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Fluorescence spectra of cardiac myosin and *in vivo* experiment: studies on daunorubicin-induced cardiotoxicity

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ABSTRACT

Objective(s): The objective of this study was to investigate the interaction of daunorubicin (DNR) and cardiac myosin (CM) and the changes in mice hearts to exhibit DNR-induced cardiotoxicity .

Materials and Methods: The interaction between DNR and CM was expressed using fluorescence quenching at pH 4.0-9.0 and 15-37 ˚C. DNR-induced cardiotoxicity was studied using *in vivo* experiment. Forty groups mice were used control group in which mice were treated with DNR orally, and three DNR-treated groups in which mice were injected intraperitoneally with DNR at seven bolus doses of 2.0, 4.0, and 6.0 mg/kg body weight, respectively. Heart indices and myocardial enzyme levels were obtained by histopathological and biochemical analysis.

Results: The fluorescence quenching mechanism of DNR-CM complex was observed to be a static procedure at 20 ˚C (pH 7.4), and weakly acidic environment (pH 4.0-6.0) or higher temperature (30-37 ˚C) promoted the interaction between DNR and CM, causing variations in conformation and normal physiological functions of CM. Thermodynamic studies demonstrated that the binding of DNR to CM was a spontaneous process driven by entropy. It also indicated that hydrophobic interaction and hydrogen bonds may play essential roles in the combination of DNR with CM. In addition, 4.0-6.0 mg/kg DNR-treated mice exhibited obvious histopathological lesion, increase in myocardial enzyme level, and reductions in blood cell count.

Conclusion: Our results are valuable for better understanding the particular mode of DNR-CM interaction, and are important to have a deeper insight into the DNR-induced cardiotoxicity.

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Introduction

Cardiac myosin (CM), the molecular motor of the heart, is the major constituent and contracting protein of the cardiac muscle. It is composed of two heavy chains (MHC, 220 kD) and two pairs of light chains, namely MLC1 (27 kD) and MLC2 (20 kD), with a molecular weight of approximately 520 kD (1). In cardiac myocyte, myosin plays a significant role in regulating myocardial contractile activity and improving cardiac performance (2). It is known that cardiomyopathy is linked with mutations in the contractile apparatus of cardiac muscle. The most common contractile protein mutations that cause heart disease are seen in myosin heavy chain (3-5). Some researchers have proven that heart failure is a response to structural and/or functional alteration of CM (6-8).

Daunorubicin (DNR, Figure 1), originally isolated from *Streptomyces peucetius varcaesitue*, belongs to the anthracycline family of compounds with intrinsic antibiotic and anti-tumor activities (9). DNR has become a mainstay in modern chemotherapy (10). Frontline administration of a higher dose of DNR might improve the outcome of acute lymphoblastic leukemia patients (11). However, DNR was observed to cause significant impairment of cardiac contractile function (2, 12). The overt cardiac toxicity of DNR could result in a late irreversible cardiomyopathy. Its cumulative toxicity and mainly drug-induced cardiotoxicity limit the administration of DNR in elderly and heavily pretreated patients (13). Therefore, the clinical value of NDR is consequently greatly compromised (14). Earlier researches proposed that cardiotoxicity was mainly related to the oxidative stress injury (15, 16), but the new studies further found that mitochondrial

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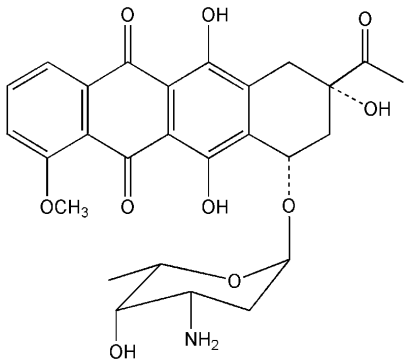

Figure 1. Molecule structure of daunorubicin (DNR)

biogenesis (17), cell energy metabolism (18), neuregulin (19), calcitonin (20, 21), and C13 alcohol metabolites (22) participate in its cardiotoxicity. To date, the exact mechanism has not been fully understood, which was the reason motivated us to further study.

Fluorescence quenching is an effective technique to study the interactions between drug and protein, which can provide important information about changes in protein conformation (23). Furthermore, the interaction between DNR and CM in the presence of metal ions has also been reported in our previous studies (24-26). However, they only performed Stern-Volmer binding parameters in a single temperature without any thermodynamic study. Therefore, to find out the pathogenesis of cardiotoxicity, further studies on the interaction between DNR and CM at different acidities and temperatures are of quite importance.

The aim of this work was to investigate the effect of DNR on CM by using fluorescence quenching to explore the interaction mechanism of DNR and CM. In addition, animal experiment was performed to study the influences of DNR on mice left ventricle, myocardial enzyme level, and hematologic parameters. These results will provide fundamental information about the pathogenesis of cardiac toxicity induced by DNR.

Materials and Methods

Reagents

DNR hydrochloride for Injection was supplied by Pfizer Italia S.r.1. Phosphate buffer was purchased from Sigma Chemical. CM was extracted and purified from pig heart, referring to our earlier work (27). Other chemical reagents were made in China and were of analytical grade. All solutions were prepared with ultrapure water (ULUP-2 Water Purification System, China).

Fluorescence spectra measurements

The fluorescence spectra of CM and DNR-CM were obtained according to the literature (25, 26). Throughout the fluorescence studies, CM solution was prepared as follows: 5.2 mg CM powder was weighed accurately, diluted it with 100 ml buffer solution at different levels of acidity, well shaken, made at the concentration of 0.1 μM, and incubated at different temperatures. The pH values were measured using a digital pH-meter (pH-3, Shanghai Lei Ci, China) with a combined glass electrode.

Calculation of fluorescence quenching constants for DNR-CM system

The fluorescence quenching of protein, caused by drug and normally divided into dynamic and static processes, is dependent on Stern-Volmer and Lineweaver-Burk double-reciprocal equations, respectively:

$$\lg\frac{(F_0-F)}{F}=\lg\left(\frac{F_0}{F}-1\right)=\lg K_{LB}+n\lg[Q] \quad (1)$$

$$\lg\frac{(F_0-F)}{F}=\lg\left(\frac{F_0}{F}-1\right)=\lg K_{LB}+n\lg[Q] \quad (2)$$

where F_0 and F are the steady-state fluorescence intensities of the biomolecule in the absence and presence of quencher, respectively. K_q is the quenching rate constant of the biomolecule, K_{SV} is the Stern-Volmer quenching constant, $[Q]$ is the concentration of quencher, τ_0 is the average fluorescence lifetime of biopolymers without quencher which is generally taken as 10^{-8} sec, K_{LB} is the binding constant and n is the number of quencher binding sites per protein molecule. The maximum scatter collision quenching constant K_{SV} of various quenchers with the biopolymer is 100 M^{-1}, otherwise, the quenching mechanism is considered as static quenching procedure (24).

Thermodynamic studies

The thermodynamic parameters, Gibbs free energy ($\Delta G°$), enthalpy change ($\Delta H°$), and entropy change ($\Delta S°$), are the main evidence for confirming the binding force of NDR and CM, and are evaluated using the equations (28):

$$\Delta H° = \frac{RT_1T_2}{T_2-T_1}\ln\frac{K_2}{K_1} \quad (3)$$

$$\Delta G° = RT\ln K = \Delta H° - T\Delta S° \quad (4)$$

where R is the gas constant, K_1 and K_2 are the binding constants of DNR and CM at 298 K and 310 K, respectively.

Animals and diet supplementation

Healthy adult male Kunming mice (weighing 22-25 g) were purchased from the Experimental Animal Center of Hubei Provincial Center for Disease Control (Reg. No. SCXK (Hubei) 2008-0003). The animals were housed at constant temperature (23±1°C) and humidity (55-60%) with a 12 hr/12 hr day/night cycle. Standard food (Wuhan Institute of Biological Products, China) and fresh water were available *ad*

Table 1. Effect of pH on the fluorescence quenching and binding constants of daunorubicin-cardiac myosin complex (20 ˚C)

pH	K_{SV} ($10^5 M^{-1}$)	R_{SV}	K_{LB}(M^{-1})	R_{LB}
4.0	2.887	0.9969	3.780×10^4	0.9942
4.5	1.966	0.9897	2.951×10^4	0.9955
5.0	1.897	0.9924	3.001×10^3	0.9934
5.5	1.844	0.9894	2.980×10^3	0.9955
6.0	1.511	0.9943	2.744×10^3	0.9964
6.5	1.302	0.9933	4.837×10^2	0.9984
7.0	1.355	0.9937	3.665×10^2	0.9922
7.4	1.319	0.9961	2.125×10^2	0.9927
8.0	1.590	0.9959	4.514×10^2	0.9950
8.5	1.709	0.9965	5.224×10^2	0.9956
9.0	1.743	0.9958	5.750×10^2	0.9978

K_{SV}, fluorescence quenching constant; R_{SV}, the linear correlation coefficient of Stern-Volmer equation; K_{LB}, binding constant; R_{LB}, the linear correlation coefficient of Lineweaver-Burk equation

libitum. All animal experiments were conducted in accordance with the internationally accepted laboratory animal use and care stated in guidelines and rules of the ethical committee, Wuchang University of Technology, China.

In vivo experimental design

Mice were randomly divided into four groups of 10 animals each. The first group (group 1) served as normal control and received DNR vehicle (normal saline) orally. The groups 2, 3 and 4 were injected intraperitoneally with DNR at seven bolus doses of 2.0, 4.0 and 6.0 mg/kg body weight to reach the cumulative doses of 14, 28 and 42 mg/kg, respectively, once every three days for a total 21 days. All animals were sacrificed by cervical dislocation 48 hr after the last dosing. Blood samples were taken from the eyeball and hearts were removed. In each group, five mice blood samples were separated from serum to analyze the level of myocardial enzyme, and the other five mice blood samples were collected to test tube containing anticoagulant for assaying hematologic parameters.

Determination of H/BW and LVM/BW

The ratio of heart to body weight (H/BW) and the ratio of left ventricular mass to body weight (LVM/BW) were obtained according to the literature (25, 26).

Biochemical analysis

The myocardial enzymes, including lactate dehydrogenase (LDH), creatine kinase (CK), creatine kinase-MB isoenzyme (CK-MB), α-hydroxybutyrate dehydrogenase (α-HBDH), and hematologic parameters including white cell count (WCC), red cell count (RCC), hemoglobin (HGB), platelet count (PLT), mean red cell volume (MCV), and mean corpuscular hemoglobin concentration (MCHC) were estimated by South-Central University for Nationalities, using automatic biochemistry analyzer (Dimension Xpand, America).

Histopathological examination of left ventricle

Histopathological analysis was performed by means of optical microscopy on paraffin material. After weighing, left ventricular tissues were excised from each heart and fixed in 10% neutral formalin and histological preparations were immediately made. Sections were cut at 5 μm thick and stained with hematoxylin and eosin (HE) for histological examination (29).

Statistical analysis

Data were presented as mean±SEM and analyzed using one-way analysis of variance (ANOVA) followed by Tukey or Bonferroni methods for *post hoc* analysis. A value of $P<0.05$ was considered statistically significant.

Results

Effect of acidity on the fluorescence spectra of DNR-CM

The fluorescence quenching of CM caused by NDR was involved in a static procedure, which formed a DNR-CM complex (24). Moreover, the conformation changes in CM were evaluated by measuring the intrinsic fluorescence intensity of protein tryptophan residues upon excitation at 295 nm (rather than 280 nm) (30). Figure 2 stacks the fluorescence spectra of DNR-CM in the pH range of 4.0-8.0, and the blue shift of emission wavelength and fluorescence quenching were found with the increase of acidity, which suggested that the acidic conditions might change the microenvironment of tryptophan residue and the tertiary structure of CM (31). Additionally, the effect of acidity on fluorescence intensity of DNR-CM complex was investigated in the pH range of 4.0-9.0 (Figure 3), the K_{SV} values, K_{LB} values, as well as correlation coefficient, R_{SV} (the linear relationship

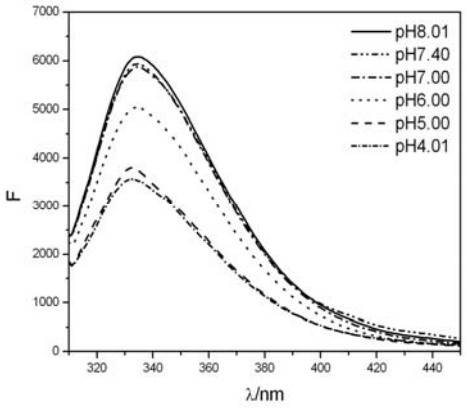

Figure 2. Effect of pH on the daunorubicin-cardiac myosin fluorescence spectra. [daunorubicin] = 0.1 μM; [cardiac myosin] = 0.1 μM; 20 ˚C; λ_{ex} = 295 nm

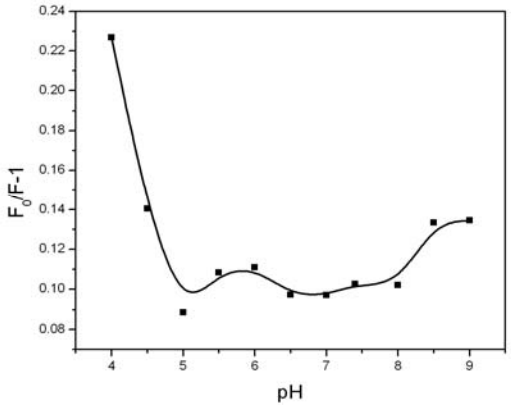

Figure 3. Effect of pH on the fluorescence intensity of daunorubicin-cardiac myosin system. Citric acid-Na_2HPO_4 buffer solution (0.01 M, pH 4.0-5.5), Tris-HCl buffer solution (0.01 M, pH 6.0-8.0) and H_3BO_3-$Na_2B_4O_7$ buffer solution (0.01 M, pH 8.5-9.0) were used and all buffers contained 0.15 M NaCl. Other conditions were similar those of Figure 2.

linear relationship between lg (F_0/F-1) and lg[Q]) in different acidities were calculated according to equations (1) and (2), respectively, and the results are listed in Table 1.

It could be found in Figure 3 and Table 1 that different acidic conditions had a great influence on fluorescence quenching degree of DNR-CM complex. At the same time, the levels of K_{LB} and K_{SV} were increased with the order of pH 6.5-7.4 (neutral), pH 8.0-9.0 (weak base) and pH 4.0-6.0 (weak acid). The results indicated that DNR was more likely to combine with CM under acidic conditions, which would give rise to conformational changes and affect the normal physiological function of CM, showing myocardial damage.

Effect of temperature on the fluorescence spectra of DNR-CM

The effects of different temperatures on fluorescence spectra of CM in the absence and presence of DNR in Tris-HCl buffer solution (pH 7.4) are shown in Figure 4. As can be seen, the fluorescence emission intensity of CM had an evident decrease with increasing temperature, and the downward trend was quite obvious (Figure 4a), particularly after the temperature reached 30°C.

Moreover, the addition of DNR did not affect the CM fluorescence spectrum, whereas more increases were seen in the reduced extent of CM fluorescence intensity (Figure 4b). Therefore, the fluorescence quenching was the common effect of DNR and temperature on CM.

To further investigate the two influencing factors, I% was defined as the ratio of CM fluorescence intensity under other conditions than that of 15 ˚C without DNR, and ΔI was the fluorescence quenching of CM caused by DNR alone, deducting the effect of temperature. The results are shown in Table 2. It could be seen that temperature had a little influence on the fluorescence intensity of DNR-CM system

from 20 ˚C to 25 ˚C, in which the quenching was mainly due to the combination of DNR with CM. Therefore, it was singularly appropriate to fix temperature at 20 ˚C to investigate the effect of other factors on the interaction between DNR and CM. Importantly, the reduction in CM fluorescence intensity was more remarkable at 30-37 ˚C, close to physiological temperature, than that of 20 ˚C. It was interpreted that higher temperature (>30 ˚C) was one of the important conditions for DNR to induce

Table 2. Effect of temperature on the fluorescence intensity of daunorubicin-cardiac myosin complex in Tris-HCl buffer solution (pH 7.4)

Fluorescence System	I (%)				
	15 ˚C	20 ˚C	25 ˚C	30 ˚C	37 ˚C
CM	100.00	95.93	88.42	66.83	68.63
DNR-CM	89.06	76.63	74.04	62.67	63.62
ΔI	10.94	19.30	14.38	4.16	5.01

CM, cardiac myosin; DNR, daunorubicin; I, the ratio of CM fluorescence intensity under other conditions to that of at 15°C without DNR; ΔI, the fluorescence quenching of CM caused by DNR alone, deducting the effect of temperature

Table 3. Fluorescence quenching constants and linear correlation coefficients of daunorubicin-cardiac myosin complex in Tris-HCl buffer solution (pH 7.4)

Temp. (˚C)	K_{SV} ($10^5 M^{-1}$)	R_{SV}
15	4.962	0.9964
20	8.560	0.9967
25	4.598	0.9977
30	3.220	0.9974
37	3.191	0.9947

K_{SV}, fluorescence quenching constant; R_{SV}, the linear correlation coefficient of Stern-Volmer equation

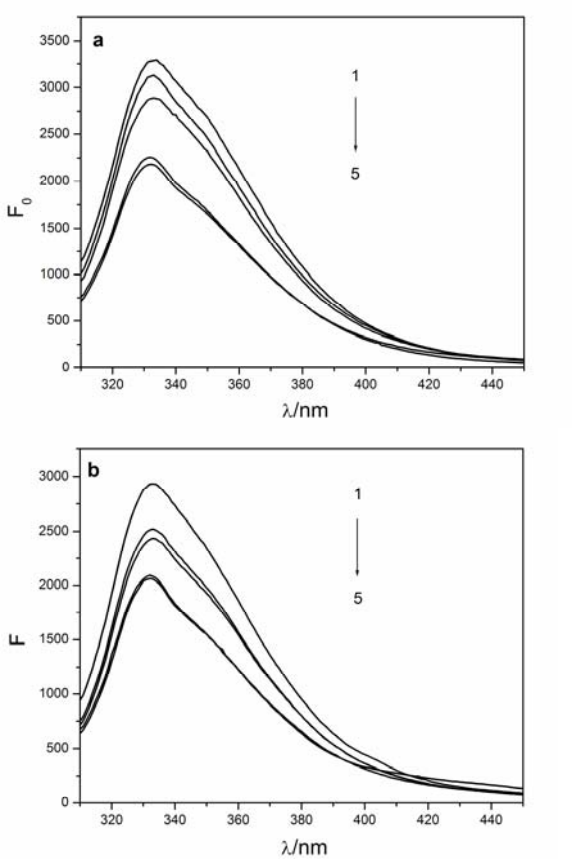

Figure 4. Effect of temperature on the daunorubicin-cardiac myosin fluorescence spectra. 1→5: 15, 20, 25, 37 and 30 °C; pH 7.4; a: [cardiac myosin] = 0.1 μM; b: [daunorubicin] = 0.1 μM, [cardiac myosin] = 0.1 μM. Other conditions as in Figure 2

cardiac toxicity.Additionally, Figure 5 enunciated the effect of temperature on Stern-Volmer curve of DNR-CM system. The K_{SV} value and correlation coefficient R_{SV} at different temperatures were calculated using equation (1), and the results are presented in Table 3. It could be observed that K_{SV} was inversely correlated with temperature ranging from 20 °C to 37 °C, which testified that the quenching mechanism of DNR-CM binding was initiated by complex formation (static quenching). However, the dynamic quenching resulted from diffusion and collision was also shown at 15-20 °C. According to the results, the fluorescence quenching of CM might be simultaneously included dynamic and static processes, indicating the complicated mechanism of interaction between DNR with CM. Some DNR molecules would closely combine with CM in terms of diffusion and collision, taking place in energy and electron transfer processes and causing dynamic quenching, whereas the other parts of DNR molecules and CM would generate the ground state complex, originating static quenching.

Table 4. Binding constants and relative thermodynamic parameters of daunorubicin-cardiac myosin complex in Tris-HCl buffer solution (pH 7.4)

Temp.(°C /K)	K(10^2M^{-1})	$\Delta H°$(KJ mol^{-1})	$\Delta G°$(KJ mol^{-1})	$\Delta S°$(J mol^{-1}K^{-1})
25/298	2.203	-8.34	-13.53	14.74
37/310	1.910	-8.34	-13.37	14.80

CM, cardiac myosin; DNR, daunorubicin; K, binding constant of DNR-CM interaction; $\Delta H°$, enthalpy change; $\Delta G°$, Gibbs free energy; $\Delta S°$, entropy change

Determination of the binding forces

The binding forces of DNR and CM were investigated by thermodynamic study, and the results are listed in Table 4. As can be seen, $\Delta G°$ was negative in the process of combination of DNR with CM, indicating that it was a spontaneous procedure with the decreases of free energy. So, it can be deduced that inserting DNR into the hydrophobic cavity of CM elements caused variation in CM molecular conformation, and released some of combined water molecules, which made $\Delta S°$ positive. Additionally, $\Delta H°$ was smaller than the product of temperature (T) and $\Delta S°$, suggesting that the contribution of $\Delta H°$ to $\Delta G°$ was relatively little. Therefore, the spontaneous process of interaction between DNR and CM was mainly driven by entropy and the binding forces were chiefly hydrophobic interaction and hydrogen bonds (32).

Variation in BW, H/BW, and LVM/BW

The effects of DNR on BW, H/BW, and LVM/BW in experimental mice are listed in Table 5. Regarding BW, no significant difference (P>0.05) was found among all DNR groups (groups 2-4) and control group (group 1), but the significant elevation (P<0.05 and P<0.01, respectively) of H/BW was observed when mice were administered with more than 2.0 mg/kg DNR (groups 3 and 4). Meanwhile, with the increase in DNR dose, DNR-treated mice (groups 2-4) showed a considerable increase (P<0.05 and P<0.01) in LVM/BW, up by 11.43%, 18.10%, and 20.95%, respectively, compared to those in group 1. The data indicated that DNR, especially at 4.0 and 6.0 mg/kg, induced cardiac lesions in a dose-dependent manner, which was associated with cardiac hypertrophy and ventricular remodeling.

Effect of DNR on myocardial enzyme level

The myocardial enzyme levels in this study were determined to evaluate the cardiac toxicity induced by DNR and the results are summarized in Table 6. Serum LDH and α-HBDH levels were increased and no statistically significant difference was seen, but CK and CK-MB levels were significantly higher (P<0.05 and P<0.01) when mice were administrated with 2.0 mg/kg DNR (group 2) as compared to the control. In addition, serum LDH, CK-MB and

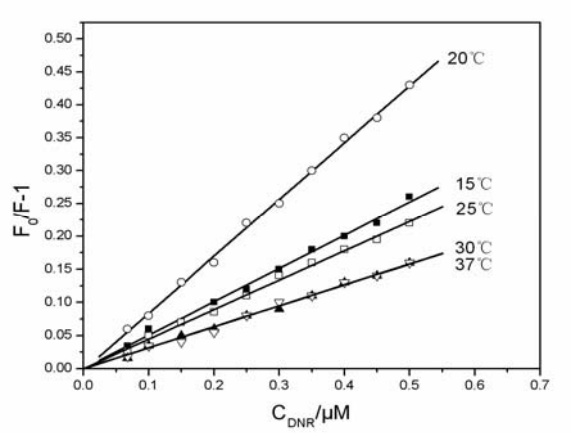

Figure 5. Effect of temperature on the Stern-Volmer curve of daunorubicin-cardiac myosin system.

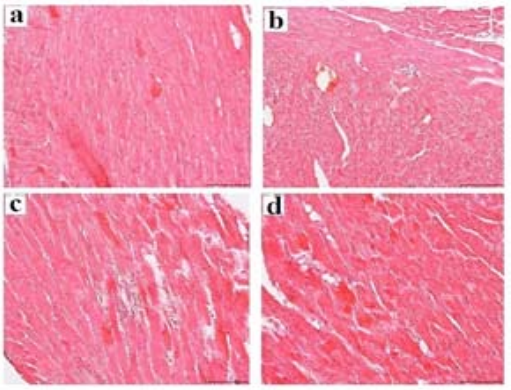

Figure 6. Histopathological changes of myocardiam tissues in mice. (HE staining, X400). a: control group; b: 2.0 mg/kg daunorubicin treatment group; c: 4.0 mg/kg daunorubicin treatment group; d: 6.0 mg/kg daunorubicin treatment group

α-HBDH, CK levels of 4.0 mg/kg DNR-administrated mice (group 3) were increased significantly *(P*<0.01 and *P*<0.001) compared with the control group levels. Similarly, mice treated with 6.0 mg/kg DNR (group 4) showed significant increases in serum LDH, α-HBDH, CK and CK-MB levels as compared to the normal control ones (*P*<0.001 and *P*<0.01). Thus, DNR treatment (4.0 and 6.0 mg/kg) impaired the myocardium in mice and the impairment occurred in a dose-related manner.

Histopathological examination of left ventricle

In order to further understand the myocardial injury caused by DNR, histopathological examination of left ventricle was carried out (Figure 6). The normal mice had no obvious vascular expansion and infiltration of inflammatory cells (Figure 6a). However, 2.0 mg/kg DNR-treated mice (group 2) showed cardiomyocytes disarray, vascular congestion, and interstitial oedema (Figure 6b). Moreover, 4.0 mg/kg DNR-treated mice (group 3) exhibited vascular dilatation, inflammatory cells infiltration, and myocardial fibrosis (Figure 6c). Here, 6.0 mg/kg DNR-treated mice (group 4) showed vascular hyperemia, hydropic degenerations, pyknotic nuclei and focal necrosis. Some foam cells were seen in the vessel wall, which suspected atherosclerosis or formed transparent thrombosis, caused vascular embolization, and led to local blood circulation disorder (Figure 6d). The results indicated that DNR caused a dose-dependent myocardial injury. Moreover, higher doses of DNR (6.0 mg/kg) might induce myocardial blood circulation barrier, and result in myocardial ischemia and heart failure.

Effect of DNR on hematologic parameters

As shown in Table 7, no significant difference in MCV and MCHC was seen among various DNR-treated groups and the control group. However, 6.0 mg/kg DNR-treated mice (group 4) displayed significant decreases (*P*<0.001 and *P*<0.01) in WCC, RCC, PLT, and HGB concentration. The reductions in WCC and HGB in this group were greater (*P*<0.01) than those in group 3. Similarly, RCC and PLT in group 4 were decreased more significantly (*P*<0.001) than those in group 3. Therefore, 6.0 mg/kg DNR treatment caused anemia and inflammation in this group.

Discussion

Fluorescence quenching refers to any processes that decrease the fluorescence intensity of a sample.

Table 5. Changes in body weight, ratio of heart to body weight, and ratio of left ventricular mass to body weight in mice treated with daunorubicin (n=10)

Experimental Group	Initial BW (g)	Final BW (g)	H/BW (mg/g)	LVM/BW (mg/g)
Group 1 (control group)	23.27±0.99	34.54±1.22	5.07±0.10	1.05±0.05
Group 2 (2.0 mg/kg DNR)	24.81±0.69	32.78±1.20	5.16±0.08	1.17±0.04*
Group 3 (4.0 mg/kg DNR)	24.03±0.88	31.64±1.07	5.44±0.12*	1.24±0.02**
Group 4 (6.0 mg/kg DNR)	23.32±0.83	31.40±1.45	5.86±0.15**	1.27±0.06**

Compared with the control group, *P<0.05, **P<0.01.
DNR, daunorubicin; BW, body weight; H, heart weight; LVM, left ventricular mass; Group 1: control group, mice treated with DNR vehicle (normal saline) orally; Groups 2-4: mice injected intraperitoneally with DNR in seven bolus doses of 2.0, 4.0 and 6.0 mg/kg BW to reach the cumulative doses of 14, 28 and 42 mg/kg, respectively

Table 6. Effect of daunorubicin on serum myocardial enzyme levels in mice (U/l, n=5)

Experimental Group	LDH	α-HBDH	CK	CK-MB
Group 1 (control group)	650±15	299±7	965±14	134±4
Group 2 (2.0 mg/kg DNR)	672±6	331±11	1058±21**	146±5*
Group 3 (4.0 mg/kg DNR)	779±4**	379±8***	1310±10***	163±8**
Group 4 (6.0 mg/kg DNR)	1078±25***	448±12***	1447±13***	178±8**

Compared with the control group, *P<0.05, **P<0.01, ***P<0.001.
DNR, daunorubicin; LDH, lactate dehydrogenase; CK, creatine kinase; CK-MB, creatine kinase MB isoenzyme; α-HBDH, α-hydroxybutyrate dehydrogenase; Group 1: control group, mice treated with DNR vehicle (normal saline) orally; Groups 2-4: mice injected intraperitoneally with DNR in seven bolus doses of 2.0, 4.0 and 6.0 mg/kg BW to reach the cumulative doses of 14, 28 and 42 mg/kg, respectively

A variety of molecular interactions can lead to quenching. These include excited state reactions, molecular rearrangements, energy transfer, collisional quenching, and ground state complex formation (33). Fluorescence quenching is divided into two categories: dynamic and static. The former is the energy or electronic transfer process; however, the latter generates new non-blooming complex due to quenching agent with protein and hence affects the tertiary structure and physiological activity of protein (24). Generally, fluorescence quenching mechanism can be studied by three methods, namely fluorescence lifetimes, dependence of quenching constant on temperature, and the absorption spectroscopy (35).

Our study showed that K_{SV} was inversely correlated with temperature from 20 °C to 37 °C (Table 3), which indicated that the probable quenching mechanism of DNR-CM binding was initiated by complex formation (static quenching) rather than by dynamic collision only (33). Moreover, acidity had an obvious influence on binding constant of DNR-CM complex (Figure 5 and Table 1). It was reported that acidity induced chain extension of protein molecules with the F configuration in an acid swelling, which could be caused changes in conformation of CM (31). Moreover, the binding constants in weakly acidic condition (pH 4.0-6.0) were higher than that of neutral (pH 6.5-7.4) and alkaline (pH 8.0-9.0) conditions, suggesting that the combination of DNR with CM occurred more easily in an acidic environment associated with an effect of CM function.

In drug-protein binding studies, $\Delta H°$ and $\Delta S°$ are the main basis to judge types of force (36). The positive value of $\Delta S°$ is frequently regarded as evidence for a hydrophobic interaction, the negative $\Delta H°$ value shows that the binding process is mainly enthalpy driven and happened by means of hydrogen binding interactions. The positive value of $\Delta S°$ and negative value of $\Delta H°$ show that both hydrophobic interaction and hydrogen bonds play a major role in the binding of drug to protein and contribute to the stability of the complex (37, 38). Hydrogen bonding is the typical character for intercalating mode of drug with macromolecule (39). However, specific electrostatic interactions between ionic species in aqueous solution are characterized by a positive $\Delta S°$ and a negative $\Delta H°$ (40). In addition, for a practical system, several forces often exist between small molecule ligands and protein because of complicated protein structure (35).

In our study, a positive value for $\Delta S°$ and a negative value for $\Delta H°$ (Table 4) were obtained, indicating that hydrophobic interactions and hydrogen bonds were present in the interaction between DNR and CM but the electrostatic interactions could not be excluded (41). Meanwhile, there had been the dipole-dipole interaction between CM and strong polar group of DNR such as hydroxyl and carbonyl groups (42).

In addition, because the formation of DNR-CM compound included an exothermic reaction accompanied by a negative $\Delta H°$, higher temperature was not conducive to the interaction between DNR and CM, which was consistent with the findings shown in Figure 4b. The quenching constants of DNR-CM were decreased with the increase of temperature associated with a reduction in the fluorescence quenching, which was in conformity with the relationship between temperature and the static quenching (35).

Originally, anthracycline-induced cardiac toxicity was described as being acute or sub-acute and was seen during or immediately after one dose or course of anthracycline. The sub-acute toxicity was manifested as a pericarditis-myocarditis syndrome or as acute left ventricular failure (43), and was particularly seen in early trials of anthracycline in

Table 7. Effect of daunorubicin on hematologic parameters in mice (n=5)

Experimental Group	WCC (10^9/l)	RCC (10^{12}/l)	HGB (g/l)	MCV(fl)	MCHC (g/l)	PLT (10^9/l)
Group 1 (control group)	4.41±0.03	8.38±0.05	137±5	52.7±0.5	314±6	852±10
Group 2 (2.0 mg/kg DNR)	4.31±0.03*	8.32±0.02	133±4	51.0±0.6	331±4	847±6
Group 3 (4.0 mg/kg DNR)	4.16±0.01**▵	7.10±0.04***▵▵▵	124±5*	52.1±0.4	329±3	686±8*** ▵▵▵
Group 4 (6.0 mg/kg DNR)	3.82±0.05***▵▵	6.46±0.03***▵▵▵	103±3**▵▵	52.6±0.3	312±9	401±10***▵▵▵

Compared with the control group, *P<0.05, **P<0.01, ***P<0.001; compared with the adjacent and lower dose DNR group, ▵P<0.05, ▵▵P<0.01, ▵▵▵P<0.001

DNR, daunorubicin; WCC, white cell count; RCC, red cell count; HGB, hemoglobin; MCV, mean red cell volume; MCHC, mean corpuscular hemoglobin concentration; PLT, platelet count; Group 1: control group, mice treated with DNR vehicle (normal saline) orally; Groups 2-4: mice injected intraperitoneally with DNR in seven bolus doses of 2.0, 4.0 and 6.0 mg/kg BW to reach the cumulative doses of 14, 28 and 42 mg/kg, respectively

adults when scheduling led to doses as high as 160 mg/m^2 being delivered over periods as short as four days (44). Therefore, changes in H/BW and LVM/BW of mice treated with DNR were evaluated the myocardial damage. In this study, LVM/BW was significantly increased in all DNR-treated groups (Table 5). Although H/BW in 2.0 mg/kg DNR treated-group was not significantly altered compared to that of controls, mice administered with 4.0 and 6.0 mg/kg DNR developed significant lesions of left ventricle.

Myocardial enzyme spectrum is a term for a variety of enzymes in myocardium, which generally consists of LDH, α-HBDH, CK and CK-MB. Under normal conditions, these enzymes were mainly distributed in the heart, bone, skeletal muscles and other tissues, especially in cardiac myocytes. When the heart damage occurs, myocardial enzyme activity increases. For example, patients suffering from acute myocardial infarction showed an increase in cardiac enzymes like CK and LDH (45).

In the present study, elevation of serum LDH, α-HBDH, CK and CK-MB activity levels are taken as a well-known quantitative index of compromised cell integrity and considered as an indicator of DNR-induced myocardial damage. Mice treated with 4.0 and 6.0 mg/kg DNR showed a marked elevation of serum LDH and CK as well as CK-MB and α-HBDH levels (Table 6), which are in accordance with those observed previously (26, 46-48). These results were confirmed by histopathological examination of ventricles of DNR-treated mice that revealed interstitial oedema, cardiomyocytes disarray and focal myocardial necrosis (Figures 6c and d).

In addition, there were significant hematologic changes after DNR treatment (Table 7). The changes included reductions in RCC and HGB concentration, which caused anemia in mice, and the result is in accordance with the data reported by Cusack *et al* (12). However, the hematological changes also included declines in WCC and PLT, which are contrary to the result obtained from rats (12). Difference in experimental animal and sample size may contribute to discrepancies. Furthermore, it can be inferred from Table 7 that 6.0 mg/kg DNR treatment caused not only anemia, but also inflammation, which are consistent with another study done by Fichtner *et al* (49). The reason might be that inflammatory cells enhanced the adhesion to vascular endothelial cells because of DNR stimulation, penetrated the vascular endothelium, entered the myocardial tissue, and finally led to local inflammatory reaction as shown in Figure 6d.\

Conclusion

In summary, DNR has strong ability to quench the CM fluorescence with a static mechanism by formation of DNR-CM complex, which was a spontaneous process, and was boosted in weakly acidic environment (pH 4.0-6.0) or higher temperature (30-37 ℃), and the interaction might influence the conformation and normal physiological functions of CM. Additionally, mice treated with 4.0 or 6.0 mg/kg DNR exhibited a serious damage in the heart. This work could be helpful for better understanding the pathogenesis of DNR cardiac toxicity.

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