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Protection of Pifithrin-α and Melatonin against Doxorubicin-Induced Cardiotoxicity.

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Protection of Pifithrin-α and Melatonin against Doxorubicin-Induced Cardiotoxicity

A dissertation presented to
the faculty of the Department of Pharmacology
East Tennessee State University

In partial fulfillment
of the requirements for the degree
Doctor of Philosophy in Biomedical Science

by
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May, 2003

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Keywords: apoptosis, cardiotoxicity, doxorubicin, 6-hydroxymelatonin, melatonin, mitogen-activated protein kinases, p53, phosphorylation, pifithrin-α, reactive oxygen species
The current studies were designed to explore the protective effects of pifithrin-α and melatonin against doxorubicin-induced cardiotoxicity. Doxorubicin was injected at a dose of 22.5 mg/kg (i.p.) in mice to induce cardiotoxic effects. Meanwhile, doxorubicin caused a significant increase of cardiac cell apoptosis following injection (14.2 ± 1.1% for doxorubicin-5 d vs. 1.8 ± 0.12% for control, \( P < 0.01 \)). Ribonuclease protection assays and Western blot analyses revealed that doxorubicin upregulated the p53-dependent genes Bax, BclxL, and MDM2 at least 2-fold. p53 was phosphorylated at Ser 15 in mouse hearts 1 h following doxorubicin injection, and p38 and ERK1/2 MAPKs mediated the phosphorylation of p53. In addition, caspases-3 and -9 were activated 24 h after doxorubicin injection. A p53 inhibitor, pifithrin-α, inhibited doxorubicin-induced apoptosis when administered at a dose of 2.2 mg/kg. Pifithrin-α abolished p53 transactivation activity but did not influence doxorubicin-induced phosphorylation at Ser 15. By effectively inhibiting the expression of p53-dependent genes, pifithrin-α blocked doxorubicin-induced activation of caspases-3 and -9, thereby preventing cardiac apoptosis. In addition, pifithrin-α attenuated doxorubicin-induced structural and functional damages, without diminishing its anti-tumor efficacy on p53-null PC-3 cancer cells. The protective effects of melatonin and its metabolite 6-hydroxymelatonin on doxorubicin-induced cardiac dysfunction were evaluated in an isolated perfused mouse hearts and \textit{in vivo} doxorubicin-treated mice. While perfusion of mouse hearts with 5 µM doxorubicin for 60 min resulted in a 50% suppression of HR×LVDP and a 50% reduction of coronary flow, pre-exposure of hearts to 1 µM melatonin or 6-hydroxymelatonin eased the cardiac dysfunction. In addition, administration of melatonin or 6-hydroxymelatonin (2 mg/kg/d) significantly attenuated doxorubicin-induced cardiac dysfunction, myocardial lesions, and cardiac cell apoptosis. Melatonin and 6-hydroxymelatonin significantly improved the survival rate of doxorubicin-treated mice. Another melatonin analog, 8-methoxy-2-propionamidotetralin, did not show any convincing protection on either animal survival or on \textit{in vitro} cardiac function, presumably due to its lack of free radical-scavenging activity. Finally, neither melatonin nor 6-hydroxymelatonin compromised the anti-tumor activity of doxorubicin in cultured PC-3 cells. These studies suggest that pifithrin-α and melatonin have significant therapeutic potential for patients suffering doxorubicin-induced cardiotoxicity.
DEDICATION

To my mother and my wife for their love and encouragement
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There are many people to thank for making my Ph. D. dream a reality.

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Cardiotoxicity of Doxorubicin

Doxorubicin (DOX) and other anthracycline anti-tumor antibiotics are a group of glycosidic antibiotics isolated from cultures of *Streptomyces peucetius var. caesieus* in the 1960s (Arcamone and others 1969; Di Marco and others 1969). Their discovery represented a major breakthrough in the fight against human cancers, as the drug was found to be unusually potent in a variety of malignancies. Today, DOX and other anthracycline antibiotics remain as major weapons in the combat of human malignant diseases (Davis and Davis 1979; Rinehart and others 1974). Owing to its broad anti-tumor spectrum and high potency, DOX is currently a first-line drug in the chemotherapy of a variety of hematopoietic tumors such as leukemias and solid tumors including breast and ovarian cancers, lymphomas, sarcomas, and gastrointestinal neoplasm (Hortobagyi 1997). In fact, all malignancies except colon cancer are responsive to DOX therapy (Davis and Davis 1979).

Unfortunately, DOX and other anthracyclines induce a number of side effects. In addition to common side effects of cancer chemotherapy such as nausea, vomiting, alopecia, nasal and oral mucosal ulceration, and hematopoietic depression due to inhibition of dividable cells (Creasey and others 1976), DOX induces a unique cardiotoxicity leading to drug-resistant congestive cardiac failure (Rinehart and others 1974; Bristow and others 1978). Indeed, it has been found that the incidence of cardiac failure increases abruptly in patients receiving more than 500 mg/m² of DOX, a fact that significantly limits DOX’s application as a therapeutic agent.

Over the past three decades, DOX-induced cardiotoxicity has been extensively investigated, and various studies have been performed to minimize the cardiac side effects of DOX administration. In clinical studies, researchers have attempted to optimize the administration schedule of DOX. In one study, cardiac toxicity was partly reduced on a weekly
therapy instead of the conventional once every 3 week injection (Torti and others 1983). In contrast, another study suggested that 500 mg/kg is a risk-cumulative dose in patients (Lefrak and others 1973; Billingham and others 1978). Thus, it is still uncertain which dosage to use for DOX administration. In terms of potential treatments, various interventions based on reducing the number of free radicals produced by DOX have displayed some encouraging effects, but this research is far from being complete. In the end, DOX-induced cardiotoxicity remains an important issue, and the optimal strategy for its prevention has yet to be defined.

Effects of DOX on Cardiac Function

The cardiotoxicity of DOX has been subdivided into acute and chronic effects, depending on their occurrence following administration of the drug. Cardiac dysfunction is one of the most important features in both acute and chronic cardiotoxicity. It occurs in humans, animal models, and isolated heart preparations after exposure to DOX.

DOX-Induced Cardiac Dysfunction in Patients

Acute cardiotoxic effects of DOX include hypotension, tachycardia, pericarditis-myocarditis syndrome, left ventricular dysfunction, and various arrhythmias, which develop within minutes after DOX administration (Bristow and others 1978; Ferrans 1978). The hypotensive effect is believed to be the consequence of peripheral vascular dilatation, caused by DOX-induced release of histamine and catecholamines (Bristow and others 1983). The pericarditis-myocarditis syndrome tends to affect patients either with or without a previous history of cardiac disease. Pericarditis can occur alone, but most patients exhibited pericarditis accompanied by significant myocardial dysfunction; in some cases, these patients died of cardiogenic shock (Starkebaum and Durack 1975).

In contrast to the acute cardiotoxic effects of DOX, chronic DOX-cardiotoxicity develops several weeks or months after treatment, sometimes even having its onset after the course of
therapy has been completed. These delayed effects manifest as cardiomyopathy or as an insidious onset of congestive cardiac failure (Henderson and others 1978). Although some studies have shown that heart failure is directly related to the amount of myocyte damage that can be evaluated by endomyocardial biopsy (Bristow and others 1978), others have found that cardiac dysfunction is not perfectly proportional to myocardial morphological changes (Isner and others 1983). Therefore, it is difficult to accurately predict the severity of DOX-induced cardiotoxicity based on the endomyocardial biopsy.

There seems to be a relationship between the occurrence of cardiac failure and the cumulative dose of DOX. When patients receive cumulative doses of DOX higher than 500 mg/m², the incidence of cardiac failure reaches 30-50% (Lefrak and others 1973). Certain changes in the drug administration schedule, such as reducing the acute dose and increasing injection frequency (thus keeping the cumulative dose unchanged), have been shown to partly suppress the cardiotoxicity of DOX (Torti and others 1983). This suggests that the development of chronic cardiomyopathy is related to the acute peak drug levels. Thus, acute and chronic cardiotoxicity could be induced by the same mechanism.

**Effects on the In Vivo Cardiac Function of Animals**

Cardiotoxicity has been successfully induced in DOX-injected animals including mice, rats, rabbits, and dogs (Rosenoff and others 1975; Doroshow and others 1979; Unverferth and others 1985). In one study, DOX injection decreased cardiac output and left ventricular (LV) peak systolic pressure in rats within a few hours by 42% and 36%, respectively (Luo and others 1997). In another study, systolic and diastolic LV functions were decreased progressively in dogs after multiple injections of DOX (1 mg/kg/wk for 8 wk, then 1 mg/kg every other wk for another 8 wk); after 16 wk of DOX treatment, maximum systolic dP/dt and cardiac index were decreased by 25% and 40%, respectively, whereas left ventricular end-diastolic pressure (LVEDP) was increased 250% (Unverferth and others 1985). While diastolic dysfunction may
be an earlier sign of DOX-induced cardiotoxicity than systolic dysfunction, high doses of DOX produce both systolic and diastolic dysfunctions. Furthermore, cardiac dysrhythmia is sometimes observed in DOX-treated animals, but it is more difficult to study due to its paroxysmal nature. DOX-induced cardiac dysrhythmia in dogs ranges from acute tachycardia to delayed development of persistent atrial and ventricular ectopic dysrhythmias together with variable depression of atrial-ventricular conductance (Kehoe and others 1978). The severity of the rhythm changes does not appear to be directly dose-dependent. Overall, DOX-induced cardiac dysfunctions during \textit{in vivo} animal studies are similar to the dysfunction characteristics of DOX-induced cardiotoxicity in patients.

**Effects on Function of Isolated Heart Preparations**

DOX impairs cardiac contractility, relaxation, and compliance in isolated animal hearts as a Langendorff preparation at various concentrations (Pelikan and others 1986; Chen and others 1987; Pouna and others 1996; Platel and others 1999). The magnitude of cardiac dysfunction depends on the duration and dose of DOX administration. In isolated rat hearts perfused with 10 $\mu$M DOX, left ventricular developed pressure (LVDP) increased slightly at 30 min and steadily decreased thereafter to 76% at 70 min; in contrast, LVEDP increased 5-fold after 70 min of perfusion (Pelikan and others 1986). Elevated coronary resistance during DOX-perfusion could result in myocardial under-perfusion and dysfunction. In non-paced isolated hearts, perfusion of DOX induces a 30% decline in heart rate after 30-min perfusion (Ganey and others 1991). Apart from the isolated whole heart, other isolated cardiac preparations have also been used. For instance, in isolated heart papillary muscle preparations, DOX dose-dependently causes suppression of both positive and negative rate of force development ($\pm dP/dt$) and increased lipid peroxidation at a dose range of 10 $\mu$M to 1 mM, although these doses are much higher than the serum peak levels in DOX-administered patients (Lee and others 1991). In isolated atria preparations, DOX inhibits both contraction frequency and contractile forces at a
dose of 0.1 mM, but overexpression of catalase in mouse hearts greatly attenuates these effects. These studies suggest that free radicals play important roles in DOX-induced cardiac dysfunction (Kang and others 1996).

The relationship between DOX’s effects on isolated cardiac preparations and its clinical cardiotoxicity is unclear, since a single dose of DOX rarely causes heart failure in patients, whereas acute exposure to DOX predictably causes dysfunction of isolated preparations. Furthermore, the concentrations required to initiate dysfunction in isolated heart preparations are 10- to 100-fold higher than plasma concentrations of DOX (0.1 µM) in patients receiving chemotherapy (Benjamin and others 1977; Pelikan and others 1986). Nevertheless, DOX-induced dysfunction in isolated heart preparations resembles the cardiac dysfunction of in vivo animal studies in many aspects. The isolated heart preparation has provided evidence of free radical generation related to its cardiac dysfunction, and it may provide more insights into DOX-induced cardiotoxicity.

Myocardial Ultrastructural Damage

The decrease in number of cardiac myocytes has been described in the postmortem of cancer patients administered DOX soon after its clinical application (Lefrak and others 1973). Further studies indicate that ultrastructural changes are dose-related as well as time-related and that ultrastructural changes persist even months after DOX administration (Jaenke 1974). The earliest change following DOX-treatment in the human heart is sarcoplasmic vacuolization, which appears to be due to the swelling of the myocyte tubular system (Unverferth and others 1981). Subsequently, the vacuolar degeneration spreads to form large spaces in the cytoplasm, eventually leading to destruction of myofibrils and other structures. Mitochondrial damages, such as swelling, vacuolization, and disruption of cristae, have also been observed (Doroshow and others 1985). Studies of endomyocardial biopsies from patients indicate that pathological damage is progressive and could be graded according to changes observed under the electron
microscope (Billingham and others 1978; Mason and others 1978). The changes are graded on a scale of “0” to “3” according to Billingham et al., where “0” is defined as no change from normal; “1” as scanty cellular changes showing early myofibrillar loss or swelling of the sarcoplasmic reticulum (SR) or both; “2” as groups of cells affected with loss of contractile elements and vacuolization of the cytoplasm; and “3” as diffuse cell damages with marked changes (total loss of contractile elements, loss of organelles, mitochondrial and nuclear degeneration).

Early and late cardiac morphological changes have been fully investigated in numerous studies (Lambertenghi-Deliliers and others 1976; Van Vleet and others 1980). Mitochondrial damage with focal clumping of cristae, densification or swelling of the matrix, and the development of nucleolar segregation were found in mice 24 h after DOX administration (Merski and others 1976). A larger number of cells are involved and contain dilation of SR cisternae, extending to all intracellular compartments within 1 to 2 wk. Mitochondrial damage was not found 1 to 3 months after DOX injection; only sarcoplasmic vacuolization, loss of myofibrils and disruption of sarcomeres and intercalated disc existed in a number of cardiac myocytes (Bellini and Solcia 1985). This is different from alterations in hearts from rats and rabbits, in which mitochondrial damages have been consistently observed following DOX-treatment (Jaenke 1974; Doroshow and others 1979; Unverferth and others 1985). The chronic cardiomyopathy may represent the cumulative damages that result from repeated acute drug exposures; however, histopathological findings are almost the same in mice receiving single dose and multiple dose injections (Bellini and Solcia 1985). Although clinical studies indicate that histopathological damage does not correspond very well to the severity of cardiac dysfunction (Isner and others 1983), it is still used as a marker of DOX-induced cardiotoxicity. The histopathological damage is believed to result from DOX-generated reactive oxygen species (ROS), since the damage is dramatically attenuated or prevented by co-administration of the antioxidant probucol (Singal and others 1995), co-administration of the ion chelator dexrazoxane
(Della and others 1996), or the overexpression of endogenous antioxidants such as catalase, metallothionein, or manganese superoxide dismutase (MnSOD) (Kang and others 1996; Yen and others 1999; Sun and others 2001).

**Subcellular Effects of DOX**

In addition to ultrastructural alterations, dysfunction of SR and mitochondria occurs in hearts from DOX-treated animals. SR releases, sequesters, and stores calcium that determines both systolic and diastolic cardiac function of cardiac myocytes; thus, SR dysfunction leads to pronounced cardiac dysfunction. Mitochondrion is the energy metabolic center of oxidative phosphorylation and ATP synthesis. As such, injury to these organelles will inevitably induce cardiac contractility impairment.

**Effects on Mitochondria**

DOX-induced mitochondrial dysfunction plays a central role in its cardiotoxicity. Heart mitochondria isolated from rats treated with DOX for 4 to 8 wk show crista damage, swelling, and decrease of Ca\(^{2+}\) loading capacity (Zhou, Starkov, and others 2001). DOX also inhibits respiration of isolated mitochondria dose-dependently (Gosalvez and others 1974). The damage of mitochondrial function is related to the downregulation of adenine nucleotide translocase-1, a protein located in the inner mitochondrial membrane that plays a key role in aerobic energy metabolism and regulation of mitochondrial membrane pore transition in cardiac myocytes (Jeyaseelan and others 1997). DOX may also impair the cardiac mitochondrial DNA that encodes some important enzymes in the mitochondria. Studies have shown that DOX induces breaking of the mitochondrial DNA helix and slows down mitochondrial DNA synthesis (Ellis and others 1987). Mitochondrial DNA deletion, therefore, might occur in the hearts of rats or mice chronically treated with DOX (Adachi and others 1993; Serrano and others 1999). The incidence of mitochondrial DNA deletion increases with the dosage and duration of DOX
administration, and it can be decreased by co-administration of the antioxidant coenzyme Q10, suggesting that ROS are involved in the DNA deletion. Indeed, it is believed that mitochondrial DNA damage results from hydroxyl radicals reacting with mitochondrial DNA to cause the preferential hydroxylation of deoxyguanosine at C8, since mitochondria are deficient in DNA repair enzymes (Palmeira and others 1997).

Effects on SR

SR has two primary functional sites: longitudinal tubules and terminal cisternae. Longitudinal tubules sequester and transport Ca\(^{2+}\) to terminal cisternae, where Ca\(^{2+}\) is stored for subsequent release to the contractile apparatus. The release of Ca\(^{2+}\) from the terminal cisternae is itself triggered by an increase in intracellular Ca\(^{2+}\). Chronic DOX-treatment impairs SR function (Shadle and others 2000; Boucek, Jr. and others 1987), and such functional impairments are associated with decreased expression of the ryanodine receptor, a calcium-induced calcium release channel in cardiac SR (Dodd and others 1993). Some studies have found that DOX suppresses SR Ca\(^{2+}\)-ATPase and Ca\(^{2+}\) uptake capacity by selectively inhibiting their gene expression. The inhibitory effects of DOX on SR Ca\(^{2+}\)-ATPase gene transcription is mediated by DOX-generated H\(_2\)O\(_2\) as well as by the downstream up-regulation of Erg-1 gene via ERK mitogen-activated protein kinases (MAPK) (Arai and others 1998; Arai and others 2000). Other studies indicate that DOX stimulates Ca\(^{2+}\) release from SR and selectively opens Ca\(^{2+}\) channels localized in terminal cisternae, but it has no effects on the SR Ca\(^{2+}\) pump or the contractile apparatus. This suggests that DOX might decrease cardiac contractility by interfering with Ca\(^{2+}\) release (Zorzato and others 1985). Moreover, enzyme activities of adenyl cyclase and guanyl cyclase were inhibited at high doses in isolated SR preparations, which was believed to be the consequence of free radical generation and lipid peroxidation (Lehotay and others 1983; Singal and Pierce 1986). Therefore, DOX might inhibit the gene expression of key enzymes in the SR and disturb Ca\(^{2+}\) handling by induction of ROS.
Free Radicals and DOX-Induced Cardiotoxicity

As discussed above, many effects of DOX on the myocardium are related to the formation of ROS. The mechanism of DOX-induced cardiac injury has been an active area of investigation in the past three decades. It is believed that the cardiotoxicity of DOX is mediated by mechanisms distinct from those responsible for its anti-tumor effects, such as DNA intercalation and interference with the activities of DNA topoisomerase II (Keizer and others 1990; Minotti and others 1999). 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, impaired Ca\(^{2+}\) handling in SR, mitochondrial DNA damage and dysfunction, and induction of apoptosis (Myers and others 1977; Kim and others 1989; Ito Hiroshi 1990; Arai and others 1998; Arola and others 2000; Tokarska-Schlattner and others 2002). The role of free radicals in DOX-induced cardiotoxicity has been extensively studied and is accepted by most researchers. The current study aims to test the protective effects of antioxidants on this cardiotoxicity.

DOX Generates Free Radicals

The free radical generation theory in DOX-induced cardiotoxicity is well documented in the literature. Several lines of evidence indicate that DOX generates free radicals via enzymatic pathways in cardiac myocytes. As shown in Fig. 1, DOX is composed of a quinone-form aglycone group and an amino sugar group (Fig. 1A). The quinone moiety can be reduced to a semiquinone form (i.e., a free radical) by single electron donors, such as NADPH, cytochrome P-450 reductase, and NADH and NADH dehydrogenase (Fig. 1B). This has been shown to occur in liver microsomes, cardiac mitochondria, cultured cardiac myocytes, and rat hearts in numerous studies (Benjamin and others 1977; Bachur and others 1979; Doroshow 1983; Doroshow and Davies 1986; Davies and Doroshow 1986; Lee and others 1991; Sarvazyan 1996).
Once formed, the semiquinone transfers an electron to molecular oxygen, generating a superoxide anion when it cycles back to the quinone form.

Fig 1. DOX generates free radicals through enzymatic pathways. A, structure of DOX; B, DOX-induced free radicals via enzymatic pathways and antioxidant defense systems. NAD, nicotinamide adenine dinucleotide; NADH, reduced NAD; NADP, nicotinamide-adenine dinucleotide phosphate; NADPH, reduced NADP; GPx, glutathione peroxidase; GSSG, glutathione; GSH, reduced glutathione; SOD, superoxide dismutase.

Free radicals may also be generated via non-enzymatic mechanisms involving DOX and iron interactions. The Fe$^{3+}$ associates with the ketone and hydroxyl groups of C-11 and C-12 of DOX (Fig. 1A), followed by an internal redox reaction, wherein an electron flows from the C-14 hydroxyl group to iron, generating an Fe$^{2+}$-DOX free radical complex. This complex reduces oxygen to a superoxide anion, after which an electron flows from Fe$^{2+}$ to DOX, resulting in an
Fe\(^{3+}\)-DOX free radical complex. The complex is then rearranged to Fe\(^{2+}\)-DOX (aldehyde), which is oxidized to Fe\(^{3+}\)-DOX (aldehyde) as another molecule of oxygen is reduced to a superoxide anion (Zweier 1985). Based on this theory, an iron-chelator, dexrazoxane, has been tested and has displayed convincing protective effects in both acute and chronic cardiotoxicity models (Voest and others 1994; Della and others 1996). It is believed that the protective effects are due to the interference of dexrazoxane with the DOX-iron complex (Malisza and Hasinoff 1996).

ROS Cause DOX-Induced Cardiotoxicity

While ROS have been recognized to play important roles in DOX-induced cardiotoxicity, questions have been raised regarding how DOX-generated ROS selectively attack the heart, rather than other organs. Studies in mice indicate that the heart has relatively low levels of endogenous antioxidant enzymes superoxide dismutase (SOD) and catalase, compared to their amounts in the liver (Doroshow and others 1980). As such, the heart is more vulnerable to DOX-generated ROS insults. Several other factors should not be ignored. First, DOX-generated superoxide anions may be converted to hydrogen peroxide by superoxide dismutase. When DOX is incubated with isolated mitochondria from rat hearts, substantial amounts of superoxide anions are formed, accompanying the damage of mitochondrial function (Doroshow 1983). Superoxide anions may also be converted to more toxic hydroxyl radicals through the Fenton reaction in the presence of Fe\(^{2+}\) (Gille and Nohl 1997). On the other hand, the peroxynitrite (ONOO\(^{-}\)) is believed to be formed from the instantaneous reaction of superoxide anion and nitric oxide, since nitrotyrosine was observed in mouse hearts following DOX treatment (Weinstein and others 2000). Both hydroxyl radicals and peroxynitrite are highly cytotoxic ROS; they can initiate protein oxidation, lipid peroxidation, and membrane damages in cardiac myocytes. For instance, peroxynitrite results in modification of protein-bound tyrosine to 3-nitrotyrosine, potentially leading to the malfunction of proteins (Beckman and Koppenol 1996). Studies have
shown that lipid peroxidation occurs in DOX-treated hearts. The malondialdehyde (MDA), a lipid peroxidation product, was increased in isolated rat LV papillary muscles exposed to 100 µM DOX for 60 min (Lee and others 1991). The serum creatine phosphokinase (CPK) and cardiac troponin T (cTnT) were elevated in DOX-treated patients and experimental animals as well (O’Brien and others 1997; Herman and others 1998; DeAtley and others 1999). Moreover, free radicals can impair sequestration of Ca\(^{2+}\) by the SR. For example, Ca\(^{2+}\) sequestration was inhibited by 70% in rat cardiac SR pre-incubated for 30 min with DOX and NAD/NADH dehydrogenase; the inhibitory effects were attenuated by incubation with catalase and free radical scavengers, N-acetylcysteine and glutathione (Harris and Doroshow 1985). The role of ROS in DOX-induced cardiotoxicity has been recently further supported by experiments using transgenic technology. DOX-induced cardiotoxicity can be largely suppressed by the overexpression of antioxidant enzymes MnSOD, metallothionein, or catalase in mouse hearts (Yen and others 1996; Kang and others 1997; Kang and others 2002). In summary, ROS play pivotal roles in DOX-induced cardiotoxicity, and antioxidants may minimize the cardiac injury induced by DOX-generated ROS.

Effects of Antioxidants on DOX-Induced Cardiotoxicity

A number of free radical scavengers have been shown to protect the heart against DOX-induced cardiotoxicity. These include α-tocopherol, N-acetylcysteine, probucol, and dexrazoxane (Wang and others 1980; Herman and others 1985; Siveski-Illiskovic and others 1995; Della and others 1996; Nazeyrollas and others 1999). Dexrazoxane (Zinecard®), which is the most effective cardioprotective thus far in DOX-induced cardiotoxicity, prevents superoxide anion formation by associating with Fe\(^{2+}\) and by blocking the Fenton reaction (Malisza and Hasinoff 1996). Unfortunately, all these compounds have pronounced clinical disadvantages. The protective effects were not consistently observed in DOX-induced cardiotoxicity (Breed and others 1980; Legha and others 1982; Wang and others 1980). Probucol, a lipid-lowering
antioxidant, confers significant protection against DOX-induced cardiotoxicity (Siveski-Iliskovic and others 1995); however, concerns about its HDL-lowering property discourage its clinical application. A cytoprotective drug, amifostine, has also been approved by the FDA to protect against DOX-induced cardiotoxicity, but it is less potent than dexrazoxane and does not prevent the mortality and weight loss caused by DOX in spontaneously hypertensive rats (Herman and others 1994). Dexrazoxane, the only cardioprotective drug currently available clinically, only reduces 50% of DOX-related cardiac complications (Hasinoff 1998). Moreover, it interferes with the anti-tumor activity of anthraeycline antibiotics and potentiates DOX’s myelosuppression (Koning and others 1991; Sehested and others 1993).

Another antioxidant, melatonin (MEL), the primary hormone of the pineal gland, has recently drawn attention (Fig. 2). In addition to its circadian rhythm regulation activity, antioxidant properties of MEL are well documented (Reiter and others 2000). It has been found that MEL acts as a powerful antioxidant and free radical scavenger of hydroxyl radicals (OH·), peroxyl radicals (ROO·), and superoxide anions (Reiter and others 1997). Indeed, MEL has been shown to be twice as potent as Trolox in removing peroxyl radicals (Pieri and others 1994), and is 5 and 14 times more effective in scavenging hydroxyl radicals than glutathione and mannitol, respectively (Hardeland and others 1993). The advantage of MEL is evident: unlike the confined distribution of vitamin C or vitamin E, MEL distributes readily in all subcellular compartments due to its water and lipid solubility. As such, it passes through the cell membrane easily and
enters cardiac cells to remove free radicals in situ. Studies have shown that MEL protects the heart and brain against apoptosis and ischemia-reperfusion injury by removing free radicals (Kaneko and others 2000; Chen and others 2001; Sun and others 2002). As a hormone, MEL may also exert its effects via MEL1a receptors in the heart. Some MEL analogs like 6-hydroxymelatonin (6-OH MEL) possess free radical scavenging capability (Dubocovich 1995), while others, such as 8-methoxy-2-propionamidotetralin (8-M-PDOT), bind to MEL receptors but have no antioxidant activities (Fig. 2). To determine if MEL protects the heart against DOX-induced cardiotoxicity and to explore the possible mechanisms involved, the effects of these compounds on mouse hearts were examined in the current study.

Apoptosis and DOX-Induced Cardiotoxicity

Until very recently, apoptosis was not known to be involved in DOX-induced cardiotoxicity (Arola and others 2000). As such, the mechanisms involved in DOX-induced cardiac apoptosis and the role of apoptosis in DOX-induced cardiac injury are far from being understood. Some studies indicate that apoptosis appears to contribute to the transition to heart failure and ischemia-reperfusion injury (Bialik and others 1997; MacLellan and Schneider 1997; Fadeel and others 1999). An important aspect of the current study is the investigation of the mechanism of DOX-induced cardiac apoptosis.

Characteristics and Pathways of Apoptosis

Apoptosis is a process of programmed cell death that plays a critical role in normal embryologic development, homeostasis, tumor surveillance, function of the immune system, and organ regression in the senescence process (Kerr and others 1972; van den Hoff and others 2000). Diseases occur as a consequence of excessive apoptosis or failure of apoptosis. In contrast to the classic necrosis resulting from an acute cellular injury, apoptosis is a genetically controlled process in which scattered single cells in a tissue actively participate in their own
demise. Morphologically, apoptotic cells “shrink abruptly” and chromatin forms “dense crescent-shaped aggregates” under the nuclear membrane (Kerr and others 1972). The plasma membrane integrity is initially preserved but subsequent convolution of the plasma membrane results in the formation of clusters of membrane-bound cellular organelles referred to as “apoptotic bodies” (Hengartner 2000; Saraste and Pulkki 2000). Unlike necrosis, apoptotic bodies are rapidly devoured by tissue macrophages or neighboring viable cells, so there is no tissue inflammation. Biochemically, apoptosis is initiated by activation of endogenous proteases that result in the cleavage of chromatin into oligonucleosome-size DNA fragments (Narula and others 1997; Hengartner 2000). DNA fragmentation is now accepted as a biochemical hallmark of apoptosis, which can be recognized in multiple experimental techniques including DNA laddering, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL), and in situ oligo ligation (ISOL) (Allen and others 1997; Didenko and others 1998; Anversa 2000; Stadelmann and Lassmann 2000). Other significant changes in apoptotic cells include phosphatidylserine translocation in the plasma membrane, initiation of caspase cascade, and activation of poly ADP-ribose polymerase (PARP) (Thornberry and Lazebnik 1998; Wieder and others 2001).

Previous studies indicate that apoptosis can be initiated in two principal pathways: intrinsic and extrinsic. The intrinsic pathway emerges from mitochondria, whereas the extrinsic pathway is activated by the stimulation of death receptors, including Fas, tumor necrosis factor-α (TNF-α)-receptor, and TNF-α-related apoptosis-inducing ligand (TRAIL)-receptor (Ashkenazi and Dixit 1998; Green and Reed 1998). While p53 and Bcl-2 family proteins play crucial roles in the intrinsic pathway, caspases relay apoptotic signals in both pathways. Numerous studies have shown that Bax and mitochondria mediate apoptosis of cardiac myocytes stimulated by hypoxia and ischemia, though other pathways have also been reported (Long and others 1997; Leri, Liu, and others 1998; Borutaite and others 2001; Kubasiak and others 2002). Activation of Bax was observed in apoptotic cardiac myocytes in human heart failure (Leri, Liu and others 2001).
1998). It has been reported that p53 mediates apoptosis of cardiac myocytes stimulated with angiotensin II (Pierzchalski and others 1997; Leri, Claudio, and others 1998). These studies strongly suggest that p53, Bax, and mitochondria play key roles in apoptosis of cardiac myocytes.

Roles of p53 in Apoptosis

The p53 tumor suppressor protein transmits signals arising from various forms of cellular stresses, including DNA damage, hypoxia, and nucleotide deprivation that induce cell cycle arrest and cell death (Yu and others 1997; Burns and el Deiry 1999; Regula and Kirshenbaum 2001). During cardiac failure, ischemia-reperfusion, and cardiac hypertrophy, p53 is heavily involved in cardiac myocyte apoptosis (Pierzchalski and others 1997; Leri, Liu, and others 1998; Fortuno and others 1999). The transcription activation of p53 appears to be an important mechanism for exerting its biological effects, though it has been demonstrated to induce apoptosis in the absence of transactivation in some cell lines (el Deiry 1998; Gupta and others 2001). The pro-apoptotic Bcl-2 family member Bax, apoptotic ligand Fas, and others have been discovered as the downstream genes (Miyashita and Reed 1995). The upstream events of p53 activation are less clear, but the endpoint of the upstream pathway is that p53 protein levels are dramatically increased through either posttranslational modifications such as phosphorylation or transcriptional regulations (Huang and others 1999; Marchenko and others 2000; Xie and others 2000).

p53 protein is composed of a conserved N-terminal transactivation domain, a proline-rich domain, a sequence-specific DNA-binding domain, a tetramerization domain, and a basic C-terminal tail (Fig. 3). There is much evidence that the transcription activities of p53 protein can be activated by phosphorylation, in addition to the elevation of the protein levels (Chernov and others 1998). In the past few years, considerable attention has been focused on the determination of phosphorylation sites within the N-terminal regulatory domain as well as the C-terminal domain. Indeed, p53 has been shown to be phosphorylated on these serine residues by a number
of kinases, including MAPKs at Ser 15, 20, and 389 (Huang and others 1999; Shieh and others 1999; She and others 2000), checkpoint kinase-2 at Ser 20 and 33 (Hirao and others 2000), and the DNA-dependent protein kinase at Ser 15 and Ser 37 (Shieh and others 1997; Smith and Jackson 1999).

On the other hand, murine double minute clone 2 (MDM2) is a key negative player in the regulation of p53 activation and degradation, which has a unique relationship with p53 (Haupt and others 1997; Oren 1999). MDM2 interacts with p53 at the N-terminal transactivation domain to inactivate the transcription activity and to induce ubiquitin-mediated proteolytic p53 degradation; on the other hand, p53 binds to the specific promoter region of MDM2 gene and stimulates its transcription (Prives 1998; Momand and others 2000). However, genotoxic or oxidative stresses induce modification of p53 and/or MDM2, particularly by phosphorylation, which blocks the interaction of MDM2 and p53, protecting p53 from degradation (Shieh and others 1997). This leads to an accumulation and elevation of intracellular p53 protein levels.

Fig. 3. Domain Structure of p53 Human Protein. Domains or regions are indicated as follows: activation and MDM2 binding domain (MDM2, TA), residues 1-60; growth suppression proline-rich region (PXXP), residues 63-97; DNA-binding domain (DBD), residues 100-300; tetramerization domain (TD), residues 323-356; and basic C-terminal domain (BD), residues 363-393. Potential phosphorylation sites at N-terminal are Ser 6, 9, 15, 20, 33, and 37, which are phosphorylated by several kinases, such as MAPKs, DNA-dependent protein kinase, and checkpoint kinase II (Bulavin and others 1999; Harris 1996; Sanchez-Prieto and others 2000; Tominaga and others 1999).

Increasing evidence indicates that p53 is involved in cardiac myocyte apoptosis (Ikeda and others 1999; Long and others 1997; Song and others 1999). The expression of ventricular
p53 and its target gene Bax increased dramatically with a significant increase of myocyte apoptosis following rapid ventricular pacing in dogs (Leri, Liu, and others 1998). On the other hand, mice can survive significantly longer after receiving heart transplantations from p53-deficient mouse donors (Hu and others 2000). Transfection of p53 into ventricular myocytes with the adenovirus induces apoptosis by initiating a mitochondria-dependent apoptotic pathway (Regula and Kirshenbaum 2001), and overexpression of Bcl-2 blocks p53-induced cardiac myocyte apoptosis (Kirshenbaum and de Moissac 1997). These studies indicate that p53 plays critical roles in myocyte apoptosis in pathophysiological conditions.

A chemical inhibitor of p53, pifithrin-α (PFT-α), has recently been found to deter p53-dependent apoptosis (Komarov and others 1999) (Fig. 4). This effect is believed to be the result of PFT-α inhibiting the p53 transactivation activity and its downstream events such as activation of caspases and PARP (Lorenzo and others 2002). Due to this action, PFT-α protects mice from lethal genotoxic stress associated with anti-cancer chemotherapy without promoting the formation of tumors (Komarov and others 1999). In addition, PFT-α protects neurons against apoptosis induced by DNA-damaging agents, amyloid beta-peptide, and glutamate (Culmsee and others 2001). The protection by PFT-α is correlated with declined p53 transcription activities and expression of Bax, the protection of mitochondrial dysfunction, and the inhibition of caspase activation.

While these data are interesting, few studies have been conducted on the roles of p53 in DOX-induced cardiac cell apoptosis and myocardial injury, and no study has been performed to examine the effects of PFT-α on DOX-induced cardiotoxicity. One of the aims of the current study is designed to examine the role of p53 in DOX-induced cardiac cell apoptosis and the effects of PFT-α on DOX-induced myocardial damages.

![Fig. 4. Structure of pifithrin-α](image)
Mitochondria and Bcl-2 Family Proteins

Recent studies indicate that roles of mitochondria in apoptosis are expanding in many types of cells (Green and Reed 1998; Wang 2001). Roles of mitochondria have been demonstrated in regard to two aspects: 1) Release of cytochrome c from mitochondria into the cytosol is one of three factors required for activation of downstream caspases in ischemic cardiac myocytes (Borutaite and others 2001). 2) Mitochondria inner membrane potential ($\Delta \psi_m$) is decreased during apoptosis (Susin and others 1998), and the loss of $\Delta \psi_m$ is mediated by the opening of a large multi-conductance channel, also known as the mitochondrial permeability transition pore (PTP). The mechanism of PTP regulation remains a mystery and is currently being investigated in many laboratories, but its regulation certainly involves the Bcl-2 family proteins (Marzo and others 1998).

Bcl-2 family proteins can be classified into anti-apoptotic and pro-apoptotic proteins according to their functions. Pro-apoptotic proteins in this family including Bax, Bad, Bid, Bak, Bcl-x, Bik, and Bim, promote cell death, while anti-apoptotic proteins, including Bcl-2, BclxL, and Bcl-w, inhibit cell death (Adams and Cory 1998). Bcl-2 proteins are located in the outer membrane of mitochondria or in the cytoplasm, and many other proteins in this family are associated with mitochondria as well (Reed and others 1998). These proteins have been implicated in the regulation of two important aspects of mitochondria pathophysiology: (1) PTP opening; and (2) release of apoptotic proteins from the mitochondria into the cytosol. Bcl-2 family proteins directly participate in the regulation of PTP. In one experiment, Bax was co-purified with the PTP protein complex from the mitochondrial membrane, implying that Bax may be closely associated with the component of the complex. When partially purified mitochondrial PTP protein complex was reconstituted in liposomes, they maintained some functions, including suppression of pore openings by recombinant Bcl-2, but not the mutant Bcl-2 protein (Zamzami and others 2000; Zamzami and others 1998).
Bcl-2 family proteins also function as channels relating to the release of cytochrome c. Their three-dimensional structures indicate that Bcl-2 or Bax can form different sized pore or ion channels. Generally, it is thought that cytoprotective proteins such as Bcl-2 tend to form small channels that assume a mostly closed conformation, whereas pro-apoptotic proteins such as Bax tend to form larger channels, assuming a mostly open conformation that prefers anions. Other mechanisms may be involved in the antagonism of these proteins; for example, Bcl-2 may be able to prevent channel formation by Bax under some conditions (Korsmeyer 1999). Regardless of the mechanism, the pro-apoptotic Bcl-2 family proteins induce the release of cytochrome c into the cytosol, initiating the activation of caspases in the presence of apoptotic protease activating factor-1 (Apaf-1) protein. Although Bcl-2 family proteins are involved in cardiac myocyte apoptosis in a myriad of insults including ischemia and hypoxic stress, their involvement in DOX-induced cardiotoxicity remains to be investigated (Kubasiak and others 2002; Kunisada and others 2002).

Caspases and Apoptosis

Caspases are a group of proteolytic enzymes that can specifically cleave the pro-enzymes into active enzymes (Thornberry and Lazebnik 1998). Caspases can be classified into two functional groups: the initiator caspases and the executioner caspases. Initiator caspases are responsible for processing and activating the executioner caspases including caspases-8 and -9. The executioner caspases such as caspases-3, -6, and -7, cleave a subset of proteins leading to characteristic morphological changes of apoptosis and DNA fragmentation. The roles of caspases in apoptosis have been greatly delineated in the past few years (Yue and others 1998; Earnshaw and others 1999; Hacker 2000; Communal and others 2002). In the intrinsic apoptotic pathway, the cytochrome c released from mitochondria functions as the caspase activator in the presence of Apaf-1. In the extrinsic pathway, death receptors mediate the activation of caspase-8, leading to the cleavage of executioner caspases such as caspase-3. One of the downstream
targets of caspases is caspase-activated DNase (CAD), which cleaves DNA into nucleosomal DNA fragments.

Studies have shown that several caspases are activated in p53-mediated apoptosis in many cell lines. Caspase-3 and caspase-9 are the major caspases activated in p53-mediated apoptosis. For example, caspase-3-like activities were increased in p53-dependent apoptosis in HEK 293 cells treated with presenilins (Alves and others 2002), caspases-3 and -9 are activated in p53-dependent apoptosis in human colon cancer cells (Bartke and others 2001), and overexpression of p53 induces an increase of caspase-1 mRNA in MCF-7 cancer cells (Gupta and others 2001). Interestingly, p53 induces apoptosis through the activation of caspases-3 and -9 in human endothelial cells exposed to DOX (Lorenzo and others 2002). Activation of caspase-3 has been demonstrated in apoptosis occurring in human cardiomyopathy (Narula and others 1999). At any rate, different caspases are activated in apoptosis, dependent on the apoptotic pathways. The current study is designed to determine which caspases are activated in DOX-induced cardiac cell apoptosis.

**DOX Induces Cardiac Cell Apoptosis**

While few studies on the roles of cardiac cell apoptosis in DOX-induced cardiotoxicity have been conducted, several lines of evidence granted an impetus to explore whether DOX induces cardiac myocyte apoptosis and whether apoptosis plays a role in its cardiotoxicity. First, superoxide anion or H$_2$O$_2$ induces cardiac myocyte apoptosis via mitochondrial pathways (von Harsdorf and others 1999). DOX is known to generate superoxide anion in cardiac myocytes as well as in intact hearts; it may initiate apoptotic pathways through ROS generation. Secondly, DOX causes mitochondrial morphological and functional damages (Lefrak and others 1973), leading to cardiac myocyte apoptosis by initiating mitochondria-dependent pathways. Mitochondria control the destination of cells and could mediate apoptosis in an intrinsic apoptotic pathway when cardiac myocytes are insulted by ROS (Bialik and others 1999; Cook
and others 1999). Finally, the inhibitory activity of DOX on topoisomerase II leads to DNA damage, activating p53 to induce cell arrest or apoptosis. In fact, DOX has been shown to induce acute cardiac myocyte apoptosis after DOX-treatment (Arola and others 2000; Nakamura and others 2000; Kalyanaraman and others 2002), although the possible involvement of apoptosis in DOX-induced cardiotoxicity remains to be determined.

Multiple mechanisms could be involved in DOX-induced apoptosis. DOX induces apoptosis through the ceramide pathway in cultured adult rat ventricular myocytes (Delpy and others 1999). Others have reported that upregulation of Bax releases cytochrome c from mitochondria, activating caspases in DOX-induced cardiac myocyte apoptosis (Gamen and others 2000; Childs and others 2002). Although p53 plays critical roles in apoptosis, its roles in DOX-induced cardiac apoptosis have not been studied. To seek an effective approach to protect against DOX-induced cardiac cell apoptosis and cardiotoxicity, one major aim of the current study is to examine the involvement of a p53-dependent apoptotic pathway in DOX-induced cardiac cell apoptosis in an in vivo murine model. In addition, the effects of PFT-α are investigated on DOX-induced cardiac cell apoptosis, ultrastructural alterations, and cardiac dysfunction.

**Rationale and Aims**

DOX-induced cardiotoxicity is an important issue, as the cardiotoxicity compromises DOX’s clinical application. Despite extensive investigation, prevention of DOX-induced cardiac injury has achieved limited success. Three approaches have been examined in previous studies: 1) Development of new anti-tumor drugs to replace the current anthracycline antibiotics; A number of new anthracyclines have emerged over the past decades, but even the better-tolerated epirubicin and highly potent idarubicin have not shown much improvement of cardiotoxicity (Arcamone and others 1997). 2) Change of the drug format by wrapping DOX in the liposome; newly developed pegylated liposomal DOX has demonstrated a lower cardiotoxicity, but
mucosal and skin toxicity increase the intolerability of this drug (Alberts and Garcia 1997; Waterhouse and others 2001). 3) Administration of cardioprotective agents along with DOX; several antioxidants such as the iron-chelator dexrazoxane have been demonstrated to slow or attenuate DOX-cardiotoxicity. Although dexrazoxane is the most effective in protecting against DOX-caused cardiac injury, its leukopenia, thrombocytopenia, and gastrointestinal toxicity when combined with DOX are of great concern (Von Hoff and others 1981; Kosty and others 2001). An ideal combination therapy would prevent DOX-induced cardiotoxicity without attenuating its anti-tumor activity or enhancing other side effects.

The current study is designed to examine the effects of PFT-α and MEL on DOX-induced cardiotoxicity. It is hypothesized that DOX induces cardiac cell apoptosis via the generation of ROS and that apoptosis contributes to DOX’s cardiotoxicity. ROS insult initiates the p53-dependent apoptotic pathway, in which pro-apoptotic protein Bax is upregulated and triggers the cytochrome c release from mitochondria to the cytoplasm. In the presence of Apaf-1, cytochrome c activates caspase cascades and eventually leads to DNA fragmentation. It is further hypothesized that cardiac apoptosis plays critical roles in DOX-induced cardiotoxicity; therefore, antioxidant MEL or p53 inhibitor PFT-α would attenuate DOX-cardiotoxicity via the blockade of cardiac cell apoptosis (Fig. 5). To test these hypotheses, an acute murine cardiotoxicity model was established as in a previous study (Weinstein and others 2000). DOX-induced cardiac cell apoptosis was detected by TUNEL or ISOL assay; and the apoptotic pathways were investigated with Western blot analysis, Ribonuclease protection assays (RPA), and gene SuperArray analyses. The protective effects of PFT-α and MEL on DOX-induced cardiac dysfunction and myocardial damages were analyzed. Finally, the anti-tumor activity of DOX in combination with PFT-α or MEL was analyzed in PC-3 prostate cancer cells.
Fig. 5. Hypothesized mechanisms of DOX-induced apoptosis and cardiotoxicity. CAD, caspase-activated DNase; ICAD, inhibitor of CAD; p38, a member of MAPKs; ERK1/2, extracellular signal-regulated protein kinase; MEK, a MAPK kinase; SAPK/JNK, stress-activated protein kinase/c-Jun N-terminal kinase.
CHAPTER 2
MATERIALS AND METHODS

Animals and Drug Treatments

Animals

Male ICR mice were purchased from Harlan (Indianapolis, IN), and C57BL/6 MEL1a receptor-deficient mice were kind gifts of Dr. Steven M. Reppert (Harvard Medical School). All mice were housed in climate-controlled facilities of the James H. Quillen College of Medicine, East Tennessee State University, accredited by the American Association for Laboratory Animal Care, and fed ad libitum. Animal protocols were approved by the Committee of East Tennessee State University on Animal Care.

Treatment of DOX, PFT-α, and MAPK inhibitors

DOX·HCl was purchased from Pharmacia and Upjohn (Kalamazoo, MI) or Sigma (St. Louis, MO). DOX solution (2 mg/ml) was injected (i.p.) at a single dose of 25 mg/kg for the survival study and 22.5 mg/kg for all other studies.

Pifithrin-α (Calbiochem; San Diego, CA) was dissolved in 1.5% dimethyl sulfoxide (DMSO) and administered 30 min before and 2 h after DOX treatment, at a dose of 2.2 mg/kg (i.p.) each. An equal volume of vehicle (1.5% DMSO in saline) was injected in control mice.

MAPK inhibitors SB 203580, PD 98059, and SP 600125 (Calbiochem, San Diego, CA) were dissolved in distilled H₂O, 1.5% DMSO, and a PPCES vehicle (30% PEG-400, 20% propylene glycol, 5% ethanol, 15% cremophor EL, and 30% normal saline), respectively. These inhibitors were administered by i.p. injection 30 min prior to DOX treatment. Control mice were injected with equivalent volumes of H₂O, 1.5% DMSO, or PPCES vehicle accordingly.
Table 1  Summary of Inhibitors Used in the Study

<table>
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<th>Inhibitor</th>
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<th>IC50</th>
<th>Effective dose</th>
<th>Solvent</th>
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<tr>
<td>SB 203580</td>
<td>p38</td>
<td>0.6 µM</td>
<td>5 mg/kg</td>
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<tr>
<td>PD 98059</td>
<td>MEK</td>
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<td>2.5 mg/kg</td>
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<td>SP 600125</td>
<td>JNK</td>
<td>90 nM</td>
<td>15 mg/kg</td>
<td>PPCES</td>
<td>(Bennett and others 2001)</td>
</tr>
<tr>
<td>Pifithrin-α</td>
<td>p53</td>
<td>-</td>
<td>2.2 mg/kg</td>
<td>1.5%DMSO</td>
<td>(Komarov and others 1999)</td>
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</table>

Treatment of MEL and Its Analogs

MEL and 6-OH MEL were purchased from Sigma (St. Louis, MO). 8-M-PDOT was a product of Tocris Cookson (Ballwin, MO). MEL and 6-OH MEL were dissolved in 10% alcohol (5 mg/ml) and 10% DMSO (5 mg/ml), respectively. They were delivered either by addition to the drinking H₂O at a concentration of 10 mg/L or via osmotic pumps (Durect, Cupertino, CA) at a constant delivery rate of 2.5 µg/h (2 mg/kg/d), 24 h before DOX administration. Control mice were administered equivalent volumes of vehicle via osmotic pumps.

Analysis of Apoptosis

DNA Laddering

Heart tissue was digested in a lysis buffer containing 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 25 mM EDTA, 0.5% SDS, and 1 mg/ml proteinase K, at 37°C overnight. The supernatant was extracted with phenol-chloroform-isoamyl alcohol (25:24:1) and genomic DNA was precipitated with isopropanol. DNA was resuspended in Tris-EDTA (ethylenediaminetetra-acetic acid) buffer and digested with RNase A (0.1 mg/ml) at 37 °C for 30 min. DNA (20 µg) was electrophoresed in 1.4% agarose gel to observe multiples of 180 – 200 bp internucleosomal DNA fragmentation. A 100 bp standard (New England Biolabs, Beverly, MA) was included as markers of DNA electrophoresis.
**TUNEL Assay**

At the end of the experiments, a section of ventricular tissue 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. To detect cardiac apoptosis, TUNEL assay was performed by a CardioTACS™ kit (Trevigen, Gathersburg, MD) according to manufacturer’s instructions. Briefly, slides were deparaffinized in xylene and dehydrated with ethanol, followed by digestion with proteinase K. The labeling reaction was conducted in the presence of TdT enzyme and dNTP at 37°C for 60 min. The horseradish peroxidase (HRP) substrate TACS Blue was applied to visualize positive nuclei, and nuclear fast red was used to counterstain normal nuclei. The percentage of TUNEL-positive cardiac cells was determined by counting 10 random fields per section under an Olympus BX40 microscope.

**ISOL Analysis**

*In situ* staining of DNA strand breaks in the serial section of each specimen was detected by the ApopTag ISOL kit using oligo A according to manufacturer’s instructions (Serologicals Corp., Norcross, GA) with some modifications. The endogenous biotin was blocked with an Avidin/Biotin blocking kit (BioGenex, San Romon, CA). As in the TUNEL assay, the peroxidase substrate TACS Blue was used to label positive nuclei, and nuclear fast red was used to counterstain normal nuclei. The percentage of ISOL-positive cardiac cells was determined by counting 10 random fields/section.

**Cell Death ELISA**

Apoptosis was quantified in mouse hearts by a Cell Death Detection ELISA Plus kit (Roche Molecular Biochemicals, Indianapolis, IN). Frozen heart tissue was pulverized in liquid nitrogen, and a portion of the heart powder was lysed in the buffer provided in the kit. Samples were processed according to manufacturer’s instructions to determine the level of cytosolic
mono- and oligonucleosomes. Protein content of the heart samples was determined by dye binding assay (Bio-Rad, Hercules, CA) to quantify the cytosolic histone-associated nucleosomes.

Analysis of Gene Expression

Isolation of Mouse Heart RNA

Mice were sacrificed at different times after treatment. Hearts were removed and rapidly frozen in liquid nitrogen until RNA isolation. The heart tissue was pulverized in liquid nitrogen and homogenized in TRI REAGENT (Molecular Research Center, Inc., Columbus, OH) using a Brinkman polytron homogenizer. After addition of 1-bromo-3-chloropropane, the homogenate was vortexed and centrifuged. The supernatant was subsequently transferred into fresh tubes and RNA was precipitated with isopropanol. Following centrifugation at 4°C, RNA was air-dried and resuspended in diethyl pyrocarbonate-treated H₂O. RNA was stored at -80°C until analysis. After spectrophotometric quantification at 260 nm, the integrity and loading of RNA were verified by agarose electrophoresis. For SuperArray analysis, RNA was treated with DNase I (1 unit/10 µg RNA), followed by re-extraction with phenol:chloroform:isoamyl alcohol (50:49:1).

RPA Assay

Apoptotic gene expression was analyzed with a RPA kit of multiple-probe templates, RiboQuant mouse apoptosis probe-2 (BD PharMingen Inc., San Diego, CA) and templates p53, Bax, BclxL, and MDM2 (Ambion, Inc., San Antonio, TX). Briefly, ³²P-labeled cRNA probes were synthesized from these templates using in vitro transcription according to manufacturer’s instructions. The probes were either extracted with phenol-chloroform-isoamyl alcohol (25:24:1) or gel-purified, and then hybridized with 5 µg total RNA at 56°C overnight, followed by digestion with RNase A and T1. Protected RNA fragments were extracted with phenol-chloroform-isoamyl alcohol (25:24:1) and separated on a 5% polyacrylamide gel containing 8 M
urea. Gels were dried and visualized by autoradiography. Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. Protected cRNA fragments were then quantified by densitometry with an AlphaImager system (Alpha Innotech Corp., San Leandro, CA) after evaluation of repeated exposures to obtain an adequate dynamic range of linear response.

SuperArray Analysis

The expression of apoptosis-related genes was profiled by a Mouse Apoptosis GEArray™ Q kit (SuperArray Inc., Bethesda, MD) according to the manufacturer’s instructions. $^{32}$P-cDNA probes were synthesized using in vitro reverse transcription from 10 µg of mouse heart RNA. Probes were subsequently hybridized with gentle agitation at 60°C overnight with cDNA fragments of apoptosis-related genes immobilized on nylon membranes provided with the kit. Membranes were washed several times and signals were visualized by autoradiography. Data were analyzed by quantifying the band density of genes as described above.

Detection of Protein Levels

Protein Extraction

Mouse hearts were homogenized with a Polytron homogenizer in a buffer containing 20 mM HEPES (pH 7.5), 150 mM NaCl, 3 mM EDTA, 3 mM EGTA, 2 mM DTT (dithiothreitol), 1% NP-40, 0.1% SDS, 2 µg/ml leupeptin, 2 µg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride. Heart homogenates were incubated on ice for 30 min, followed by centrifugation at 12,000 × g for 20 min. The supernatant was transferred, aliquoted, and stored at -80°C. Protein concentrations were determined by a BCA protein assay using BSA as a standard (Pierce Chemical Co., Rockford, IL).
Western Blot Analysis

The heart homogenate was resuspended in 2X sample buffer containing 125 mM Tris-HCl (pH 6.8 at 25°C), 3% SDS, 20% glycerol, 100 mM DTT and 0.03% bromophenol blue. Samples were subsequently heated at 95°C for 5 min. Proteins were separated by 7.5% - 12% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane by using a Trans Blot® SD Semi-Dry Electrophoretic Transfer Cell System (BioRad, Richmond, CA). Membranes were blocked by gentle agitation in a TBS buffer (20 mM Tris-HCl, pH 7.6 at 25°C, 150 mM NaCl) containing 0.1% Tween-20 and 5% non-fat milk for 60 min at room temperature. Blots were then incubated with specific antibodies (Table 2) at 4°C overnight, followed by incubation with corresponding secondary antibodies. After washing 15 min 3 times, blots were detected with the ECL™ Western blot analysis detection reagent (Amersham Biosciences, Piscataway, NJ).

Table 2  Antibodies Used in the Study

<table>
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<th>Antibodies</th>
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<tr>
<td>Bax</td>
<td>Oncogene Res. Products (Cambridge, MA)</td>
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<tr>
<td>p53, BclxL, MDM2</td>
<td>Santa Cruz Biotech (Santa Cruz, CA)</td>
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<tr>
<td>p38, p-p38 (Thr180/Tyr182), ERK, p-ERK (Thr202/Tyr204), JNK, p-JNK (Thr183/Tyr185) p-p53 (Ser 6, 9, 15, 20, or 389)</td>
<td>Cell Signaling Tech (Beverly, MA)</td>
</tr>
<tr>
<td>actin</td>
<td>Sigma (St. Louis, MO)</td>
</tr>
</tbody>
</table>

Electron Microscopy Analysis

Ultrastructural changes of mouse hearts were analyzed as previously described (Liu and others 2002). Briefly, hearts were fixed with 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) at room temperature for 4 h. Heart tissues were rinsed with cold buffer, post-fixed with 1% osmium tetroxide, dehydrated in a series of ethanol and propylene oxide, and
embedded in an Araldite/DDSA (dodecenyl succinic anhydride) mixture (Electron Microscopy Sciences, Fort Washington, PA). Ultra-thin sections of the left ventricular wall were obtained with a Leica Ultracut UCT ultramicrotome (Leica Inc., Deerfield, IL) and placed on copper grids. Samples were double stained with uranyl acetate and lead citrate and observed under a Philips TECNAI 10 electron microscope. Two samples from each group were examined to detect the alterations of myocardial structures.

Mouse Survival Study

Mice were randomly assigned to eight groups (6 mice/group): 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, i.p.) 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 prior to DOX injection and continuously until the mice were euthanized. Mouse general condition was observed and body weight was recorded daily until termination of experiments. Survived mice were euthanized by injection of 120 mg/kg pentobarbital on the 5th day following DOX injection.

Analysis of Enzyme Activities

Detection of Myocardial Caspase Activities

Caspases-3 and -9 activities were measured by a colorimetric caspase activity assay kit (BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA). Heart tissue was homogenized in a lysis buffer (50 mM HEPES, pH 7.4, 0.1% CHAPS, 5 mM DTT, 0.1 mM EDTA, 0.1% NP-40) followed by centrifugation. The supernatant was stored at -70°C. Caspase activity assays were initiated by incubating 75 µg of supernatant with specific colorimetric caspase-3 substrate (Ac-DEVD-pNA) or caspase-9 substrate (Ac-LEHD-pNA) in 100 µl of assay buffer in 96-well
microplates at 37°C for 2 h, and then read at 405 nm in a microplate reader. Results were expressed as arbitrary unit (absorbance at 405 nm/mg protein).

**Measurement of Serum CPK Activities**

Total CPK activities of the mouse serum were assayed using a CPK test kit (Sigma, St Louis, MO) according to manufacturer’s instructions with modifications. CPK activity was evaluated by the rate of the reaction, in which CPK catalyzes the transfer of a phosphate to ADP to form ATP leading to the reduction of NAD to NADH, a product that increases the absorbance at 340 nm (Szasz and others 1976). One unit of CPK was defined as the reduction of 1 µmole NAD⁺ to NADH per min in a BIS-TRIS buffer (pH 6.9) at 25°C.

**Measurement of Serum MEL Concentrations**

Serum was separated by centrifugation of blood at 4°C and stored at -20°C until use. Serum MEL level was measured using a radioimmunoassay kit (ALPCO Diagnostics, Windham, NH) according to manufacturer’s instructions. Briefly, an equal volume of appropriately diluted serum was incubated with anti-MEL antibody and [¹²⁵I]-MEL at 4°C overnight. The solid phase secondary antibody was added and incubated for 15 min. After centrifugation and aspiration of the supernatant, pellets were counted in a RIASTAR gamma counter (Packard Instrument Co., Meriden, CT).

**Cardiac Function Analysis**

**In Vitro Cardiac Function Analysis**

Isolated heart perfusion was performed using a modified Langendorff technique as previously described (Pouna and others 1996). Mice were injected with sodium heparin (500 units/kg, i.p.) 20 min prior to anesthetization with sodium pentobarbital (120 mg/kg, i.p.). The
mouse chest was opened and the heart was rapidly removed and soaked in ice-cold Krebs-Henseleit buffer (Appendix A). Hearts were cannulated and perfused retrogradely with Krebs-Henseleit buffer at 37°C. A balloon was introduced into the left ventricle via the left auricle. LV pressure and heart rate were 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 Inc., Akron, OH). To better evaluate the cardiac function, LVDP (maximal LV pressure – minimal LV pressure) was calculated, and HR×LVDP was used to assess the left ventricular performance, as it reflects both chronotropic and inotropic activities of the heart. Coronary effluent was collected and measured every 15 min.

**In Vivo Cardiac Function Analysis**

*In vivo* cardiac function was analyzed with an ultrasound-based technique as described previously (Geisterfer-Lowrance and others 1996; Kubota and others 1998). Five days after DOX injection, mice were injected with heparin (500 units/kg, i.p.) and anesthetized with chloral hydrate (360 mg/kg, i.p.). Mice were intubated with a 22-gauge soft catheter and ventilated with a rodent ventilator (Columbus Instruments, 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, aortic root, anterior, and posterior walls of the heart. The inter-crystal distance, LV pressure, and electrocardiogram were recorded on a beat-to-beat basis. Cardiac function parameters, including LVEDP, LV end-systolic pressure (LVESP), LVDP, HR×LVDP, 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 functional analysis, animals were sacrificed with an
overdose of sodium pentobarbital (120 mg/kg, i.p.). Hearts were removed and perfused for 2 min as Langendorff preparations to remove the remaining blood. Portions of the mid-ventricle were fixed for morphological and apoptosis studies. The remaining parts were stored in liquid nitrogen until use.

Cell Culture and Treatments

PC-3 prostate cancer cells were obtained from the American Type Culture Collection (Manassas, VA), and cultured in F12-K media supplemented with 10% fetal bovine serum. Approximately 3000 cells were plated into each well in a 96-well culture plate and allowed to attach overnight before they were subjected to further treatment.

DOX was diluted with F-12K media and added to culture dishes at appropriate concentrations. Experiments were terminated 4 d following treatment by washing with cold PBS. To examine the effect of MEL or 6-OH MEL on DOX’s anti-tumor activity in PC-3 cells, MEL or 6-OH MEL was diluted with F-12K medium to appropriate concentrations. Cells were pre-incubated with different concentrations of MEL (0.1 M-100 µM) or 6-OH MEL (0.01-10 µM) for 1 h prior to the exposure to DOX. Equivalent volumes of solvents (alcohol or DMSO) were added to the control cells. To detect the effects of PFT-α on DOX’s anti-tumor activity, cells were treated with various concentrations of PFT-α (0.1-50 µM) or an equal volume of DMSO for 1 h and subsequently incubated with DOX.

Measurement of Cell Density

A sulforhodamine B colorimetric assay was used to measure the cell density as previously described (Skehan and others 1990). Experiments were terminated by washing cells with warm PBS 4 d after treatment. Cells were then fixed with a 10% ice-cold trichloroacetic acid. A 0.4% sulforhodamine B solution was subsequently added and incubated for 20 min followed by washing with 1% acetic acid. After overnight drying, the stain was dissolved in 10
mM Tris (pH 10.0) and read on a microplate reader at 490 nm. Relative cell density was expressed as absorbance at 490 nm/well.

**Statistical Analysis**

All values except survival rate were expressed as means ± SEM. Statistical differences between each pair of groups were determined by Chi-square 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 the groups. $P < 0.05$ was considered significantly different.
DOX-Induced Cardiac Cell Apoptosis

Cardiac cell apoptosis increased significantly following a single injection of DOX (22.5 mg/kg, i.p.) in a time-dependent manner. DNA laddering began to emerge in mouse hearts 3 d after DOX injection, and persisted until 5 d following treatment (Fig. 6). This observation was confirmed by more quantitative approaches including TUNEL and ISOL analyses. TUNEL analysis showed that positive myocardial nuclei increased 5-fold 3 days after DOX-treatment and 6-fold 5 days after DOX-treatment (Fig. 7). Similar results were achieved in the ISOL assay, a more specific DNA fragmentation staining procedure, in which 14.2% of cardiac cells showed ISOL-positive nuclei in hearts from mice 5 days after DOX-treatment, compared to 1.8% from control mice (Fig. 8). Thus, DOX does induce cardiac cell apoptosis in the acute stages of its cardiotoxicity.

Fig. 6. DNA laddering formation in mouse hearts following DOX injection. Mice were treated with DOX or an equal volume of saline and sacrificed at different time points. Heart DNA was isolated and electrophoresed on 1.4% agarose gel. Lanes 1 and 2, control; 3 and 4, DOX-treated 3 d; 5, DOX-treated 5 d; 6, 100 base pair DNA marker.
Fig. 7. DOX-induced cardiac cell apoptosis as analyzed by TUNEL. Mice were treated with DOX (22.5 mg/kg, i.p.) or an equal volume of saline. Hearts were removed at the indicated times and analyzed with TUNEL assay. A, representative TUNEL staining showing positive nuclei. a, control; b, DOX-treated 1 d; c, DOX-treated 3 d; d, DOX-treated 5 d. B, percentage of TUNEL-positive nuclei. \(^*P < 0.05\), vs. control and DOX-treated 1d, \(n = 6\).

Fig. 8. ISOL assay showing DOX-induced apoptosis in mouse hearts. DOX or an equal volume of saline was injected i.p. in mice. Mice were sacrificed and hearts were removed 5 d after injection. Ventricular sections were stained with an ISOL kit. A, representative ISOL staining showing positive nuclei. a, control; b, DOX-treated 5 d. B, percentage of ISOL-positive nuclei in mouse hearts treated with saline or DOX for 5 d. \(^*P < 0.05\), vs. CON, \(n = 6\).
Mechanisms of DOX-Induced Apoptosis

To determine the signal pathways of DOX-induced cardiac apoptosis, mRNA levels and protein expression of apoptosis-related genes were examined. It was found that p53 was phosphorylated at Ser 15 by p38 and ERK MAPKs, followed by the expression of p53-mediated downstream genes in mouse hearts after DOX injection.

DOX Induces p53 Phosphorylation

The p53 protein level was upregulated by DOX in mouse hearts as early as 4 h after DOX-treatment, despite a lack of upregulation of p53 mRNA levels (Fig. 9). To delineate how DOX led to elevation of p53 protein, phosphorylation of p53 at Ser 6, 9, 15, 20, and 389 was analyzed by Western blot analysis, since posttranslational modification such as phosphorylation is the major regulatory mechanism resulting in the elevation of p53 protein (Lakin and Jackson 1999; Lambert and others 1998; Gottlieb and others 2002). Phosphorylated p53 (p-p53) at Ser 15 was identified in mouse hearts as early as 1 h and peaked at 2 h after DOX treatment. The level of p-p53 at Ser 20 was also increased, but the change was not significant (data not shown). On the other hand, phosphorylation of Ser 6, 9, 20, and 389 was not detected. Phosphorylation of p53 at Ser 15 was followed by the elevation of p53 protein levels (Fig. 9). This suggests that a relationship exists between the elevation of p53 protein and p-p53 at Ser 15.

Fig. 9. Phosphorylation of p53 at Ser 15 and elevation of p53 levels in DOX-treated mouse hearts. Phosphorylation of p53 at Ser 15 and p53 levels were detected by Western blot analysis with specific p-p53 at Ser 15 and p53 antibodies. Actin was included as a loading control.
MAPKs Mediate the Phosphorylation of p53

To determine whether MAPKs play a role in the phosphorylation of p53, phosphorylation of MAPKs was measured in DOX-treated mouse hearts. Phosphorylation of p38 began as early as 1 h after DOX treatment and declined after 2 h; phosphorylation of ERK occurred as early as half an hour in mouse hearts following DOX injection (Fig. 10). In contrast, JNK MAPK was not phosphorylated in mouse hearts after DOX-treatment. These results indicate that ERK and p38 are likely to mediate the phosphorylation of p53 at Ser 15.

![Fig. 10. Phosphorylation of p38 and ERK1/2, but not JNK MAPK, in DOX-treated mouse hearts. Mice were injected with DOX (22.5 mg/kg, i.p.) and sacrificed at designated times. Hearts were subjected to Western blot analysis using specific p-p38 (Thr180/Tyr182), p38, p-ERK (Thr202/Tyr204), ERK, p-JNK (Thr183/Tyr185), and JNK antibodies.](image)

MAPK inhibitors were used to confirm roles of p38 and ERK MAPKs in mediating phosphorylation of p53. Western blot analysis showed that SB 203580, a p38 inhibitor, partially inhibited the phosphorylation of p53 at Ser 15 but not p38 itself (Fig. 11A). Also, the ERK inhibitor PD 98059 blocked the phosphorylation of ERK and partially inhibited the phosphorylation of p53 at Ser 15 at a dose of 2.5 mg/kg (Fig. 11B). In contrast, SP 600125, a
JNK inhibitor, did not significantly inhibit the phosphorylation of p53 at Ser 15 at a dose of 15 mg/kg (Fig. 11C). These data indicate that p38 and ERK MAPKs mediate the phosphorylation of p53 at Ser 15.

**Fig. 11. Effects of MAPK inhibitors on the phosphorylation of MAPKs and p53.** Mice were injected with DOX and/or MAPK inhibitors and sacrificed 2 h after DOX injection. Hearts were subjected to Western blot analysis with specific antibodies against p-p53 (Ser 15), p53, p-p38 (Thr180/Tyr182), p38, p-ERK (Thr202/Tyr204), ERK, p-JNK (Thr183/Tyr185), and JNK. A, effects of p38 inhibitor SB 203580 (5 mg/kg) on the phosphorylation of p38 and p53. B, effects of ERK inhibitor PD 98059 (2 mg/kg) on the phosphorylation of ERK and p53. C, effects of JNK inhibitor SP 600125 (15 mg/kg) on the phosphorylation of JNK and p53. SB, SB 203580; PD, PD 98059; SP, SP 600125.
DOX Upregulates Expression of Apoptosis-Related Genes

RPA was used to evaluate changes of apoptosis-related genes in mouse hearts following DOX injection. As shown in Fig. 12, RPA results indicate that the message levels of Bax, BclxL and MDM2 were induced as early as 12 h, peaked at 24 h after DOX-treatment, and declined at 48 h. Although these genes are p53 target genes, the mRNA level of p53 was not upregulated by DOX treatment.

Fig. 12. Upregulated mRNA levels of apoptosis-related genes in mouse hearts following DOX treatment. Total heart RNA was extracted and subjected to RPA analysis as described in Materials and Methods. The mRNA levels of apoptosis-related genes were determined by RPA with a RiboQuant Probe mAPO-2 (A), and p53 and MDM2 probes (B).
To confirm the above findings and to obtain a more complete profile of genes involved in DOX-induced apoptosis, mouse heart RNA was further examined by SuperArray analysis. Because most genes were upregulated within 24 h following DOX injection, gene expression levels were determined at this time point. The pro-apoptotic genes Bax and TNFR2, as well as the anti-apoptotic gene BclxL, were upregulated at least 2-fold in mouse hearts (Fig. 13).

![SuperArray analysis showing gene expression of apoptosis-related proteins. Mice were treated with DOX or an equal volume of saline and sacrificed 24 h later. The expression of apoptosis-related genes was detected in mouse hearts by SuperArray assay. A, representative gene expression analyzed by SuperArray; bottom panels represent shorter exposures of internal control gene β-actin. B, densitometric analysis of gene expression. ^aP < 0.05, CON vs. DOX, n = 3.](image)
DOX Induces Protein Expression of Apoptosis-Related Genes

Since DOX upregulated Bax, BclxL, and MDM2, all of which are p53 downstream genes, the protein levels of these genes were determined by Western blot analysis. As shown in Fig. 14, protein levels of BclxL were increased as early as 2 h following DOX injection, while Bax and MDM2 were upregulated 2-3 fold in mouse hearts 24 h after DOX treatment. These data are consistent with the results of RPA and SuperArray analysis.

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</table>

Fig. 14. DOX-induced protein expression of p53 target genes. Mice were injected with DOX and sacrificed at indicated time points. Heart protein was extracted and analyzed by Western blot analysis with specific antibodies against Bax, BclxL, MDM2, and actin as described in Materials and Methods.

PFT-α Inhibits DOX-Induced Apoptosis and Cardiotoxicity

The above data (Fig. 9-14) indicate that p53-dependent apoptotic genes play a crucial role in DOX-induced apoptosis. To test this possibility, the effects of the p53 inhibitor PFT-α on DOX-induced cardiac cell apoptosis, ultrastructural alterations, and cardiac dysfunction were determined.

PFT-α Blocks DOX-Induced Cardiac Cell Apoptosis

ISOL analysis was performed to determine whether PFT-α blocks DOX-induced cardiac cell apoptosis. As shown in Fig. 15, DOX gave rise to a 5-fold increase of ISOL-positive nuclei
in cardiac cells at a single dose of 22.5 mg/kg; however, administration of PFT-α along with DOX almost abolished DOX-induced increase of ISOL-positive nuclei (DOX 13.2% vs. DOX + PFT-α 2.56%, \( P < 0.05, n = 6 \)). These data confirm that DOX induces cardiac cell apoptosis in a p53-dependent pathway and this apoptosis can be blocked by PFT-α.

Fig. 15. Effects of PFT-α on DOX-induced apoptosis in mouse hearts. Mice were treated with DOX and/or PFT-α and sacrificed 5 days later. Heart sections were stained with a modified ISOL kit. \(^aP < 0.05\) DOX vs. CON, DOX + PFT-α, and PFT-α treated, \( n = 6 \).

**Effects of PFT-α on DOX-Induced Phosphorylation of p53**

Western blot analysis showed that PFT-α had no significant effect on DOX-induced phosphorylation of p53 at Ser 15. Thus, it did not inhibit the elevation of p53 protein levels in hearts of DOX-treated mice (Fig. 16 and 18).

Fig. 16. Effects of PFT-α on the phosphorylation of p53 at Ser 15 in DOX-treated mouse hearts. Mice were injected with DOX and/or PFT-α, and hearts were removed 2 h after treatment. Heart protein was extracted and subjected to Western blot analysis with specific antibodies against p53 and p-p53 at Ser 15.
PFT-α Inhibits DOX-Induced Expression of p53 Target Genes

RPA was employed to determine the effects of PFT-α on DOX-induced gene expression of p53 and its target genes. As shown in Fig. 17, PFT-α blocked DOX-upregulated mRNA levels of Bax, BclxL, and MDM2 in mouse hearts 24 h following DOX treatment. Neither DOX nor PFT-α had any effect on p53 gene expression in mouse hearts. Along with the above data, this finding indicates that PFT-α targets the expression of p53 downstream genes in the DOX-induced apoptotic pathway.

Fig. 17. Inhibitory effects of PFT-α on the expression of p53 target genes in DOX-treated mouse hearts. Mice were treated with DOX and/or PFT-α and were sacrificed 24 h later. Total heart RNA was subjected to RPA analysis. PFT-α inhibited the expression of BclxL, Bax (A), and MDM2, but not p53 (B). Lane 1, CON; lane 2, DOX-treated, lane 3, DOX and PFT-α-treated; lane 4, PFT-α-treated mice. Intensity of protected bands was quantified by densitometric analysis (C). *P < 0.05, DOX vs. CON, PFT-α, DOX + PFT-α, n = 3.

PFT-α Inhibits Protein Expression of p53 Target Genes

To confirm the above data, the effects of PFT-α on DOX-induced protein expression of p53 and its target genes in mouse hearts were analyzed by Western blot analysis. While PFT-α significantly inhibited DOX-stimulated protein expression of Bax, BclxL, and MDM2 in mouse
hearts, it did not reduce DOX-upregulated protein levels of p53 (Fig. 18). These results are consistent with the RPA data, in which PFT-α inhibited the gene expression of Bax, BclxL, and MDM2. Since PFT-α did not affect the phosphorylation of p53, it did not block the increase in p53 protein level due to MDM2-mediated p53 degradation.

Fig. 18. Effects of PFT-α on the protein expression of apoptosis-related genes. Mice were sacrificed 24 h following DOX and/or PFT-α injection. Protein expression of Bax, BclxL, and MDM2, and p53 were analyzed in mouse hearts by Western blot analysis. A, heart proteins were subjected to Western blot analysis with specific antibodies against p53, Bax, BclxL, MDM2, and actin. B, densitometric analysis of protein expressions. \( ^a P < 0.01 \), vs. CON; \( ^b P < 0.05 \), vs. DOX + PFT-α; \( ^c P < 0.05 \), DOX + PFT-α vs. CON, n = 5.

PFT-α Inhibits Caspase Activation

Activities of caspases-3 and -9 were measured in hearts from mice treated with DOX and/or PFT-α. DOX induced 3-fold increases of caspases-3 and -9 activities in mouse myocardium. PFT-α markedly inhibited DOX-induced activation of caspases-3 and -9 (Fig. 19). This indicates that caspases-3 and -9 play important roles in DOX-induced p53-dependent apoptosis, and that PFT-α inhibits apoptosis by blocking the activation of caspases-3 and -9.
Fig. 19. PFT-α blocked DOX-induced activation of caspases-3 and -9. Mice were sacrificed 24 h after DOX and/or PFT-α injection and hearts were removed for the measurement of caspases-3 and -9 activities using a colorimetric caspase activity assay. *P < 0.05 vs. other groups, n = 5.

PFT-α Attenuates Myocardial Ultrastructural Alterations

Administration of DOX led to myocardial ultrastructural damage in the current study, a result that is consistent with previous reports (Lefrak and others 1973; Rosenoff and others 1975; Lambertenghi-Deliliers and others 1976). Myocardial changes, characterized as cytoplasmic vacuolization, mitochondrial swelling, and dense bodies in cardiac mitochondria, were focally distributed in some myocytes but merged when groups of cells were affected. More severe changes such as myofibrillar disorganization or loss were diffuse in some cardiac myocytes (Fig. 20B). Treatment with PFT-α dramatically suppressed the damage, particularly the degree of cytoplasmic vacuolization, mitochondrial swelling, and cristae disappearance. Myofibrillar disorganization without myofibrillar loss was still present (Fig. 20C). In contrast, no damage was found in hearts from PFT-α-treated mice (Fig. 20D). These electron microscopic studies suggest that PFT-α protects mitochondrial function and membrane integrity of cardiac myocytes.
Fig. 20. PFT-α protected against DOX-induced myocardial ultrastructural alterations. A, control; B, DOX; C, DOX + PFT-α; D, PFT-α. DOX caused cytoplasmic vacuolization (v), mitochondrial swelling (m), and myofibril disarrangement and loss (f). Administration of PFT-α dramatically attenuated DOX-induced myocardial damage.

PFT-α Inhibits DOX-Induced Serum CPK Release

Since DOX causes disruption of the cardiac myocyte membrane, the release of intracellular CPK into the serum has been used to evaluate the existence and extent of cardiac myocyte injury (Lee and others 1991; DeAtley and others 1999). The present study showed that mouse serum CPK was elevated 5 days after DOX treatment. PFT-α partially inhibited the
DOX-induced increase of serum CPK (Fig. 21), implying that it protected the cardiac myocyte membrane integrity.

Fig. 21. Inhibition of DOX-induced elevation of serum CPK. CPK activities were measured by a CPK test assay. \(^a\)P < 0.01, DOX vs. CON, DOX + PFT-\(\alpha\), PFT-\(\alpha\); \(^b\)P, DOX + PFT-\(\alpha\) vs. CON and PFT-\(\alpha\), n = 6.

PFT-\(\alpha\) Protects Against DOX-Induced Cardiac Dysfunction

\textit{In vivo} cardiac function was analyzed in mice treated with DOX and/or PFT-\(\alpha\) (Liu and others 2002). Mice were randomly assigned to four groups: control, DOX, DOX + PFT-\(\alpha\), and PFT-\(\alpha\). Heart weight and body weight were measured daily. At the end of the experiments, the heart weight to body weight ratio (mg/g) was slightly increased in DOX-treated mice, compared to control mice 5 days after treatment (4.7 \(\pm\) 0.13, vs. 4.4 \(\pm\) 0.14, \(P > 0.05\)). This change was not statistically significant and was primarily attributed to the progressive weight loss observed in DOX-treated mice. Although DOX-treatment did not result in cardiac hypertrophy and dilatation within 5 days, it greatly suppressed cardiac function, as shown in Table 3. DOX-treatment results in a significant elevation of LVEDP and a suppression of LVDP, indicating a decline of LV contractility. Due to the suppression of LV contractility, a reduction of SV and CO was observed in DOX-treated mice. In contrast, mice administered with DOX and PFT-\(\alpha\) showed marked improvement of cardiac function. Compared to the control, PFT-\(\alpha\) did not show
any effects on the cardiac function. These data demonstrate that PFT-α improved LV contractility in DOX-injected mice.

Table 3. Effects of PFT-α on DOX-Induced Cardiac Dysfunction

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>DOX</th>
<th>DOX + PFT-α</th>
<th>PFT-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (beats/min)</td>
<td>461 ± 24</td>
<td>416 ± 29</td>
<td>457 ± 14</td>
<td>443 ±20</td>
</tr>
<tr>
<td>LVDP (mmHg)</td>
<td>83.7 ± 4.1</td>
<td>64.4 ± 5.2a</td>
<td>81.4 ± 3.2</td>
<td>90.5 ± 4.0</td>
</tr>
<tr>
<td>HR×LVDP</td>
<td>38,620 ±2,924</td>
<td>25,780 ±2,272a</td>
<td>37,320 ±2,260</td>
<td>40,590 ±3,382</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>1.86 ± 0.9</td>
<td>8.4 ± 0.72a</td>
<td>1.02 ± 0.42</td>
<td>0.99 ± 0.4</td>
</tr>
<tr>
<td>+dP/dt (mmHg/s)</td>
<td>3582 ± 217</td>
<td>1993 ± 299a</td>
<td>3297 ± 225</td>
<td>4291 ± 399</td>
</tr>
<tr>
<td>-dP/dt (mmHg/s)</td>
<td>2581 ± 182</td>
<td>1493 ± 223a</td>
<td>2365 ± 321</td>
<td>3376 ± 182</td>
</tr>
<tr>
<td>SV (µl)</td>
<td>9.73 ± 0.65</td>
<td>6.12 ± 0.49a</td>
<td>8.68 ± 0.59</td>
<td>9.70 ± 0.75</td>
</tr>
<tr>
<td>CO (ml)</td>
<td>4.29 ± 0.35</td>
<td>2.32 ± 0.23a</td>
<td>4.01 ± 0.35</td>
<td>4.83 ± 0.52</td>
</tr>
</tbody>
</table>

Mice were injected with DOX and/or PFT-α. Six out of nine DOX-treated mice survived. *In vivo* cardiac function was measured 5 d after DOX injection by a SonoMetrics data acquisition and analysis system. HR, heart rate; LVDP, left ventricular developed pressure; LVEDP, left ventricular end-diastolic pressure; ±dP/dt, first derivatives of left ventricular pressure; SV, stroke volume; CO, cardiac output. aP < 0.01 vs. CON, DOX + PFT-α, and PFT-α.

Effects of PFT-α on the Anti-Tumor Activities of DOX

To examine the effects of PFT-α on the anti-tumor activities of DOX, a p53-null prostate cancer cell line, PC-3, was treated with DOX. The results indicate that DOX killed about 50% of PC-3 cells at a concentration of 80 nM (LC₅₀), as determined by a sulforhodamine-stained cell density test (Fig. 22A). Pre-incubation of cells with PFT-α at a concentration less than 10 µM did not have significant effects on the inhibition of PC-3 cells by DOX. In contrast, PFT-α had toxicity at 50 µM and enhanced the cytotoxicity of DOX.
Fig. 22. Effects of PFT-α on the anti-tumor potency of DOX. Sulforhodamine B colorimetric assay was performed following treatment of PC-3 cells with DOX and/or PFT-α. A, dose-effect study showing that the IC50 of DOX on PC-3 cells is 80 nM. B, effects of PFT-α on the growth of PC-3 cells in the absence (open bars) or presence of DOX treatment (solid bars). \(^aP<0.05\) vs. treatments at other concentrations of PFT-α in the absence of DOX; \(^bP<0.05\) vs. treatments at other concentrations of PFT-α in the presence of DOX, \(n=6\).

**MEL Protects the Heart against DOX-Induced Cardiotoxicity and Apoptosis**

Previous studies indicate that DOX induces cardiotoxicity by the generation of ROS. This series of experiments were designed to investigate whether MEL, an antioxidant, protects against DOX-induced cardiotoxicity and cardiac cell apoptosis.

**MEL and 6-OH MEL Improve Survival of DOX-Treated Mice**

The general appearances of mice from each group (CON, DOX, MEL, DOX + MEL, 6-OH MEL, DOX + 6-OH MEL, 8-M-PDOT, and DOX + 8-M-PDOT) were observed following DOX injection. At the time of sacrifice, 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 the other groups. The survival rates of treated mice from each group are shown in Table
4. While 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.

Table 4 Effects of MEL, 6-OH MEL, and 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>CON</td>
<td>8/8</td>
</tr>
<tr>
<td>DOX(^a)</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(^a)</td>
<td>8/16</td>
</tr>
</tbody>
</table>

Mice were injected with a single dose of DOX (25 mg/kg, i.p.) or an equivalent volume of saline. The mice were given MEL, 6-OH MEL, or 8-M-PDOT in drinking water (10 mg/liter) 24 h prior to injection and until sacrifice. The number of surviving mice was observed for the next 5 d following DOX injection. \(^a\)P < 0.05 vs. other groups.

MEL and 6-OH MEL Protect Cardiac Function in DOX-Perfused Mouse Hearts

A dose-response study indicates that DOX significantly depressed cardiac function at concentrations higher than 1 \(\mu\)M (data not shown). Perfusion of hearts with 5 \(\mu\)M DOX for 60 min resulted in a 40% reduction of HR and a 30% suppression of dP/dt (Fig. 23, A and B). Perfusion of 5 \(\mu\)M DOX resulted in a 50% decrease of HR×LVDP as compared with the control group (Fig. 23C). Exposure of mouse hearts to 1 \(\mu\)M MEL or 6-OH MEL 5 min prior to DOX treatment, followed by perfusion of these hearts with a buffer containing 5 \(\mu\)M DOX and 1 \(\mu\)M MEL, restored HR×LVDP to 85% of the basal level (Fig. 23C).
Fig. 23. Effects of MEL, 6-OH MEL, and 8-M-PDOT on the cardiac function of 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. Functional parameters were expressed as the percentage of the baseline values measured after 30 min of stabilization. A, heart rate; B, +dP/dt; C, HR×LVDP. \(^aP < 0.05,\) DOX vs. other groups except DOX + 8-M-PDOT-treated; \(^bP < 0.05,\) DOX + 8-M-PDOT vs. other groups except DOX-treated, n = 5.

In addition, DOX caused a 45% decline of coronary flow rate (CFR) after 60 min of perfusion (Fig. 24A). Treatment of the hearts with MEL or 6-OH MEL almost abolished DOX-induced decline of CFR (Fig. 24B). 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 CFR, these results indicate that MEL or 6-OH MEL, but not 8-M-PDOT, improve the cardiac performance of DOX-treated mouse hearts by restoring coronary circulation.
Fig. 24. Effects of MEL, 6-OH MEL, and 8-M-PDOT on DOX-induced reduction of coronary flow. Hearts were perfused 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. Coronary perfusate was collected every 15 min and expressed as the percentage of basal levels. A, DOX-perfusion resulted in a reduction of CFR; B, effects of MEL, 6-OH MEL, and 8-M-PDOT on the coronary flow rate of DOX-perfused hearts. \(^{a}P < 0.05,\) DOX vs. other groups except DOX + 8-M-PDOT-treated, n = 5.

Role of MEL1a Receptor in the Cardioprotective Effects of MEL

To study if MEL acts on mouse hearts through its receptors, MEL1a receptor-deficient mouse hearts were perfused as described above. The data show that MEL protects the cardiac function of both MEL1a receptor-deficient mice and wild-type mice (Fig. 25). Pre-exposure of mouse hearts to 1 µM MEL 5 min prior to DOX perfusion, followed by perfusion of the heart with a buffer containing 5 µM DOX and 1 µM MEL, protected cardiac function and coronary flow of hearts from both wild-type and MEL1a receptor-deficient mice. These results indicate that the protective effects of MEL are not mediated by MEL1a receptor.
Plasma MEL Concentration in MEL-Administered Mice

The RIA results showed that plasma MEL concentration varies from 10.0 ng/ml to 22.3 ng/ml with a mean concentration at 14.8 ± 1.5 ng/ml (0.06 µM) in osmotic pump-implanted mice. On the other hand, the plasma MEL concentration was significantly lower in mice with MEL in the drinking water (4.7 ± 0.4 ng/ml (0.02 µM), P < 0.05). Despite the difference, effective protection was obtained in mice administered MEL in their drinking water. This indicates that the lower MEL concentration (4.7 ng/ml) in orally-administered mice, is sufficient to protect against DOX-cardiotoxicity. Since the water consumption of each mouse (30 g) is about 5 ml/d, it was estimated that approximately 50 µg/d of MEL (or 1.67 mg/kg/d) would be adequate to protect animals against the acute toxicity of DOX.

MEL and 6-OH MEL Protect In Vivo Cardiac Function

Table 5 summarizes the hemodynamic indices (HR, LVEDP, 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. LVEDP was higher in the DOX group than in the CON group ($P < 0.05$), indicating that DOX impaired ventricular diastolic properties of the heart. LVESP and $±dP/dt$, 2 parameters of cardiac contractility, were significantly decreased in the DOX group. There was no change in the LV end-diastolic dimensions among the groups, which indicates that acute DOX treatment does not induce cardiac dilatation. Although HR in the DOX group was also not significantly lower than that in other groups, there was a 62% reduction in CO, which can be attributed to a significant decline 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.

Table 5  Effects of MEL and 6-OH MEL on the 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 ($\mu$l)</th>
<th>CO (ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON</td>
<td>442±25</td>
<td>4.8±0.7</td>
<td>64.7±3.8</td>
<td>2638±127</td>
<td>2010±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 a</td>
<td>37.3±5.5 a</td>
<td>904±156 a</td>
<td>727±153 a</td>
<td>4.1±0.4 a</td>
<td>1.6±0.3 a</td>
</tr>
<tr>
<td>MEL</td>
<td>451±29</td>
<td>3.0±0.8</td>
<td>63.2±3.2</td>
<td>2664±120</td>
<td>1978±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>1914±95</td>
<td>1629±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>2384±334</td>
<td>1863±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>1774±208</td>
<td>1586±121</td>
<td>7.7±0.6</td>
<td>3.0±0.5</td>
</tr>
</tbody>
</table>

Mice were injected with DOX (22.5 mg/kg, i.p.) or saline. MEL or 6-OH MEL was administered via a micro-osmotic pump at a constant delivery rate of 2.5 $\mu$g/h 24 h prior to DOX treatment and continuously until sacrifice. Six out of 8 DOX-treated mice survived. In vivo cardiac function was measured by the SonoMetrics system as described in Materials and Methods. $aP < 0.05$ vs. all other groups, n = 6.
MEL and 6-OH MEL Protect Myocardial Ultrastructure

Electron and light microscopic analyses of left ventricles 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 when 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 vacoulation, and focal myofilament disarray (Fig. 26 B and C). Cellular damage was reduced dramatically by the administration of MEL or 6-OH MEL. No myofilament 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. 26 D and E). MEL- and 6-OH MEL-treated mice showed normal ultrastructure of the heart. The results indicate that MEL or 6-OH MEL is able to protect the integrity of subcellular structure of DOX-treated mouse hearts.

Fig. 26. Representative electron micrographs showing morphological alterations of hearts from treated mice. A) control; B) and C) DOX-treated; D) DOX + MEL-treated; E) DOX + 6-OH MEL-treated. v, vacoulation; m, mitochondrial damages; f, myofibril disarrangement and loss.
MEL and 6-OH MEL Attenuate DOX-Induced Elevation of Serum CPK

While DOX-treatment resulted in a 3-fold increase of serum CPK activity as compared with the CON group (Fig. 27), 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.

Fig. 27. MEL and 6-OH MEL protected DOX-induced CPK release. Mouse serum CPK was measured as described in Materials and Methods. aP < 0.05, DOX vs. all other groups, n = 6.

MEL and 6-OH MEL Inhibit DOX-Induced Cardiac Cell 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. Since the TUNEL assay may stain DNA fragments deriving from non-apoptotic processes, experiments were performed in the serial sections from each heart to determine myocyte apoptosis by the ISOL method. 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. 28). Co-treatment of MEL or 6-OH MEL significantly attenuated apoptosis of cardiac myocytes in DOX-treated mice. The effects of MEL and 6-OH MEL were further determined by a cell death ELISA assay. As shown in Fig. 29, whereas DOX-treated mouse hearts showed a fourfold increase of cytosolic nucleosomes compared with control mouse hearts, MEL or 6-OH MEL significantly inhibited DOX-induced DNA fragmentation.
Fig. 28. Protective effects of MEL and 6-OH MEL on apoptosis in DOX-treated mouse hearts. Heart sections were stained by TUNEL (A) or ISOL (B). Immunolabeled nuclei of cardiac cells were determined by random counting of 10 fields per section. $^{a}P < 0.05$, DOX vs. CON, DOX + MEL vs. MEL, or DOX + 6-OH MEL vs. 6-OH MEL, n = 6; $^{b}P < 0.05$, DOX vs. DOX + MEL or DOX + 6-OH MEL; n = 6.

Fig. 29. Protective effects of MEL and 6-OH MEL on DNA fragmentation. The cytosolic mono- and oligonucleosomes of mouse hearts were quantified by a Cell Death ELISA$^\text{plus}$ kit as described in Materials and Methods. $^{a}P < 0.05$, DOX vs. all other groups, n = 5.
Effects of MEL and 6-OH MEL on the Anti-Tumor Potency of DOX

The effects of MEL and 6-OH MEL on the anti-tumor potency of DOX were performed in DOX-sensitive PC-3 cells. Treatment of DOX at 80 nM results in the inhibition of growth of 50% cells when cell density was measured with a sulforhodamine B colorimetric assay. Treatment of MEL (0.1-100 µM, which is far higher than the 0.06 µM in serum of MEL-administered mice) or 6-OH MEL (0.01-10 µM) had no effect on the growth of PC-3 cells, nor the LC₅₀ of DOX (Fig. 30). Thus, MEL or 6-OH MEL did not change the potency of DOX in PC-3 cancer cells.

Fig. 30. Effects of MEL and 6-OH MEL on the growth of PC-3 cells. 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) was added 3 h before the application of DOX. The number of surviving cells 4 d after addition of DOX (solid bar) or medium (open bar) was measured by sulforhodamine B colorimetric assay and was expressed as optical density per well. aP < 0.05, DOX vs. CON; bP < 0.05, DOX vs. DOX + MEL or DOX + 6-OH MEL, n = 6.
In the current study, the protective effects of PFT-$\alpha$ and MEL against DOX-induced cardiac cell apoptosis and cardiotoxicity were investigated. These studies indicate that PFT-$\alpha$ and MEL may protect the heart against DOX-induced cardiotoxicity in two different but related mechanisms: anti-apoptosis and antioxidant properties. The major novel findings in the current study include: 1) DOX induces cardiac cell apoptosis mainly through the expression of p53-dependent gene Bax and activation of caspases. 2) Activation of p53 by DOX is related to its phosphorylation at Ser 15 and is mediated by p38 and ERK1/2 MAPKs. 3) By blocking the expression of p53 downstream genes, PFT-$\alpha$ abolishes DOX-induced cardiac cell apoptosis and attenuates DOX-induced structural and functional damages. 4) MEL protects hearts against DOX-induced cardiotoxicity, at least partially by blocking cardiac cell apoptosis, which is related to its antioxidant activities.

**DOX Induces Apoptosis in a p53-Dependent Pathway**

Identifying cellular events and signaling pathways of cardiac apoptosis are essential for understanding the mechanisms of DOX-induced cardiotoxicity. Because therapeutic approaches can be achieved through the inhibition of critical cellular events and blockade of signaling pathways. The current study indicates that DOX induces cardiac cell apoptosis via a p53-dependent pathway, in which p53 is regulated by phosphorylation at serine residues, followed by the elevation of p53 protein and expression of p53 target genes such as Bax, a gene whose product initiates cardiac cell apoptosis. Revelation of these events in DOX-induced cardiac cell apoptosis will provide insights into the mechanisms, prevention, and treatment of DOX-induced cardiotoxicity.
DOX Induces Cardiac Cell Apoptosis

The percentage of apoptotic cells was significantly increased in the early stage following DOX injection. These results are consistent with a previous report that DOX induces cardiac cell apoptosis within a few days following DOX injection (Arola and others 2000). Recently, numerous studies have indicated that apoptosis may be an important contributing factor to cardiovascular disease. Apoptosis is involved in cardiac ischemia, and overexpression of Bcl-2 attenuates ischemia-reperfusion injury through inhibition of cardiac apoptosis (Bialik and others 1997; Chen and others 2001). In human cardiomyopathy, mitochondria-mediated cardiac apoptosis is related to cardiac function (Narula and others 1999). The current study indicates that DOX-induced cardiac apoptosis may play an important role in DOX-induced acute cardiotoxicity. Previous histopathological studies revealed a striking reduction of cardiac cells in DOX-induced cardiomyopathy in patients (Lefrak and others 1973). Loss of cardiac cells is one of the major features of DOX-induced cardiomyopathy and is an important cause of cardiac dilatation and failure. Our studies indicate that cardiac apoptosis in DOX-treated mice may account for the cardiac cell loss.

The mechanism involved in DOX-induced cardiac apoptosis is important because the elucidation of apoptotic pathways may be helpful in drug discovery. For instance, inhibitory apoptotic proteins (IAPs) are thought to be the potential targets of drug development (Reed 2001). However, the mechanisms involved in DOX-induced cardiac apoptosis have not been thoroughly explored. The current study has explicated the possible pathways involved in DOX-induced cardiac apoptosis. As shown in previous studies, apoptosis can be mediated through death receptors such as Fas or TNFR, or initiated by mitochondrial dysfunction (Ashkenazi and Dixit 1998; Kroemer and Reed 2000). Two important events triggering mitochondria-dependent apoptotic pathways include DNA damage and free-radical generation. It is known that DOX can induce DNA damage as well as free radical generation (Davies and Doroshow 1986; Lee and others 1991; Bagchi and others 1995; Zhou, Palmeira, and others 2001). Therefore, it is very
likely that DOX induces apoptosis in mitochondria-dependent pathways, which involve the upregulation of Bax and release of cytochrome c. The current study has shown that the upregulation of Bax is dependent upon the phosphorylation of p53. The results strongly suggest that DOX induces cardiac cell apoptosis in a p53-dependent pathway.

Roles of p53 in DOX-Induced Apoptosis

Tumor suppressor gene p53 plays a pivotal role in cell differentiation and apoptosis (Burns and el Deiry 1999; Hirao and others 2000). Activation of p53 initiates cell growth arrest or apoptosis in response to a variety of stress signals in order to eliminate damaged or potentially dangerous cells. As such, p53 is extremely important in the surveillance of possible tumor cell formation in normal functioning. Many chemotherapeutic drugs exert their anti-tumor actions by activating p53 to induce cancer cell apoptosis, and these include DNA damaging drugs, anti-metabolites, microtubule-active drugs, and inhibitors of the proteosome (Blagosklonny 2002). Functionally deficient p53 has been observed in about 50% of cancers and heavily contributes to the multiple drug resistance observed in certain cancer therapies (Harris 1996). Given all this, activation of p53 is a desirable goal during cancer chemotherapy.

On the other hand, p53 is highly expressed in several normal tissues (such as hematopoietic tissues, intestinal epithelia, and testes) that are most commonly injured in cancer chemotherapy (Schwartz and others 1993; Komarova and others 1997). The activation of p53 in normal tissues during chemo- or radio-therapy may cause apoptosis and cell injury. For example, p53-dependent apoptosis in the intestinal epithelia could result in gastrointestinal side effects during cancer chemotherapy. Indeed, overexpression of p53 facilitates cancer cell death, which is good to cancer chemotherapy; however, it increases normal cell death as well (Blagosklonny and el Deiry 1998). Despite the relatively low expression of p53 in heart tissue, p53 has been reported to be responsible for cardiac myocyte apoptosis in various diseases (Long and others 1998; Ikeda and others 1999). The mechanism of this apoptosis is believed to involve
the upregulation of pro-apoptotic genes by p53. For instance, in cultured human endothelial cells, DOX induces apoptosis by a p53-dependent mechanism that involves triggering of mitochondrial cytochrome c release, activation of caspase-3, and downregulation of Bcl-2 protein expression (Lorenzo and others 2002).

The importance of p53 in DOX-induced cardiac cell apoptosis has been favored by the result that p53 inhibitor blocked the apoptosis by inhibiting the expression of p53 downstream genes, such as Bax, BclxL, and MDM2. This result is consistent with a previous study which showed that p53 is a transcriptional activator of the Bax gene (Miyashita and Reed 1995), and it supports the notion that DOX-induced apoptosis involves upregulation of pro-apoptotic genes by p53. Other studies indicate that the transcriptional activation of p53 may be an important mechanism in DOX-induced apoptosis. In fact, p53 protein levels and transcriptional activities were upregulated in cells by genotoxic or hypoxic stresses (Sanchez-Prieto and others 2000; Koumenis and others 2001). A recent research has shown that overexpression of a transcription factor, ATF3, inhibits cardiac myocyte apoptosis by downregulating p53 expression (Nobori and others 2002). In contrast, the current study found that p53 protein levels are determined by phosphorylation of p53 at Ser 15. Although the reasons for this discrepancy are unclear, it may be partly due to the different cell systems used in the two studies.

**Activation of p53**

An accumulating body of evidence suggests that the stability and transcriptional activity of p53 is regulated by phosphorylation at multiple serine residues (Chernov and others 1998; Sakaguchi and others 1998; Huang and others 1999). Phosphorylation at Ser 15 or Ser 20 promotes the stability of p53 by blocking the interaction of p53 with its negative regulator MDM2, which plays a critical role in ubiquitin-dependent p53 degradation (Shieh and others 1997). In the current study, p53 was phosphorylated at Ser 15 in mouse hearts several hours after DOX injection. This phosphorylation was followed by an elevation of p53 levels and
upregulation of downstream gene expression, indicating that p53 protein levels and activities can be upregulated via phosphorylation. Although this relationship was not explored in the current study, one possible mechanism is the abolishment of the interaction between p53 and MDM2, as discussed above. Alternatively, phosphorylation of p53 may increase the interaction of p53 with p300/CBP, an acetyl transferase that acetylates lysine residues located at the C-terminal of p53. This acetylation initiates the binding of p53 to specific gene promoters (Lambert and others 1998). In our unpublished experiments, it was found that p300/CBP was induced in mouse hearts after DOX injection. Presumably, this result could be due to DOX-induced phosphorylation of p53, which upregulates p53 levels by facilitating the activity of p300/CPB. Regardless of whether p53 upregulation and activation occur via a decrease of p53-MDM2 interaction, an increase of p53-p300/CPB interaction, or both, it is clear that phosphorylation of p53 enhances the transcriptional activity of p53 and results in the expression of downstream genes.

It is worthy to mention that the regulation of p53 is complicated because it has multiple phosphorylation sites. Ser 15 and Ser 20 are the most common phosphorylation sites when cells are attacked by genotoxic stresses (Huang and others 1999; Sanchez-Prieto and others 2000; She and others 2000). The regulation of p53 is further complicated by the fact that phosphorylation is not the only mechanism of regulation; for instance, researchers have recently demonstrated that regulation of p53 involves the interaction of MDM2, Akt, and PTEN by either phosphorylation, translocation, or degradation (Gottlieb and others 2002; Mayo and Donner 2002). Despite the complexity of p53 regulation, it is certain from past studies and from the current study that at the very least, the activation and stability of p53 is regulated through phosphorylation at Ser 15 (Chernov and others 1998; She and others 2000). Indeed, in this study, increased expression of Bax, BclxL, and MDM2 followed the phosphorylation and elevation of p53 in hearts from DOX-treated mice, implying that phosphorylation at Ser 15
initiates p53 transcriptional activation. In summary, phosphorylation of p53 at Ser 15 plays a
critical role in p53 activation in mouse hearts exposed to DOX.

**ERK1/2 and p38 MAPKs Mediate p53 Phosphorylation**

Previous studies have demonstrated that members of the MAPK family play major roles
in the phosphorylation and activation of p53 in response to ischemia/reperfusion and UV
radiation (Bulavin and others 1999; Huang and others 1999; Keller and others 1999). At least
three subfamilies have been recognized in this family: p38, ERK1/2, and JNK MAPKs. The
current study shows that DOX induces the phosphorylation of MAPK p38 and ERK1/2 prior to
phosphorylation of p53 at Ser 15 in mouse hearts. The role of p38 and ERK1/2 MAPKs in the
phosphorylation of p53 has been examined using specific inhibitors. Both the p38 inhibitor SB
203580 and the ERK1/2 inhibitor PD 98059 partially inhibited the phosphorylation of p53 at Ser
15. This was not true for SP 6000125, a JNK inhibitor, which indicates that only p38 and
ERK1/2 MAPKs are involved in the phosphorylation and activation of p53 in DOX-treated
mouse hearts.

We here for the first time show that MAPKs mediate the phosphorylation of p53 in
DOX-treated mouse hearts. The conclusion that phosphorylation of p53 relies on DOX-induced
phosphorylation of MAPKs is supported by a previous study indicating that another
anthracycline antibiotic, daunorubicin, induces phosphorylation of MAPKs in cultured cardiac
myocytes, although the phosphorylation of p53 was not examined in that study (Zhu and others
1999). One difference between the daunorubicin study and the current study is that daunorubicin
was shown to phosphorylate all three members of the MAPK family, while DOX was shown to
phosphorylate only p38 and ERK1/2, as discussed above. It is not surprising that daunorubicin
and DOX have different pathways in their actions on the heart, given that they are substantially
different compounds.

DOX probably induces the phosphorylation of MAPK through the generation of ROS,
since a past study showed that DOX-initiated p38 phosphorylation was suppressed in cardiac
myocytes by the overexpression of antioxidant metallothionein (Kang and others 2000). This indicates that ROS at least mediate DOX-induced phosphorylation of p38; more evidence is required to show that ROS also mediate the phosphorylation of ERK1/2 MAPK.

Expression of p53 Downstream Genes

In this study, DOX enhanced expression of p53 downstream genes Bax, BclxL, and MDM2 in mouse hearts, transducing signals from p53 to the downstream events of cardiac apoptosis. It is known that both Bax and BclxL are members of the Bcl-2 family proteins. Bax is known to promote apoptosis, and it is involved in cardiac myocyte apoptosis of right ventricles from rats following pulmonary artery-banding (Liu and others 1998; Ikeda and others 1999). In contrast, BclxL is an anti-apoptotic protein. At first glance, it seems inconsistent that both a pro-apoptotic gene (Bax) and an anti-apoptotic gene (BclxL) could be upregulated by p53. However, it is known that the absolute level of expression of BclxL is not as important as changes in the Bax/BclxL ratio in determining apoptotic activity. Furthermore, it has been reported that the loop domain of BclxL could be cleaved by caspases-1 and -3, and the C-terminal cleavage product is potently pro-apoptotic (Clem and others 1998). Taking these into consideration, we think the upregulation of BclxL is not contradictory, its cleavage product may contribute to DOX-induced cardiac apoptosis, although it remains to be determined if BclxL is cleaved by activated caspases in DOX-treated mouse hearts.

MDM2 appears to have a very different function than Bax and BclxL. Sequence analysis of the MDM2 promoter reveals the existence of a p53 DNA-binding consensus sequence, and p53 is known to augment MDM2 expression (Wu and others 1993). As discussed previously, MDM2 is an important negative regulator of p53 that plays a major role in controlling the p53 protein degradation rate (Haupt and others 1997; Honda and Yasuda 1999). Specifically, MDM2 binds to the N-terminal domain of p53 and acts as a ubiquitin ligase leading to p53 proteolysis by the 26S proteosome (Honda and Yasuda 1999). As such, there is a negative feedback loop involved in the regulation of p53: p53 augments MDM2 expression, which in turn augments the
degradation of p53. In this study, MDM2 was upregulated in the hearts of DOX-treated mice. Presumably, this would decrease p53 levels. However, there was an increase in p53 levels due to the phosphorylation of p53 and subsequent upregulation of p53. These seemingly contradictory findings might be due to the fact that phosphorylation of p53 at Ser 15 hinders its interaction with MDM2 (Shieh and others 1997), thus preventing MDM2 from augmenting p53 degradation. Further studies on this feedback loop may be helpful in finding approaches to block DOX-induced cardiac cell apoptosis.

**PFT-α Protects the Heart against DOX-Cardiotoxicity Partly Due to Anti-Apoptosis**

To confirm the hypothesis that DOX induces cardiac apoptosis in a p53-dependent pathway, the effects of PFT-α on DOX-induced cardiac cell apoptosis and cardiac injury were explored. It was found that PFT-α almost abolished DOX-induced cardiac cell apoptosis. PFT-α achieved the blockade by inhibiting the p53 transcriptional activity and therefore the activation of caspases-3 and -9. These effects of PFT-α may play crucial roles in its preserving myocardial structural integrity and protecting against DOX-induced cardiac dysfunction and release of CPK.

**Anti-Apoptosis of PFT-α and Protection against DOX-Induced Cardiotoxicity**

As shown in previous and current studies, DOX treatment results in acute cardiac dysfunction in mice (Weinstein and others 2000; Pacher and others 2002). Severe depression of left ventricular function has been shown to develop 5 days following DOX-treatment. This depression manifests as reduced LVDP and elevation of LVEDP, thereby decreasing SV and CO, despite a lack of cardiac dilatation. The acute cardiotoxicity and heart failure following DOX-administration in the mouse model observed in the current studies are consistent with clinical observations of DOX-induced cardiotoxicity. In this study, administration of PFT-α along with DOX improved mouse cardiac function, with an accompanying dramatic reduction of
cardiac cell apoptosis. Mice injected with DOX and PFT-α showed a relatively normal LVDP and LVEDP, indicating an improvement of cardiac contractility.

The mechanisms involved in the protective effects of PFT-α are only partially understood. It is likely that PFT-α improves cardiac function due to its inhibition of cardiac apoptosis. Apoptosis has been shown to contribute to the development of cardiac failure in paced dogs (Leri, Liu, and others 1998); as such, inhibition of cardiac cell apoptosis would presumably protect cardiac function. Indeed, inhibition of cardiac cell apoptosis by a p38 inhibitor SB 203580 greatly improved the recovery of cardiac function in ischemia-reperfusion injury (Yue and others 2000). Furthermore, overexpression of caspase-3 suppresses cardiac function recovery and exacerbates ischemia injury by enhancing cardiac cell apoptosis, indicating that protection of cardiac injury could be achieved by inhibiting cardiac myocyte apoptosis (Condorelli and others 2001). In addition, activation of PARP, a substrate of caspases and a nuclear enzyme that mediates apoptosis and ROS damage by transferring ADP ribose units from NAD$^+$ to nuclear proteins, promotes cardiac damage and development of heart failure in mice after DOX injection (Pacher and others 2002). Hence, PFT-α attenuates DOX-induced cardiotoxicity at least partly due to its inhibitory effects on cardiac cell apoptosis.

PFT-α Blocks DOX-Induced Gene Expression

PFT-α did not inhibit the elevation of p53 levels caused by DOX-induced phosphorylation of p53, but it blocked the p53-dependent expression of Bax, BclxL, and MDM2. These data indicate that PFT-α blocks transcriptional activities of p53. It is only partly understood how PFT-α accomplishes this action. In one study, PFT-α suppressed the translocation of p53 into nuclei and reduced the stability of nuclear p53, but it did not affect protein expression or sequence-specific DNA binding activity of p53 (Komarov and others 1999). In contrast, another study reported that PFT-α eliminates the elevation and DNA-binding activity of p53 protein in cultured human endothelial cells (Lorenzo and others 2002). Given the
different cell system and dose of PFT-α in these studies, it is possible that the action of PFT-α on the phosphorylation and expression of p53 varies in different conditions and cell systems, thus explaining these contradictory results. Regardless of which result is correct, all studies to date, including the current study, support the notion that PFT-α inhibits apoptosis through the blockade of a p53-dependent pathway.

**PFT-α Blocks DOX-Activated Caspases**

In this study, caspases-3 and -9 were activated in DOX-treated mouse hearts. This observation is consistent with previous studies in which activation of caspase-3 occurs following DOX treatment (Kumar and others 2001; Negoro and others 2001). The upregulation of Bax seen in this study indicates that cytochrome c may be released from mitochondria to initiate the activation of caspase-9, which cleaves the pro-caspase-3 into active caspase-3. Indeed, DOX has been shown to induce cytochrome c release in cultured cardiac myocytes and animal hearts (Wang and others 2001; Childs and others 2002).

Several studies suggest that upregulation of Bax and activation of caspase-3 are critical events in DOX-induced cardiac cell apoptosis. For example, inhibition of Bax by overexpression of BclxL has been shown to abolish DOX-induced caspase-3 upregulation (Kunisada and others 2002). Furthermore, overexpression of caspase-3 is sufficient to induce cardiac myocyte apoptosis (Wu and others 2000). Given all this, inhibition of Bax or caspase-3 would likely be adequate to block DOX-induced cardiac cell apoptosis. In the current study, the finding that treatment of mice with PFT-α dramatically reduces expression of Bax and activities of caspases-3 and -9 indicates that PFT-α may be effective in blocking DOX-induced cardiotoxicity by blocking DOX-induced apoptosis.
PFT-α Protects against DOX-Induced Myocardial Damage

DOX is known to cause ultrastructural changes of myocytes including cytoplasmic vacuolization, mitochondrial damage, and myofibrillar loss (Lefrak and others 1973; Rosenoff and others 1975; Billingham and others 1978). Because DOX triggers membrane peroxidation and disruption, release of CPK from myocytes is a marker of myocardial damage, which can therefore be quantified by measuring the CPK levels in the blood or culture medium (DeAtley and others 1999). In the current study, ultrastructural changes were observed shortly after DOX treatment in mice at a high dose of 22.5 mg/kg. Pre-treatment of mice with PFT-α significantly attenuated both these myocardial ultrastructural changes as well as the release of CPK. These experiments indicate that PFT-α inhibits DOX-induced myocardial damage.

It is not clear how PFT-α protects myocardial lesions in DOX-administered mice. However, the cardioprotection is possibly related to the protection of mitochondrial function. Mitochondria play key roles in myocardial damage, as they control cell destination by dominating energy metabolism and by initiating apoptosis. There is much evidence that DOX induces mitochondrial dysfunction. First, mitochondrial swelling and cristae disappearance have been observed in hearts from animals injected with DOX (Ferrans 1978). Second, DOX causes loss of mitochondrial membrane potential and activates caspases-3 and -9 in Jurkat cells (Gamen and others 2000). Third, treatment of isolated rat heart mitochondria with DOX leads to cytochrome c release, indicating that mitochondrial membrane function is damaged after exposure to DOX (Green and Leeuwenburgh 2002). Finally, cytochrome c release and cardiac myocyte apoptosis have been observed in animals and cultured cardiac myocytes exposed to DOX (Wang and others 2001; Childs and others 2002). In this study, PFT-α blocked Bax protein expression and activation of caspase-3. In addition, PFT-α blocks DOX-induced activation of caspase-12 (data not shown), which in turn activates caspase-9 (Morishima and others 2002). These results suggest that PFT-α inhibits DOX-induced Bax expression to protect mitochondria function, thereby preventing the activation of caspases.
Effects of PFT-α on the Anti-Tumor Potency of DOX

PC-3 is a DOX-sensitive prostate carcinoma cell line widely used to investigate the cytotoxic effects of various anti-cancer drugs, including DOX and other anthracycline derivatives (Carroll and others 1993; Polin and others 1997; Teicher and others 1997). DOX inhibits PC-3 cell growth and proliferation via a p53-independent apoptotic pathway; indeed, these cells lack expression of functional p53 (Wu and others 2002). In this study, PFT-α did not show any negative effects on the anti-tumor activities of DOX; in fact, it actually enhanced this activity at higher concentrations. Such an enhancing activity would be an especially valuable advantage in a combination PFT-α and DOX treatment in cancer chemotherapy.

Since p53 plays key roles in the checkpoint of cell growth and replication, the inhibition of p53 by PFT-α has raised concerns about its potential influence on normal cell life. A study has recently demonstrated that PFT-α decreases genetic stability in combination with another anti-neoplastic drug etoposide (Bassi and others 2002). If this finding is confirmed, secondary malignancies could emerge during long-term combination PFT-α and DOX treatment. Clearly, further research is required before any application of PFT-α can be initiated. Such research would be extremely justified, as this study suggests that PFT-α could be developed to prevent cardiotoxicity without decreasing the potency of DOX in p53-mutant cancer patients.

MEL Protects the Heart Against DOX-Induced Cardiotoxicity

Another aim of the current study was to investigate the protective effects of MEL on DOX-induced cardiotoxicity and apoptosis. In the study, MEL dramatically improved survival rates in mice treated with an acute high dose of DOX. Moreover, MEL was able to attenuate the acute effects of DOX-induced cardiac dysfunction and myocardial damages in mouse hearts. The cardioprotective effect of MEL could be attributed in part to the suppression of DOX-induced apoptosis of myocytes.
Many studies have shown that MEL has free radical scavenging capability (Beyer and others 1998; Antunes and others 1999; Karbownik and Reiter 2000). For instance, MEL can protect rat hearts against ischemia-reperfusion injury by scavenging hydroxyl radicals (Kaneko and others 2000). Since MEL has a relatively short half-life, its metabolite 6-OH MEL is of great interest. Past studies have examined the antioxidant activities of 6-OH MEL and a MEL analog, 8-M-PDOT. It was found that 6-OH MEL is almost as potent as MEL, but that 8-M-PDOT does not show any free radical scavenging ability (Liu and others 2002).

MEL or 6-OH MEL protects against DOX-induced cardiac dysfunction in both *in vitro* and *in vivo* studies. MEL or 6-OH MEL reversed DOX-induced functional suppression (including impaired contractility and diastolic properties), decreased coronary flow rate, and reduced HR in perfused mouse hearts. The *in vivo* study revealed a pronounced elevation of LVEDP, decline of LVESP, and a consequent reduction of SV and CO in DOX-administered mice, despite the lack of 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.

Previous work suggests that MEL’s cardioprotective effect can be attributed mainly to its antioxidant activity. For instance, MEL has recently been shown to attenuate DOX-induced changes in both the GSH/GSSG ratio and lipid peroxidation in the brain, heart, lung, and kidney (Agapito and others 2001). The present study expands on this work by providing more evidence for the importance of MEL’s antioxidant activity in its cardioprotective effect. Specifically, administration of MEL or 6-OH MEL dramatically improved survival rates of DOX-treated mice; in contrast, administration of 8-M-PDOT did not show any effects. Furthermore, it was demonstrated that MEL protects MEL1a receptor-deficient mice as well as their wild-type littermates against DOX-induced cardiotoxicity, excluding a significant role of the MEL1a
receptor in the actions of MEL. Taking all these observations into consideration, the improved survival rates for animals treated with MEL or 6-OH MEL is primarily due to their antioxidant activities, not to their receptor-mediated activities.

MEL or 6-OH MEL, but not 8-M-PDOT, reversed DOX-induced impairments of cardiac function in the perfused mouse heart. DOX caused suppression of cardiac contractility and reduction of HR, results that have been repeatedly observed in isolated mouse heart perfusion (Chen and others 1987; Ganey and others 1991; Platel and others 1999). The cardiac contractility injury has convincingly been shown to be related to free radical generation (Rajagopalan and others 1988). In contrast, the mechanism of HR reduction remains less clear due to the unavailability of effective research methods. One possibility is that DOX or DOX-induced ROS formation causes disturbances in calcium homeostasis, leading to a reduction of heart rate due to a decrease in intracellular calcium that reduces excitability of pacemaker cells in the sinoatrial node and other cells in the cardiac conduction system. This speculation was partly supported by the fact that DOX prolonged the duration of ventricular action potentials in isolated papillary muscle fibers by generating free radicals, as well as by the fact that the antioxidant vitamin E protects against this effect (Venditti and others 1998). It is very likely that MEL and 6-OH MEL reversed the reduction of heart rate in DOX-perfused mouse hearts via their antioxidant activities in this study.

The reduction of coronary flow seen in DOX-treated mice may have resulted from DOX-induced coronary vascular constriction. DOX-generated superoxide radicals are able to form peroxynitrite in the presence of NO (Weinstein and others 2000), 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 DOX-perfused rat hearts (Pelikan and others 1986). 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.

In Vivo vs. In Vitro Cardiac Function

The cardiac function studies were 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. In the in vitro studies, cardiac dysfunction occurs in the absence of structural changes because DOX-induced superoxide anions are known to compromise mitochondrial function by inhibiting mitochondrial complex I activity as well as by disrupting the mitochondrial permeability transition pore (Yen and others 1999). The ensuing increase in apoptosis occurred 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, the model used in this study is different from DOX-induced chronic cardiomyopathy in patients or experimental animals.

In the in vivo study, there were no remarkable histopathological lesions observed under the light microscope, an observation that was in agreement with a previous study in mice overexpressing MnSOD (Yen and others 1996). However, apoptosis and ultrastructural damage of the mitochondria and myofibrils was very evident, and these changes are certainly a major factor in cardiac dysfunction.

MEL Protects Against DOX-Induced Myocardial Lesions

As described above, DOX induces myocardial damage, characterized by cytoplasmic vacuolization, mitochondrial swelling, and myofibril disarrangement, which is consistent with results observed in previous studies (Rosenoff and others 1975; Billingham and others 1978; Bellini and Solcia 1985). 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 (Yen and others 1996). This suggests that the improvement of cardiac function by MEL or 6-OH MEL in the present 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

The current studies have shown that MEL and 6-OH MEL dramatically attenuated DOX-induced cardiac apoptosis. Previous studies have reported that cardiac injury induced by anthracycline antibiotics involves apoptosis of cardiac myocytes, but the mechanisms involved remain to be elucidated (Kumar and others 1999; Zhu and others 1999; Arola and others 2000; Wang and others 2001). The most probable mechanism of DOX-induced apoptosis involves the production of DOX-generated ROS. Several groups have shown that DOX induces apoptosis in both cultured cardiac myocytes and hearts from treated animals (Maulik and others 1998; Kumar and others 1999; Nakamura and others 2000). Moreover, DOX-induced apoptosis in cultured cardiac myocytes and endothelial cells was inhibited by free radical scavengers, implying that free radicals at least partly contribute to DOX-induced cardiac apoptosis (Kotamraju and others 2000). These studies suggest that DOX induces apoptosis through free radical production, and that apoptosis contributes to DOX-induced cardiotoxicity. This may explain how MEL or 6-OH MEL reduces cardiac apoptosis in DOX-injected mice; their antioxidant activities apparently play important roles.

Due to their free radical scavenging capability, MEL or 6-OH MEL diminished serum CPK elevation in DOX-treated mice. As shown in previous studies, DOX induces lipid peroxidation through generation of free radicals, leading to release of CPK from myocytes (Lee
and others 1991; Luo and others 1997; DeAtley and others 1999). The results of the current study indicate that MEL or 6-OH MEL may protect myocytes against membrane damage induced by DOX, thereby protecting their structure and function.

Effects of MEL or 6-OH MEL on the Potency of DOX

PC-3 cells have been used to observe the effects of MEL or 6-OH MEL on anti-tumor activities of anthracycline antibiotics like DOX, as discussed above. In this 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. These results are inconsistent with previous reports that MEL can enhance anti-tumor activities of DOX in certain tumor-bearing mice and that MEL can increase the efficacy of other cancer chemotherapy in cancer patients (Lissoni and others 1999; Wahab and others 2000). This discrepancy may be attributed to differences in experimental models and conditions, or they may be due to a difference in the response of different tumors to MEL. Although MEL did enhance the anti-neoplastic effects of DOX in the current study, the results clearly show that MEL or 6-OH MEL does not interfere with DOX’s anti-tumor activity.

Future Directions

This study has shown that DOX induces acute cardiotoxicity at a single dose injection. p53-dependent cardiac cell apoptosis plays an important role in DOX-induced cardiotoxicity. Both the p53 inhibitor PFT-α and the antioxidant MEL can attenuate DOX-induced cardiotoxicity, partially because of their inhibition of DOX-induced cardiac apoptosis.

However, all of these results used an acute cardiotoxicity model. The effects of PFT-α and MEL on DOX-induced chronic cardiomyopathy should be further investigated. In addition, the mechanism involved in PFT-α and MEL should be further explored in a cultured cardiac myocyte system.
CHAPTER 5
SUMMARY AND CONCLUSION

1. DOX induces significant increase of cardiac cell apoptosis, myocardial lesions, and cardiac dysfunction in ICR mice 3 days after administration.

2. p53 protein is phosphorylated at Ser 15 in mouse hearts several hours after DOX injection, followed by the expression of downstream genes Bax, BclxL, and MDM2, indicating that the p53-dependent pathway plays a major role in DOX-induced cardiac cell apoptosis.

3. The upstream events of the p53-dependent pathway are phosphorylation of ERK1/2 and p38 MAPKs, which phosphorylate p53 at Ser 15 in DOX-treated mouse hearts.

4. PFT-α blocked DOX-induced cardiac cell apoptosis by inhibiting the expression of p53 target genes and the activation of caspases-3 and -9.

5. PFT-α has a protective ability against DOX-induced myocardial ultrastructural changes, serum CPK elevation, and cardiac dysfunction.

6. MEL and 6-OH MEL protect cardiac function in DOX-perfused mouse hearts, as well as in mice 5 days after administration of DOX; they also improves the survival rate and attenuate the myocardial structural damages and CPK release in mice treated with DOX.

7. MEL and 6-OH MEL significantly reduce cardiac cell apoptosis in DOX-treated mice.

8. MEL protects MEL1a receptor-deficient as well as wild-type mice against DOX-induced cardiac dysfunction in isolated hearts, indicating that the MEL1a receptor is not important in its cardioprotective effect.

9. Because 8-M-PDOT has no protective effects due to a lack of antioxidant activity, MEL and 6-OH MEL exert their actions primarily through their free radical scavenging ability.

10. Neither PFT-α nor MEL compromises the anti-tumor potency of DOX on p53-null PC-3 cancer cells.
BIBLIOGRAPHY


APPENDIX A

Components of Krebs-Henseleit Buffer Used in Heart Perfusion

Table 6  Components of Krebs-Henseleit Bicarbonate Buffer

<table>
<thead>
<tr>
<th>Compounds</th>
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<th>Mass (g/L)</th>
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<tr>
<td>NaCl</td>
<td>58.4</td>
<td>118</td>
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<td>Glucose</td>
<td>180.2</td>
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<td>NaHCO₃</td>
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<td>KCl</td>
<td>74.56</td>
<td>4.7</td>
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<tr>
<td>KH₂PO₄</td>
<td>136.09</td>
<td>1.2</td>
<td>0.16</td>
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<tr>
<td>MgSO₄</td>
<td>264.47</td>
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<tr>
<td>CaCl₂</td>
<td>147.02</td>
<td>2.0</td>
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APPENDIX B

Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Apaf-1</td>
<td>apoptotic protease activating factor-1</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>CFR</td>
<td>coronary flow rate</td>
</tr>
<tr>
<td>CO</td>
<td>cardiac output</td>
</tr>
<tr>
<td>CPK</td>
<td>creatine phosphokinase</td>
</tr>
<tr>
<td>d</td>
<td>day</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA-PK</td>
<td>DNA-dependent protein kinase</td>
</tr>
<tr>
<td>DOX</td>
<td>doxorubicin</td>
</tr>
<tr>
<td>±dP/dt</td>
<td>first derivatives of LV pressure over time</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal regulated kinase</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>HR</td>
<td>heart rate</td>
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<td>6-OH MEL</td>
<td>6-hydroxymelatonin</td>
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<td>i.p.</td>
<td>intraperitoneal injection</td>
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<tr>
<td>ISOL</td>
<td><em>in situ</em> oligo ligation</td>
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<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
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<tr>
<td>LV</td>
<td>left ventricle</td>
</tr>
<tr>
<td>LVDP</td>
<td>left ventricular developed pressure</td>
</tr>
<tr>
<td>LVEDP</td>
<td>left ventricular end-diastolic pressure</td>
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<tr>
<td>LVESPV</td>
<td>left ventricular end-systolic pressure</td>
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<tr>
<td>Term</td>
<td>Definition</td>
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<td>-----------------------------------------------------------------------------</td>
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<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase family</td>
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<td>MDM2</td>
<td>murine double minute clone 2</td>
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<td>MEK</td>
<td>MAPK kinase</td>
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<td>MEL</td>
<td>melatonin</td>
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<td>8-M-PDOT</td>
<td>8-methoxy-2-propionamidotetralin</td>
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<td>MnSOD</td>
<td>manganese superoxide dismutase</td>
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<tr>
<td>NO</td>
<td>nitric oxide</td>
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<tr>
<td>PARP</td>
<td>poly ADP-ribose polymerase</td>
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<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
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<tr>
<td>PFT-α</td>
<td>pifithrin-α</td>
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<tr>
<td>PTP</td>
<td>permeability transition pore</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>RPA</td>
<td>ribonuclease protection assay</td>
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<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
</tr>
<tr>
<td>SV</td>
<td>stroke volume</td>
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<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-α</td>
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<tr>
<td>TNFR</td>
<td>TNF-α receptor</td>
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<tr>
<td>TUNEL</td>
<td>in situ terminal deoxynucleotidyl transferase mediated nick-end labeling</td>
</tr>
<tr>
<td>wk</td>
<td>week</td>
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</tbody>
</table>

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VITA

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- 2001 Students’ Choice Award in 17th Annual Student Research Forum, East Tennessee State University
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