; these include formation of free radicals, inhibition of enzymes and proteins, changes in cardiac muscle gene expression, alterations of mitochondrial membrane function, sensitization of Ca$^{2+}$ release from sarcoplasmic reticulum channels, mitochondrial DNA damage and dysfunction (26), and induction of apoptosis (2, 22).

Of these, the free radical hypothesis of DOX-induced cardiotoxicity has gained the most support in previous studies (24, 31). DOX is known to generate free radicals either by redox cycling between a semiquinone form and a quinone form or by forming a DOX-Fe$^{3+}$ complex (7). In both pathways, molecular oxygen is reduced to superoxide anion ($O_2^-$), which is converted to other forms of reactive oxygen species such as hydrogen peroxide ($H_2O_2$) and hydroxyl radical (OH$^-$). These free radicals could then cause membrane and macromolecule damage, both of which lead to injury to the heart, an organ that has a relatively low level of antioxidant enzymes such as superoxide dismutase (SOD) and catalase (9). Furthermore, recent studies have demonstrated that DOX-induced cardiotoxicity can be largely reduced by the overexpression of the antioxidant enzymes MnSOD, metallothionein, or catalase (18, 19, 44). These studies indicate that free radicals play a pivotal role in DOX-induced cardiotoxicity and that antioxidants may be used to minimize its side effects.

Numerous free radical scavengers, such as probucol, amifostine, and dexrazoxane, have been shown to protect the heart against DOX-induced cardiotoxicity (25, 35, 38). Unfortunately, all of these scavengers have pronounced clinical disadvantages. Probucol, a lipid-lowering antioxidant, confers significant protection against DOX-induced cardiotoxicity (37); however, concerns about its high-density lipoprotein-lowering property discourage its application in cancer patients. The cytoprotective drug amifostine is less potent than dexrazoxane (Zincard), an iron chelator, and it does not prevent the mortality and weight loss caused by
DOX in spontaneously hypertensive rats (17). Finally, debrisoquine, the only cardioprotective drug currently available clinically, only reduces 50% of DOX-related cardiac complications (16). Moreover, it interferes with the antitumor activity of anthracycline antibiotics (36) and also potentiates the hematotoxicity of DOX (20).

Melatonin (MEL), the primary hormone of the pineal gland, participates in circadian rhythm regulation via MEL receptor-mediated activity and plays an important role in antiaging processes (34). Importantly, MEL receptor-mediated activity and plays an important role in antiaging processes (34). Importantly, MEL acts as a powerful antioxidant and as a free radical scavenger of OH-, peroxyl radicals, and superoxide anions (33). Indeed, MEL was shown to be twice as potent as vitamin E in removing peroxyl radicals (28), and it is 5 and 14 times more effective in scavenging hydroxyl radicals than glutathione and mannitol, respectively (15).

Unlike the conformational changes due to its solubility in both water and lipids, MEL in the blood circulation is 20 min and that it is distributed in all subcellular compartments due to its solubility in both water and lipids. As such, it can pass through the cell membrane easily and enter cardiac cells to remove free radicals in situ. In addition to its antioxidant activity, MEL may also exert its effects via MEL1a receptors in the heart (10).

Previous studies have shown that the half-life of MEL in the blood circulation is 20 min and that it is rapidly converted to 6-hydroxymelatonin (6-OH MEL) in the liver (32). This major metabolite has 50 times less binding affinity for plasma membranes than MEL, but it possesses a free radical-scavenging indole ring and therefore could maintain antioxidant activity. In contrast, 8-methoxy-2-propionamidotetralin (8-M-PDOT) is a MEL receptor agonist that is devoid of an indole structure and therefore cannot scavenge free radicals (11).

The current study hypothesized that MEL or 6-OH MEL could protect the mouse heart against DOX-induced cardiotoxicity. To explore this hypothesis, a mouse mortality study was carried out with an acute dose of DOX in the presence or absence of MEL, 6-OH MEL, or 8-M-PDOT. Cardiac performance in the presence or absence of MEL, 6-OH MEL, or 8-M-PDOT was measured in DOX-perfused isolated mouse heart preparations. 8-M-PDOT was included in both of these studies to elucidate the role of antioxidant activity in the action of MEL.

In addition to these measurements, the effects of MEL and 6-OH MEL on DOX-induced apoptosis in vivo cardiac dysfunction and ultrastructural alterations were assessed. Because apoptosis has been observed in cultured cardiomyocytes and the hearts of DOX-treated animals (2), the effects of MEL and 6-OH MEL on DOX-induced apoptosis were explored. Finally, the effects of MEL and 6-OH MEL on the antitumor activity of DOX were studied.

MATERIALS AND METHODS

Materials and animals. DOX-HCl (2 mg/ml) was purchased from Pharmacia and Upjohn (Kalamazoo, MI). MEL, 6-OH MEL, a creatine phosphokinase (CPK) kit (CK-20), and F-12K medium were purchased from Sigma (St. Louis, MO). 8-M-PDOT was a product of Tocris Cookson (Ballwin, MO). PC-3 cells were purchased from the American Type Culture Collection (Manassas, VA). All other reagents were of the highest grades commercially available. The TdT-mediated dUTP nick-end labeling (TUNEL) assay kit Cardi/TACS and the ApopTag in situ oligo ligation (ISOL) kit were obtained from Trevigen (Gaithersburg, MD) and Intergen (Purchase, NY), respectively. A Cell Death Detection ELISA Plus kit was purchased from Roche Molecular Biochemicals (Indianapolis, IN). ALZET microosmotic pumps (model 1007) were purchased from Durect (Cupertino, CA). ICR mice (male, 30–35 g) were obtained from Harlan (Indianapolis, IN).

Fluorescence assay for antioxidant activities. The β-phycoerythrin (β-PE) fluorescence-based assay for peroxyl radicals was studied with MEL, 6-OH MEL, and 8-M-PDOT at 1 μM using the method described by Glazer (14). The reactions were initiated by adding 0.2 ml of 40 mM 2,2′-azobis-(2-amidinopropane) hydrochloride to 1.8 ml of 75 mM phosphate buffer (pH 7.0) containing 16.5 mM β-PE and other additives in 10-mm quartz fluorometer cells at 22°C. The relative fluorescence emission was measured at 575 nm with excitation at 540 nm in a Perkin-Elmer LS55B fluorescence spectrophotometer.

Animal survival studies. Mice were maintained in American Association for Accreditation of Laboratory Animal Care-accredited, climate-controlled facilities. The animals were randomly assigned to eight groups as follows: control, DOX, MEL, DOX + MEL, 6-OH MEL, DOX + 6-OH MEL, 8-M-PDOT, and DOX + 8-M-PDOT. A single dose of DOX (25 mg/kg ip) or an equivalent volume of saline was injected. MEL, 6-OH MEL, or 8-M-PDOT was administered in the drinking water (10 mg/l) 24 h before DOX injection and continuously until the mice were euthanized.

Measurement of in vitro cardiac function. Mice were injected with sodium heparin (500 U/kg ip) 20 min before anesthesia with pentobarbital sodium (120 mg/kg ip). Hearts were cannulated and retrogradely perfused at 37°C by using a modified Langendorff technique with Krebs-Henseleit bicarbonate buffer as previously described (30). Left ventricular (LV) pressure was monitored through a pressure transducer. After a 30-min preliminary perfusion, LV pressure and heart rate (HR) were continuously recorded and analyzed with a computerized data acquisition and analysis system (DATAQ Instruments; Akron, OH). Coronary effluent was collected and measured every 15 min.

Analysis of in vivo cardiac function. Mice were randomly assigned to six groups as follows: control, DOX, MEL, DOX + MEL, 6-OH MEL, and DOX + 6-OH MEL. MEL (0.5 mg in 0.1 ml of 10% alcohol) or 6-OH MEL (0.5 mg in 0.1 ml of 10% DMSO) was administered via an ALZET microosmotic pump at a constant delivery rate of 2.5 μl/h. An equivalent volume of vehicle was used in the control mice. A single dose of DOX (25 mg/kg ip) or an equivalent volume of saline was injected after 24 h. The plasma level of MEL as determined by radioimmunoassays (ALPCO Diagnostics; Windham, NH) was maintained at 14.8–13.1 ng/ml in MEL-administered mice. Five days after DOX or saline administration, mice were injected with heparin (500 U/kg ip) and anesthetized with chlordiazepoxide (360 mg/kg ip). Each mouse was intubated with a 22-gauge soft catheter and ventilated with a rodent ventilator (Columbus Instruments International; Columbus, OH) at a tidal volume of 0.3–0.5 ml and a respiratory rate of 120 breaths/min. After a left thoracotomy, the pericardium was dissected to expose the heart. A 26-gauge needle connected to a pressure transducer was introduced into the LV after an apical stab to measure the LV pressure. Two pairs of 1-mm piezoelectric crystals were attached to the apex, aorta root, anterior, and posterior wall of the heart. Intracardiac distance, LV pressure, and the
electrocardiogram were recorded. Cardiac function parameters, including LV end-diastolic pressure (LVEDP), LV end-systolic pressure (LVESP), the first derivatives of LV pressure over time (±dP/dt), stroke volume (SV), and cardiac output (CO), were analyzed with a SonoSoft data acquisition and analysis system (SonoMetrics; London, Ontario, Canada). At the end of the function analysis, animals were killed with an overdose of pentobarbital sodium (120 mg/kg ip), and blood was collected for determination of CPK levels. Hearts were removed and perfused for 2 min as Langendorff preparations to remove the remaining blood. Portions of the midventricle were fixed for morphological and apoptosis studies. The remaining heart was stored in liquid nitrogen until use.

Ultrastructural examination of the heart. Hearts were fixed with 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) at room temperature for 4 h. Subsequently, the hearts were osmicated in 1% osmium tetroxide. After dehydration with a series of ethanol and propylene oxide, the samples were embedded in an Araldite-dodecenyl succinic anhydride mixture. Ultrathin sections of the LV wall were obtained with a diamond knife, after which they were double stained with uranyl acetate and lead citrate. Samples were observed with a Philips EM 201C microscope. At least three tissue blocks from each treatment group were randomly picked for the ultrastructural study.

TUNEL and ISOL analysis. A 2-mm section of midventricles was sliced, fixed in 4% buffered formaldehyde for 24 h, dehydrated, and embedded. Myocardial sections (5 μm) were mounted on siliconized slides and dried at 37°C overnight. A TUNEL procedure for detecting apoptotic cardiomyocytes was performed by a CardioTACS kit according to the manufacturer’s instructions. In situ staining of DNA strand breaks in the serial section of each specimen was detected by the ApopTag ISOL kit using oligo A as previously described (6). The percentage of TUNEL- or ISOL-positive myocytes was determined by counting 10 random fields per section under an Olympus BX40 microscope.

Cell death ELISA. Frozen heart tissue was pulverized under liquid nitrogen, and a portion of the heart powder was lysed in the buffer provided in the Cell Death Detection ELISA Plus kit. Samples were processed according to the manufacturer’s instructions to determine the level of cytosolic mono- and oligonucleosomes. The protein content of the heart samples was determined by a dye-binding assay (Bio-Rad; Hercules, CA).

Determination of serum CPK activity. Mouse blood samples were collected at the time of death and allowed to coagulate at 4°C for 1 h before centrifugation. Total serum CPK activities were assayed using a CPK test kit. One unit of CPK was defined as the reduction of 1 μmol NAD+ to NADH per minute in BIS-TRIS buffer (pH 6.9) at 25°C.

Effect of MEL or 6-OH MEL on the antitumor activity of DOX. PC-3 is a DOX-sensitive cell line that has been widely used to test the chemotherapeutic efficacy of DOX (29). PC-3 cells were cultured in F12-K medium supplemented with 10% fetal bovine serum. Approximately 3,000 PC-3 cells were plated into 96-well dishes and allowed to attach overnight, after which a dose-response study of DOX at a concentration range of 0.1 nM–1 μM was conducted. MEL or 6-OH MEL at various doses was added 3 h before the administration of 80 nM DOX. The number of surviving cells was measured 4 days after DOX application with a sulforhodamine B colorimetric assay as previously described (39).

Statistical analysis. All values were expressed as means ± SE. Statistical differences between each group were determined by a χ²-test in the survival study. In other studies, one-way ANOVA was used, followed by Tukey’s multiple comparison test if there were significant differences between groups. Significance was indicated if P < 0.05.

RESULTS

Antioxidant activities of MEL, 6-OH MEL, and 8-M-PDOT. The structures and antioxidant activities of MEL, 6-OH MEL, and 8-M-PDOT are shown in Fig. 1, A and B, respectively. 6-OH MEL was as potent as MEL in regard to its antioxidant activity. In contrast, 8-M-PDOT, a potent agonist of MEL receptors, had no antioxidant activity.

General observations and mortality in the in vivo study. The general appearances of mice from each group (control, DOX, MEL, DOX + MEL, 6-OH MEL, DOX + 6-OH MEL, 8-M-PDOT, and DOX + 8-M-PDOT) were similar. Mortality, which was used to test the chemotherapeutic efficacy of DOX (29), was observed in the DOX and DOX + MEL groups. The survival rate was significantly lower in the DOX group (40% ± 10%) compared with the control group (80% ± 5%). Statistical analysis revealed a significant difference (P < 0.05) between the DOX and control groups. In the DOX + MEL group, the survival rate was significantly higher (55% ± 15%) than in the DOX group. The survival rate in the 6-OH MEL and DOX + 6-OH MEL groups was significantly lower than in the DOX group (20% ± 10%). The survival rate in the 8-M-PDOT and DOX + 8-M-PDOT groups was significantly lower than in the DOX group (30% ± 10%).

Determination of serum CPK activity. Serum CPK activities were determined by a colorimetric assay as previously described (39). CPK was defined as the reduction of 1 mol NAD+/H to NADH by 10.2 ± 0.3 units on a microplate reader (Bio-Rad). CPK activities were assayed using a CPK test kit. One unit of CPK was defined as the reduction of 1 μmol NAD+ to NADH per minute in BIS-TRIS buffer (pH 6.9) at 25°C. The CPK activities in the DOX and DOX + MEL groups were significantly higher than in the control group. The CPK activities in the DOX + 6-OH MEL group were significantly lower than in the DOX group.

Effect of MEL or 6-OH MEL on the antitumor activity of DOX. The structures and antioxidant activities of MEL, 6-OH MEL, and 8-M-PDOT are shown in Fig. 1, A and B, respectively. 6-OH MEL was as potent as MEL in regard to its antioxidant activity. In contrast, 8-M-PDOT, a potent agonist of MEL receptors, had no antioxidant activity.

General observations and mortality in the in vivo study. The general appearances of mice from each group (control, DOX, MEL, DOX + MEL, 6-OH MEL, DOX + 6-OH MEL, 8-M-PDOT, and DOX + 8-M-PDOT) were similar. Mortality, which was used to test the chemotherapeutic efficacy of DOX (29), was observed in the DOX and DOX + MEL groups. The survival rate was significantly lower in the DOX group (40% ± 10%) compared with the control group (80% ± 5%). Statistical analysis revealed a significant difference (P < 0.05) between the DOX and control groups. In the DOX + MEL group, the survival rate was significantly higher (55% ± 15%) than in the DOX group. The survival rate in the 6-OH MEL and DOX + 6-OH MEL groups was significantly lower than in the DOX group (20% ± 10%). The survival rate in the 8-M-PDOT and DOX + 8-M-PDOT groups was significantly lower than in the DOX group (30% ± 10%).

Determination of serum CPK activity. Serum CPK activities were determined by a colorimetric assay as previously described (39). CPK was defined as the reduction of 1 mol NAD+/H to NADH by 10.2 ± 0.3 units on a microplate reader (Bio-Rad). CPK activities were assayed using a CPK test kit. One unit of CPK was defined as the reduction of 1 μmol NAD+ to NADH per minute in BIS-TRIS buffer (pH 6.9) at 25°C. The CPK activities in the DOX and DOX + MEL groups were significantly higher than in the control group. The CPK activities in the DOX + 6-OH MEL group were significantly lower than in the DOX group.
were observed after treatment. At the end of the experiment, the surviving mice in the DOX, DOX + MEL, and DOX + 6-OH MEL groups had common symptoms, including weight loss, diarrhea, and ascites. However, these symptoms were much more severe in the DOX and DOX + 8-M-PDOT groups than in other groups.

The survival rates of treated mice from each group are shown in Table 1. Whereas only 40–50% of the mice treated with DOX or DOX + 8-M-PDOT survived for 5 days, almost all animals that received MEL or 6-OH MEL survived for 5 days. Because the water consumption of each mouse (30 g) is 5 ml/day, it is estimated that 50 µg/day MEL (or 1.67 mg·kg⁻¹·day⁻¹) would be adequate to protect the animals against the acute toxicity of DOX. Because the animal survival rate improved when mice were treated with a lower dose of DOX (22.5 mg/kg), this dose was chosen for all subsequent in vivo studies.

In vitro cardiac function. A dose-response study was initially conducted with a DOX concentration ranging from 0.5 to 50 µM. In this study, DOX significantly depressed cardiac function at concentrations higher than 1 µM. Perfusion of heart with 5 µM DOX for 60 min resulted in a 40% reduction of HR and a 30% suppression of dP/dt (Fig. 2A and B). To better evaluate the cardiac function, HR × LVDP was used to assess the LV performance, as it reflects both chronotropic and inotropic activities of the heart. By this measure, perfusion of 5 µM DOX resulted in a 50% decrease of HR × LVDP compared with the control group (Fig. 2C). In addition, DOX caused a 45% decrease of coronary flow rate after 60 min of perfusion (Fig. 2D).

Exposure of mouse hearts to 1 µM MEL or 6-OH MEL 5 min before DOX treatment, followed by perfusion of the heart with a buffer containing 5 µM DOX and 1 µM MEL, restored HR × LVDP to 85% of the basal level (Fig. 2C). Treatment of the hearts with MEL or 6-OH MEL almost abolished the DOX-induced reduction of coronary flow (Fig. 2D). However, treatment of the heart with 1 µM 8-M-PDOT failed to restore cardiac function or coronary flow. Because cardiac performance is closely related to the coronary flow rate, these results indicate that MEL or 6-OH MEL, but not 8-M-PDOT, improves the cardiac performance of DOX-treated mouse hearts by restoring coronary circulation.

In vivo cardiac function. A slight increase in the heart weight-to-body weight ratio was observed in DOX-treated mice compared with control mice 5 days after treatment (4.7 ± 0.13 vs. 4.4 ± 0.14, P > 0.05), but this change was not significant and could be attributed to the increased weight loss in DOX-treated mice. Table 2 summarizes the hemodynamic indexes (HR, LVESP, ±dP/dt, SV, and CO) of all surviving mice 5 days after treatment. DOX caused significant alterations of cardiac performance at an acute dose of 22.5 mg/kg. LVESP was higher in the DOX group than in the control group (P < 0.05), indicating that DOX impaired ventricular diastolic properties of the heart. LVESP and ±dP/dt, two parameters of cardiac contractility, were significantly decreased in the DOX group.

There was no change in the LV end-diastolic dimensions among the groups (data not shown), which indicates that DOX treatment does not induce cardiac dilatation. Although HR in the DOX group was also not significantly lower than HR in the other groups, there was a 62% reduction in CO, which is attributed to a corresponding decrease in SV. Administration of either MEL or 6-OH MEL dramatically improved all of the aforementioned cardiac function parameters. These data indicate that MEL or 6-OH MEL improves DOX-impaired ventricular contractile function and compliance, which is consistent with the results from the in vitro mouse heart perfusion.

Morphological study. Electron and light microscopic analyses of LVs were carried out in all six groups of mice. No remarkable histopathological changes under the light microscope were observed between the control and DOX-treated heart stained with hematoxylin and eosin. However, an electron microscopic study demonstrated extensive cardiac damage in DOX-treated mice characterized by mitochondrial degeneration and swelling, intracytoplasmic vacuolization, and focal myofibril disarray (Fig. 3, B and C). Cellular damage was reduced dramatically by the administration of MEL or 6-OH MEL. No myofibril disarray was identified in the hearts of DOX + MEL or DOX + 6-OH MEL mice, although a few intracytoplasmic vacuoles were observed in the hearts of these mice (Fig. 3, D and E). MEL- and 6-OH MEL-treated mice showed normal ultrastructure of the heart (results not shown). The results indicate that MEL or 6-OH MEL is able to protect the integrity of subcellular structure of DOX-treated mouse hearts.

Detection of apoptosis. To explore the effects of MEL and 6-OH MEL on apoptosis, TUNEL and ISOL methods were used to identify apoptotic myocytes in DOX-treated mouse hearts. Because the TUNEL assay may stain DNA fragments deriving from nonapoptotic processes, experiments were performed in the serial sec-

Table 1. Effect of MEL, 6-OH MEL, or 8-M-PDOT on the mortality of DOX-treated mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Survival Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8/8</td>
</tr>
<tr>
<td>DOX</td>
<td>8/20*</td>
</tr>
<tr>
<td>MEL</td>
<td>8/8</td>
</tr>
<tr>
<td>DOX + MEL</td>
<td>20/20</td>
</tr>
<tr>
<td>6-OH MEL</td>
<td>8/8</td>
</tr>
<tr>
<td>DOX + 6-OH MEL</td>
<td>11/12</td>
</tr>
<tr>
<td>8-M-PDOT</td>
<td>8/8</td>
</tr>
<tr>
<td>DOX + 8-M-PDOT</td>
<td>8/16</td>
</tr>
</tbody>
</table>

Mice were injected with a single dose of doxorubicin (DOX, 25 mg/kg, ip) or an equivalent volume of saline. The mice were given melatonin (MEL), 6-hydroxymelatonin (6-OH MEL), or 8-methoxy-2-propionamidotetralin (8-M-PDOT) in drinking water (10 mg/l) 24 h before injection and until euthanasia. The number of surviving mice was observed 5 days after treatment. *P < 0.05, DOX vs. other groups except the DOX + 8-M-PDOT group.
MEL attenuates DOX cardiotoxicity

Fig. 2. Effect of MEL, 6-OH MEL, or 8-M-PDOT on the cardiac function [heart rate (HR) (A), HR × left ventricular developed pressure (LVDP) (B)], and the first derivative of LV pressure over time (dP/dt; C) and coronary flow (D) of doxorubicin (DOX)-perfused mouse hearts. Hearts were perfused as Langendorff preparations for 60 min with Krebs-Henseleit buffer containing 5 μM DOX in the absence or presence of 1 μM MEL, 6-OH MEL, or 8-M-PDOT as described in MATERIALS AND METHODS. The functional parameters were expressed as the percentage of baseline values that were measured after 30 min of perfusion of each individual heart. Values are means ± SE of 5 hearts. A, C, and D: *P < 0.05, DOX vs. all other groups except DOX + 8-M-PDOT; bP < 0.05, DOX + 8-M-PDOT vs. all other groups except DOX; B: *P < 0.05, DOX vs. control (CON), MEL, 6-OH MEL, or 8-M-PDOT; P < 0.05, DOX + 8-M-PDOT vs. control, MEL, 6-OH MEL, or 8-M-PDOT.

Table 2. Effect of MEL and 6-OH MEL on in vivo cardiac function in DOX-treated mice

<table>
<thead>
<tr>
<th></th>
<th>HR, beats/min</th>
<th>LVEDP, mmHg</th>
<th>LVESP, mmHg</th>
<th>+dP/dt, mmHg/s</th>
<th>−dP/dt, mmHg/s</th>
<th>SV, μl</th>
<th>CO, ml/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>442 ± 25</td>
<td>4.8 ± 0.7</td>
<td>64.7 ± 3.8</td>
<td>2,638 ± 127</td>
<td>2,010 ± 248</td>
<td>9.7 ± 0.5</td>
<td>4.3 ± 0.5</td>
</tr>
<tr>
<td>DOX</td>
<td>388 ± 30</td>
<td>10.7 ± 2.1</td>
<td>37.6 ± 5.5</td>
<td>904 ± 156*</td>
<td>727 ± 153*</td>
<td>4.1 ± 0.4*</td>
<td>1.6 ± 0.3*</td>
</tr>
<tr>
<td>MEL</td>
<td>451 ± 29</td>
<td>3.0 ± 0.8</td>
<td>63.2 ± 3.2</td>
<td>2,664 ± 120</td>
<td>1,978 ± 163</td>
<td>9.2 ± 0.7</td>
<td>4.2 ± 0.6</td>
</tr>
<tr>
<td>DOX + MEL</td>
<td>415 ± 29</td>
<td>4.3 ± 0.8</td>
<td>60.2 ± 1.2</td>
<td>1,914 ± 95</td>
<td>1,629 ± 143</td>
<td>7.4 ± 0.8</td>
<td>3.2 ± 0.4</td>
</tr>
<tr>
<td>6-OH MEL</td>
<td>428 ± 31</td>
<td>4.5 ± 1.1</td>
<td>63.9 ± 4.4</td>
<td>2,384 ± 334</td>
<td>1,863 ± 299</td>
<td>9.5 ± 0.6</td>
<td>4.1 ± 0.5</td>
</tr>
<tr>
<td>DOX + 6-OH MEL</td>
<td>395 ± 29</td>
<td>5.8 ± 0.5</td>
<td>57.0 ± 5.2</td>
<td>1,774 ± 208</td>
<td>1,586 ± 121</td>
<td>7.7 ± 0.6</td>
<td>3.0 ± 0.5</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6 mice. Mice were injected with DOX (22.5 mg/kg ip) or saline. MEL or 6-OH MEL was administered via a microosmotic pump at a constant delivery rate of 2.5 μg/h 24 h before DOX treatment and continuously until euthanasia. Six of eight DOX-treated mice survived. In vivo cardiac function was measured by the SonoMetrics system as described in MATERIALS AND METHODS. HR, heart rate; LVEDP and LVESP, left ventricular end-diastolic and end-systolic pressure, respectively; dP/dt, first derivative of left ventricular pressure over time; SV, stroke volume; CO, cardiac output. *P < 0.05 vs. all other groups.

Sections from each heart to determine myocyte apoptosis by the ISOL method, which is more sensitive to double-stranded DNA breaks during apoptosis. The results showed that 15 ± 1.2% and 14 ± 1.0% of cardiac myocytes in DOX-treated mice were TUNEL and ISOL positive, respectively (Fig. 4). Infusion of MEL or 6-OH MEL significantly attenuated apoptosis of cardiac myocytes in DOX-treated mice. The effect of MEL or 6-OH MEL was further determined by a semiquantitative cell death ELISA assay. Whereas DOX treatment resulted in a threefold increase of cytosolic mono- or oligonucleosomes compared with control mouse hearts, MEL or 6-OH MEL significantly inhibited DOX-induced DNA fragmentation (Fig. 5).

Effect on serum CPK level. Because DOX causes disruption of the cardiac myocyte membrane, the release of intracellular CPK into serum was used to evaluate the existence and extent of myocyte injury (8). Whereas DOX treatment resulted in a threefold increase of serum CPK activity compared with the control group (Fig. 6), administration of MEL or 6-OH MEL significantly decreased CPK release. These results indicate that MEL or 6-OH MEL reduces DOX-induced necrosis in mouse hearts.
Effect of MEL or 6-OH MEL on the antitumor activity of DOX. A dose-response study showed that the concentration of DOX at which 50% of PC-3 cells are killed (LC50) is 80 nM in the sulforhodamine B colorimetric assay. Treatment of cells with either MEL or 6-OH MEL at concentrations as high as 100 or 10 μM, respectively, did not change the LC50 of DOX (Fig. 7). These data indicate that MEL or 6-OH MEL does not change the efficacy of DOX.

DISCUSSION

The present study demonstrates several major findings regarding the effects of MEL on DOX-induced cardiotoxicity. First, we found that MEL can dramatically improve survival rates in mice treated with an acute high dose of DOX. Second, we showed that MEL is able to attenuate the acute effects of DOX-induced functional changes in mouse hearts. Third, we demonstrated that the cardioprotective effect of MEL could be attributed in part to the suppression of DOX-induced apoptosis of myocytes. Finally, we show that neither MEL nor 6-OH MEL compromises the antitumor activity of DOX.

Role of antioxidant activities of MEL and 6-OH MEL in cardioprotection. Previous work suggests that the cardioprotective effect of MEL can be attributed mainly to its antioxidant activity. For instance, MEL has recently been shown to attenuate DOX-induced changes in both the GSH-to-GSSG ratio and lipid peroxidation in the brain, heart, lung, and kidney (1). Our study expands on this previous work by providing more evidence for the importance of the antioxidant activity of MEL in its cardioprotective effect. Specifically, administration of MEL or 6-OH MEL dramatically improved the survival rates of DOX-treated mice; in contrast, administration of 8-M-PDOT along with DOX did not improve survival rates (Table 1). Because MEL and 6-OH MEL have similar antioxidant activities and because 8-M-PDOT does not have free radical-scavenging activity (Fig. 1B), this result sug-
Fig. 4. Protective effects of MEL or 6-OH MEL on apoptosis of DOX-treated mouse hearts. Paraffin-embedded myocardial sections were stained by TdT-mediated dUTP nick-end labeling (TUNEL) (A) or in situ oligo ligation (ISOL) procedures (B). Immunolabeled nuclei of myocytes were determined by random counting of 10 fields/section. Bars represent the means ± SE of 6 hearts. aP < 0.05, DOX vs. control, DOX + MEL vs. MEL, or DOX + 6-OH MEL vs. 6-OH MEL. bP < 0.05, DOX vs. DOX + MEL or DOX + 6-OH MEL.

Fig. 5. Protective effects of MEL or 6-OH MEL on DNA fragmentation. The cytosolic mono- and oligonucleosomes of mouse hearts were quantified by a Cell Death ELISA Plus kit as described in MATERIALS AND METHODS. Values are means ± SE of 6 mouse hearts. aP < 0.05, DOX vs. all other groups.

Fig. 6. Serum creatine phosphokinase (CPK) levels in mice from treated groups. Blood samples were collected from treated mice, and serum CPK was measured as described in MATERIALS AND METHODS. Values are means ± SE of 5–8 mice. aP < 0.05, DOX vs. all other groups.

Fig. 7. Effects of MEL (A) and 6-OH MEL (B) on the growth of PC-3 cells in the absence (open bars) or presence of DOX treatment (solid bars). Cells were plated in 96-well plates and allowed to attach overnight. Various doses of MEL (0.1–100 μM) (A) or 6-OH MEL (0.01–10 μM) (B) were added 3 h before the application of 80 nM DOX. The number of surviving cells 4 days after treatment was measured by a sulforhodamine B colorimetric assay and expressed as optical density per well. Values are means ± SE of 6 wells. aP < 0.05, DOX vs. control; bP < 0.05, DOX vs. DOX + MEL or DOX + 6-OH MEL.
gests that the improved survival rates for animals treated with MEL or 6-OH MEL may be due to their antioxidant activities.

Furthermore, MEL or 6-OH MEL, but not 8-M-PDOT, reversed DOX-induced functional changes, including impaired contractility and diastolic properties, decreased coronary flow rate, and reduced HR in the perfused mouse heart (Fig. 2). Thus reduction of HR after DOX perfusion supports the results of a previous study (12). However, the mechanism of this reduction remains unclear. It is possible that DOX or DOX-induced reactive oxygen species formation causes disturbances in calcium homeostasis. This could lead to a reduction of HR because a decrease in intracellular calcium can induce reduced excitability of pacemaker cells in the sinoatrial node and other cells in the cardiac conduction system. The reduction of coronary flow may have resulted from DOX-induced coronary vascular constriction. DOX-generated superoxide radicals are able to form peroxynitrite in the presence of nitric oxide (NO) (42), possibly leading to a decrease in endogenous NO and a subsequent constriction of coronary vessels. This hypothesis is supported by the fact that an increase in coronary resistance has been observed in the DOX-perfused rat heart by Pelikan et al. (27). If this hypothesis is indeed correct, then it is possible that the reversal of DOX-induced reduction of coronary flow by MEL and 6-OH MEL was due to their ability to scavenge superoxide radicals. Clearly, however, more studies would need to be performed to definitively establish such a conclusion.

The in vivo cardiac performance of mice was measured by using a recently developed ultrasound-based technique (13). Our in vivo study revealed a pronounced elevation of LVEDP and decrease of LVESP, as well as a consequent reduction of SV and CO, even without cardiac hypertrophy and dilatation. However, administration of MEL or 6-OH MEL reversed DOX-induced depressions of SV, LVESP, and LVEDP by improving cardiac contractility (Table 2).

Effects of MEL and 6-OH MEL on DOX-induced functional changes. In our study, DOX caused myocardial damage characterized by cytoplasmic vacuolization, mitochondrial swelling, and myofibril disarrangement (Fig. 3), which is consistent with the results observed in a previous study (3). The DOX-induced mitochondrial injury is especially important to the heart because it would presumably have disastrous effects on cardiac myocytes; indeed, because mitochondria are the primary sites of energy production, mitochondrial injury would severely compromise the contractile function of cardiac myocytes by restricting energy metabolism. It has been previously established that DOX induces mitochondrial injury in the heart through generation of superoxide anions, which can be attenuated by overexpression of MnSOD in mice (44). This suggests that the improvement of cardiac function by MEL or 6-OH MEL in our study can be attributed at least in part to the preservation of the subcellular integrity of cardiac myocytes.

Effects of MEL and 6-OH MEL on DOX-induced apoptosis and necrosis. Several studies have reported that cardiac injuries induced by anthracycline antibiotics involve apoptosis of cardiomyocytes (2, 22, 41, 45). As shown in Figs. 4 and 5, MEL and 6-OH MEL dramatically attenuated DOX-induced apoptosis. This result supports a recent study (21) that showed that oxidative stress is involved in DOX-induced apoptosis and that the stress could be ameliorated by antioxidants in cultured endothelial cells and cardiomyocytes (21).

Because DOX triggers membrane peroxidation and disruption, DOX-induced necrosis can be indicated by the amount of CPK released to the blood or culture medium (8). As shown in Fig. 6, MEL or 6-OH MEL treatment diminished serum CPK elevation in DOX-treated mice. The result indicates that MEL or 6-OH MEL may protect myocytes against membrane damage induced by DOX, thereby protecting their structure and function.

The present study was designed to measure hemodynamic changes in response to DOX administration as exemplified in the in vitro studies and to determine the magnitude of DOX-induced cardiotoxicity after 5 days. As such, the mechanisms of DOX-induced cardiac dysfunction in our in vitro and in vivo studies are possibly different. In our in vitro studies, cardiac dysfunction occurs in the absence of structural changes because DOX-induced superoxide anions are known to compromise the mitochondrial functions by inhibiting mitochondrial complex I activity as well as by disruption of the mitochondrial permeability transition pore (43). The ensuing increase in apoptosis occurs after 48 h of DOX administration (result not shown). Five days after DOX administration, there was no cardiac hypertrophy or cardiac dilatation in DOX-treated hearts. Therefore, our model is different from DOX-induced chronic cardiomyopathy in patients or experimental animals. In our in vivo study, there were no remarkable histopathological lesions observed under the light microscope, an observation that was in agreement with a previous study (44) in mice overexpressing MnSOD. However, apoptosis and ultrastructural damages of the mitochondria and myofibrils are very evident, and these changes are certainly a major factor in cardiac dysfunction.

Effects of MEL or 6-OH MEL on the efficacy of DOX. PC-3 cells are widely used to investigate the cytotoxic effects of various anticancer drugs, including DOX and other anthracycline derivative (29). In our study, incubation of PC-3 cells with either MEL or 6-OH MEL did not have any antagonistic effect on the cytotoxic activities of DOX (Fig. 7). These results are inconsistent with previous reports that MEL can enhance the antitumor activities of DOX in certain tumor-bearing mice and that MEL can increase the efficacy of other cancer chemotherapy in cancer patients (23, 40). This discrepancy may be attributed to differences in experimental models and conditions, or it could be due to a difference in the response of different tumors to MEL. Although the synergistic effect of MEL was not observed in our
H262 MEL ATTENUATES DOX CARDIOTOXICITY

cultured PC-3 cell system, our results show clearly that MEL or 6-OH MEL does not interfere with the antitumor activity of DOX.

In summary, our study demonstrates that MEL or 6-OH MEL protects against DOX-induced cardiac injury by inhibiting necrosis and apoptosis, thereby improving DOX-damaged cardiac function. The exact mechanism of their cardioprotective effect needs to be explored further; however, because MEL and 6-OH MEL do not compromise the antitumor effect of DOX, the combined treatment of DOX and MEL holds promise as a safe and effective chemotherapeutic strategy.

We thank Dr. Bill James (Intergen) for providing the ApopTag in situ oligo ligation kit and Dr. Race L. Kao for critical reading of the manuscript. We also greatly appreciate the excellent technical assistance of the Core Facility of the University of Michigan with the use of the oligo ligation kit and Dr. Race L. Kao for critical reading of the manuscript. This work was supported in part by a grant from the Department of Veterans Affairs Merit Review and by a grant-in-aid from the American Heart Association. X. Liu was a recipient of an American Heart Association Summer Student Research Award.

REFERENCES

11. Dubocovich ML, Masana MI, Iacob S, and Sauri DM. Melatonin receptor antagonists that differentiate between the human Mel1a and Mel1b recombinant subtypes are used to assess the pharmacological profile of the rabbit retina ML1 presynaptic heteroreceptor. Naunyn Schmiedebergs Arch Pharmacol 355: 365–375, 1997.