

Protective effect of ocotillol against doxorubicin-induced acute and chronic cardiac injury

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Abstract. Doxorubicin (Dox) has been clinically observed to exert marked anticancer activity. However, it is severely restricted by its associated dose-dependent cardiotoxicity, which may be attenuated by decreasing the cumulative dosage via combining with a non-toxic 'sensitizer'. We previously reported that ocotillol is capable of enhancing the antitumor activity of Dox; however, the effects of ocotillol on its cardiotoxicity remain unclear. In the current study, the effects of ocotillol on the toxicity of Dox were investigated, particularly its role in cardiotoxicity. In the acute injury model, pre-administration of ocotillol prolonged the survival time. In the chronic animal model, pre-administration of ocotillol decreased the elevated levels of plasma creatine kinase (CK) and CK-MB, as well as attenuated the pathological changes that occurred. Pre-treatment with ocotillol ameliorated the decreased glutathione level and reduced the cumulated malondialdehyde in the heart tissue. In addition, pre-treatment with ocotillol restored the lowered white blood cell count. The results indicate that Dox co-treatment with ocotillol may effectively alleviate its associated toxic injury, particularly cardiotoxicity. Thus, co-administration of Dox with ocotillol may be a potential therapeutic strategy.

Introduction

Doxorubicin (Dox), isolated from cultures of *Streptomyces peuce-tius var. caesius*, is a cytotoxic anthracycline antibiotic and is commonly used for cancer treatment (1). Although it has been

well demonstrated that Dox exerted robust antitumor activity, the associated dose-dependent cardiotoxicity restricted its clinical use (1-3). A novel therapeutic regime, in which one compound is employed to enhance its antitumor activity while decreasing the severe side effects, may improve the care of cancer patients.

The mechanism of cardiac injury induced by Dox, which may involve free radical stress, calcium overloading, mitochondrial dysfunction or dysregulation of iron hemostasis, has been investigated (1,4). The exact molecular mechanisms, however, in which reactive oxygen species (ROS) are considered to be central remain unclear (1,5). It was reported that Dox has been observed to transform into a semiquinone radical, which then reduces oxygen to produce superoxide and reacts with polyunsaturated fatty acids to yield lipid hydroperoxide (6). The antioxidants, which cleaved the generated ROS, were hypothesized to exhibit protective effects against Dox-induced cardiomyopathy (7,8). Notably, a number of antioxidants, including thrombopoietin, schisandrin B and probucol, as well as the FDA-approved dexrazoxane, have been shown to prevent and treat Dox induced-cardiomyopathy (7-10). Thus, novel antioxidants, which enhance the anticancer activity of Dox, may be a potential candidate for a novel combination therapeutic regime.

The ginsengs have been employed in Asian societies for thousands of years and used as herbal medication for a variety of disorders (11). The major pharmacological properties of ginseng were attributed to the ginsenosides, the active ingredients, which have been documented to possess diverse activities, including neuroprotective, cardioprotective, antioxidant and anticancer properties (11,12). Ocotillol (Fig. 1), a derivative of pseudoginsenoside F11 from American ginseng, was recently reported to potentiate the anticancer activity of Dox (13). However, the effect of ocotillol on Dox-induced cardiac injury, which is the most severe and lethal toxic effect of Dox, remains to be fully understood. In the current study, an *in vivo* model was developed to determine the effect of ocotillol on Dox-induced acute and chronic cardiomyopathy.

Materials and methods

Materials. Ocotillol was isolated from American Ginsengs by the Shandong Luye Pharmaceutical Company (Yantai, China)

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and obtained as white powder with the molecular formula $C_{30}H_{52}O_5$ and a molecular weight of 492. The purity of the compound was checked by high-performance liquid chromatography and was observed to be >98.5%. *In vivo*, ocotillol and Dox (Beyotime Institute of Biotechnology, Hangzhou, China) were dissolved in 1% carboxymethylcellulose sodium (CMCS; Shandong Luye Pharmaceutical Company) and 0.9% sodium chloride as the proposed doses, respectively.

Animals. Male Swiss mice (weight, 18-22 g) were obtained from Shandong Luye Pharmaceutical Company. The animals were housed in a light- and temperature-controlled room (21-22°C; humidity, 60-65%) and had *ad libitum* access to a standard diet and water. All the experiments were performed in accordance with the Guideline for Care and Use of Experimental Animals of Experimental Animal Research Committee of Yantai University.

Model of Dox-induced acute cardiomyopathy. Male Swiss mice were randomly divided into two groups (n=10 per group). The control group was administered with one dose of Dox dissolved in 0.9% NaCl intraperitoneally (i.p.) at 20 mg/kg on day 1 and 10 doses of CMCS gavage (i.p.) daily. The pretreated groups received a total of 10 doses of ocotillol at 10 mg/kg daily with the first administration 24 h prior to administration of one dose of Dox. The animals were checked twice daily and the number of dead mice were recorded continuously for 12 days post-dose administration. The survival curve was presented and the difference was compared between the two groups.

Model of Dox-induced chronic cardiomyopathy. Male Swiss mice were randomly divided into four groups (n=10 per group). The control group was administered a total of six doses of 0.9% NaCl i.p. every other day and eight doses of CMCS daily orally (p.o.). The Dox group was administered a total of six doses Dox dissolved in 0.9% NaCl i.p. at 3 mg/kg (accumulative dose 18 mg/kg) every other day, and a total of eight doses of CMCS p.o. daily. The pretreated groups received a total of eight doses ocotillol at 5 mg/kg and 10 mg/kg daily with the first administration 24 h prior to Dox injection.

Blood sampling and tissue preparation were performed under anesthesia with ketamine (Peking Union Medical College, Beijing, China) at 50 mg/kg and xylazine (EnoGene, Nanjing, China) at 20 mg/kg 24 h after the final dose. Blood samples were drawn into heparinized tubes and divided into two parts. One was for the assays of white blood cell counts (WBC), red blood cell counts (RBC) and platelets (PLT). The other part was immediately centrifuged (2,500 × g for 10 min at 4°C), and the plasma was stored at -80°C, and the content of creatine kinase (CK) and CK MB fraction (CK-MB) in plasma were measured with a Hitachi 7060 Fully Automated Biochemistry Analyzer (Hitachi, Tokyo, Japan).

Following the sacrifice of the mice by carbon dioxide asphyxiation, the hearts were rapidly removed. Half the tissue was weighed and homogenized in ice-cold normal saline (1/9, w/v) and centrifuged (5,000 × g for 10 min at 4°C). The suspension was stored at -80°C and the content of glutathione (GSH) and malondialdehyde (MDA) in the heart tissue were determined using commercial kits provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The protein concen-

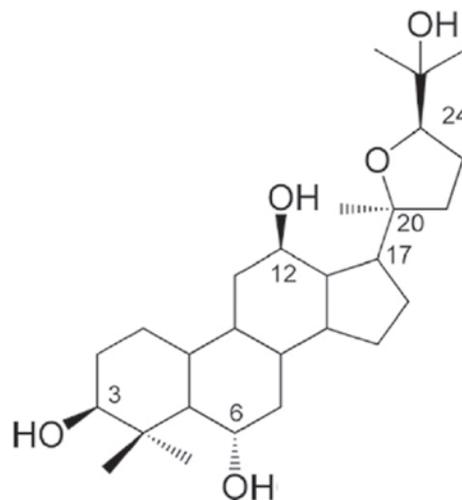


Figure 1. Chemical structure of ocotillol.

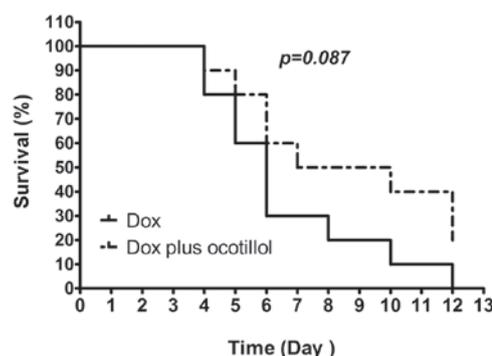


Figure 2. Effect of ocotillol on mice survival in model of Dox-induced acute cardiomyopathy. Mice were assigned to two groups (n=10 per group): Dox alone (20 mg/kg) and Dox (20 mg/kg) plus ocotillol (10 mg/kg), and administered with the indicated compounds. The difference in survival times between groups was assessed using the Kaplan-Meier test. Dox, doxorubicin.

tration was determined by the bicinchoninic acid kit (Beyotime Institute of Biotechnology) and was used to normalize the data.

The other sections of heart tissue were fixed with 4% formaldehyde overnight, dehydrated in ascending grades of alcohol and embedded in paraffin. Serial sections were sliced at 5 μm and stained with hematoxylin and eosin. The sections were analyzed and images were captured using a Nikon Eclipse 50i microscope (Nikon, Chiyoda, Japan) by two pathologists with blind investigation and the representative images are presented.

Statistical analysis. Survival rates were compared by Kaplan-Meier log-rank test. Other data in this study are expressed as the mean ± standard deviation and analyzed by one-way analysis of variance. The difference between two groups was determined by Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Ocotillol prolongs the survival time in a model of acute Dox-induced cardiomyopathy. The mice were injected once

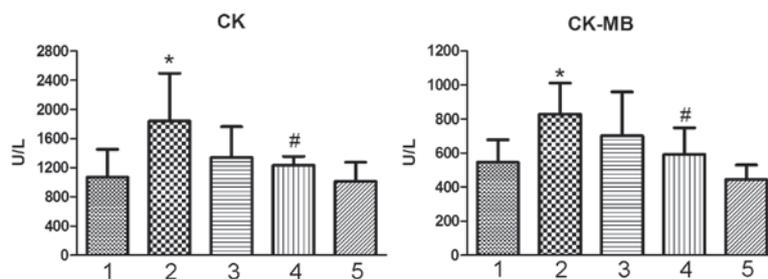


Figure 3. Effect of ocotillol on serum CK and CK-MB in the model of Dox-induced chronic cardiomyopathy. Animal serum was prepared and the serum CK and CK-MB were detected. 1, control group; 2, Dox group (3 mg/kg); 3, Dox (3 mg/kg) plus ocotillol group (5 mg/kg); 4, Dox (3 mg/kg) plus ocotillol group (10 mg/kg); and 5, ocotillol group (10 mg/kg). All data are expressed as the mean \pm standard deviation (n=10). *P<0.05, vs. the control group; #P<0.05, vs. the Dox group. Dox, doxorubicin; CK, creatine kinase; CK-MB, creatine kinase MB fraction.

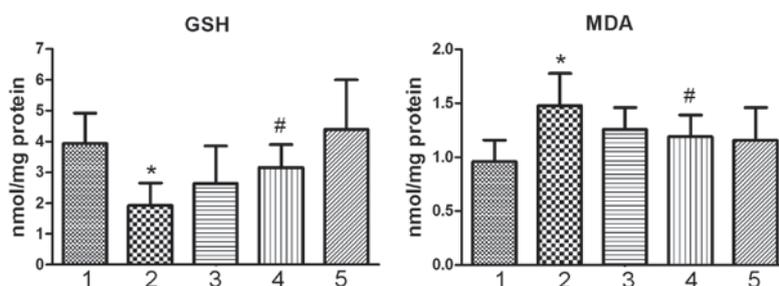


Figure 4. Effect of ocotillol on the content of GSH and the content of MDA in heart tissue. The tissues were cooled, homogenized, and then determined for GSH and MDA. 1, control group; 2, Dox group (3 mg/kg); 3, Dox (3 mg/kg) plus ocotillol group (5 mg/kg); 4, Dox (3 mg/kg) plus ocotillol group (10 mg/kg); and 5, ocotillol group (10 mg/kg). All data are expressed as the mean \pm standard deviation (n=10). All data are expressed as means \pm SD (n=10). *P<0.05, vs. control group; #P<0.05, vs. Dox group. Dox, doxorubicin; GSH, glutathione; MDA, malondialdehyde.

with Dox at dosage of 20 mg/kg and the morbidity was observed twice daily. As shown in Fig. 2, all animals in the Dox group succumbed within 12 days post-dose. However, co-treatment with ocotillol at a dosage of 10 mg/kg, prolonged the survival rate ($p=0.087$, compared with the Dox group), in which 2 of 10 animals remained alive at the end of the experiment.

Ocotillol exerts a protective effect in a model of chronic Dox-induced cardiomyopathy

Plasma CK and CK-MB. The levels of CK and CK-MB are biomarkers of heart tissue damage (14). As a cardiotoxic agent, Dox significantly increased the level of CK and CK-MB in the treated animals (Fig. 3; $P<0.05$, vs. the control group), which indicated the occurrence of heart tissue injury. Co-treatment with ocotillol at a dose of 10 mg/kg was observed to significantly decrease the elevated levels of CK and CK-MB (Fig. 3, $P<0.05$, vs. Dox group). Ocotillol alone exhibited no marked effect at the tested dosage.

Tissue GSH and MDA. Tissue GSH is a significant antioxidant biomolecule against oxidative stress (15). Following treatment with Dox, the contents of GSH in the heart tissue was significantly decreased (Fig. 4; $P<0.05$, vs. the control group). The content of MDA was significantly increased in the animals treated with Dox (Fig. 4; $P<0.05$, vs. control group). Co-treatment with ocotillol, however, significantly alleviated the reduction of GSH and elevation of MDA (Fig. 4; $P<0.05$, vs. Dox group). Ocotillol

alone exhibited no marked effect on the content of GSH and MDA in heart tissue at the tested dosage.

Histological examination. Histological examinations for the left ventricles were performed as previously described (15). The animals in the control and ocotillol groups were observed with normal cardiomyocyte morphology. However, in animals treated with Dox, disorganization of myofibrillar arrays and cytoplasmic vacuolization were observed (Fig. 5). When pre-treated with different dosage of ocotillol, less histopathological changes were observed. Ocotillol alone had no marked effect at the tested dosage.

Ocotillol attenuates decreased WBC count. Following two weeks of administration, Dox markedly reduced the WBC count (Table I; $P<0.05$, vs. control group). However, co-treatment with ocotillol at a dosage of 10 mg/kg restored the lowered WBC to a greater degree (Table I; $P<0.05$, compared with Dox group). Dox in the presence or absence of ocotillol had no marked effects on RBC and PLT counts (Table I).

Discussion

In previous studies, repeated administration of Dox lead to frequent and devastating cardiomyopathy, and complications commonly lead to a reduced quality of life for the patient and/or morbidity (1,3). A non-toxic 'sensitizer', which enhanced the potential of Dox without increasing its toxic

Table I. Effects of ocotillol and Dox on WBC, RBC and PLT counts (mean \pm standard deviation).

Group (n=10)	WBC (10^6 /ml)	RBC (10^9 /ml)	PLT (10^6 /ml)
Control	11.45 \pm 1.74	12.86 \pm 1.40	555.00 \pm 145.56
Dox 3 mg/kg	3.24 \pm 0.33 ^a	10.48 \pm 1.49	406.67 \pm 80.40
Dox 3 mg/kg+Ocotillol 5 mg/kg	5.13 \pm 1.10 ^b	11.03 \pm 1.20	476.52 \pm 188.43
Dox 3 mg/kg+Ocotillol 10 mg/kg	7.13 \pm 1.67 ^b	10.18 \pm 2.04	576.33 \pm 298.49
Ocotillol 10 mg/kg	11.2 \pm 2.89	12.76 \pm 1.08	658.00 \pm 135.01

^aP<0.05, vs. the control group; ^bP<0.05, vs. the Dox group. Dox, doxorubicin; WBC, white blood cells; RBC, red blood cells; PLT, platelet.

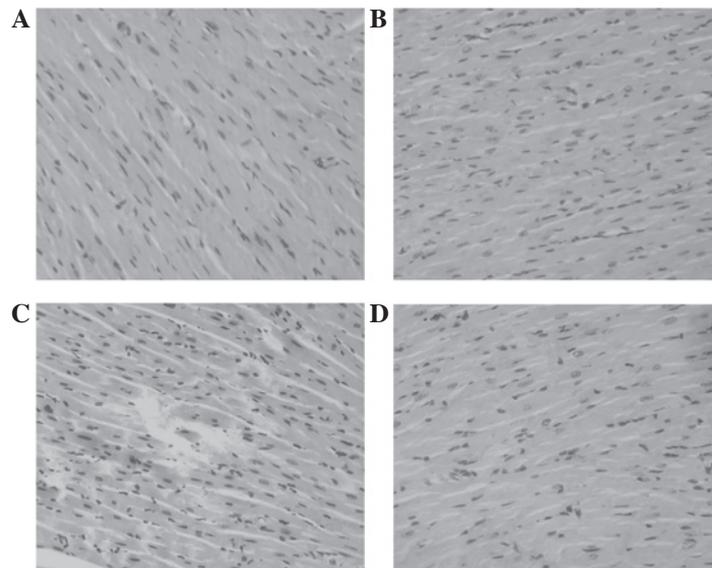


Figure 5. Effect of ocotillol on the pathological changes in left ventricles. Half of the heart was fixed stained with hematoxylin and eosin. Light microscopy-observations were conducted and the representative fields are shown. (A) Control group, (B) ocotillol group (10 mg/kg), (C) Dox group (3 mg/kg) and (D) Dox (3 mg/kg) plus ocotillol group (10 mg/kg). Magnification, x400. Dox, doxorubicin.

effect, is likely to improve the treatment of cancer patients. Ocotillol was recently reported to enhance the potential of Dox (15) and in the current study, evidence is provided that ocotillol may also exert cardioprotective effects against Dox-induced cardiomyopathy.

Since ocotillol had been shown to enhance the antitumor activity of Dox, it was important to determine whether ocotillol may also increase its toxicity, particularly for its dose-dependent and irreversible cardiotoxicity. In the model of Dox-induced acute cardiomyopathy, co-treatment with ocotillol did not decrease the survival time, but exhibited protective activity against Dox-induced morbidity. Dox-ocotillol combination therapy, therefore, may not only increase the antitumor effects, but may also decrease the toxic effects, which in turn may provide clinical benefits.

The chronic cardiac injury model was performed to determine the effect of ocotillol on the Dox-induced cardiomyopathy. CK and CK-MB are well established diagnostic markers for myocardial function (14). During cardiac myocyte injury, these enzymes were leaked into the serum, which was easily detected in the blood samples. In the current study, cumulative doses of Dox (18 mg/kg) caused a significant increase in CK and CK-MB, which indicated that Dox exhib-

ited severe cardiotoxicity. The increased plasma enzymes were suppressed by pre-treatment with ocotillol, which indicated that ocotillol was capable of attenuating Dox-induced cardiac injury. Notably, the histological examination of the heart tissue also showed that pre-treatment with ocotillol could significantly alleviate the Dox-induced histopathologic lesion.

It has been well documented that the cardiotoxicity of Dox was mediated by ROS (5). In view of the importance of oxidative stress to cardiac injury, a number of studies have suggested that the cardioprotective effects of ginseng ingredients, including ginsenoside Rg1 and Rh2, were associated with the reduction in oxidative stress by enhancing endogenous antioxidant reserve (15,16). Based on our previous results, ocotillol is capable of exerting cardioprotective effects on myocardial injury induced by isoproterenol in rats by enhancing the antioxidative potency of the heart (17). Pre-treatment with ocotillol may increase the content of GSH in heart tissue and as a consequence, the MDA may be cleared by these anti-oxidant biomolecules.

Bone marrow suppression is a major toxic property of cytotoxic drugs, including Dox and paclitaxel, which has been primarily observed with leukopenia and neutropenia (18,19). In the current study, the decreased extents of the leukopenia

in the mice treated with Dox were significantly attenuated by co-treatment with ocotillol, which indicated that ocotillol was capable of alleviating the bone marrow toxicity of Dox.

The effect of ocotillol on the toxicity of Dox was completely different to its effect on the potency of Dox, the exact mechanism of which remains unknown. One possible interpretation is that this occurred in a cell/tissue-dependence manner (20), in which these selective characteristics are likely to further benefit its co-administration clinically. This finding, which was also observed in a number of published compounds, including Rh2 and schisandrin (8,15), supported the further investigation for ocotillol as a protector against Dox-induced cardiotoxicity.

In conclusion, the present study showed the protective effect of ocotillol against Dox-induced cardiomyopathy, which may be associated with the role of ocotillol in the maintenance of the endogenous anti-oxidant status in heart tissue. Combined with the previous findings, ocotillol was capable of enhancing the antitumor activity of Dox, the data implied that use of ocotillol with Dox may be an improved therapeutic strategy.

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