

Pretreatment with Statin Attenuates the Cardiotoxicity of Doxorubicin in Mice

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Abstract

Cardiotoxicity, which may result from intense cardiac oxidative stress and inflammation, is the main limiting factor of the anticancer therapy using doxorubicin. Because statins might exert beneficial pleiotropic cardiovascular effects, among other things, by anti-inflammatory and antioxidative mechanisms, we investigated whether or not fluvastatin pretreatment can attenuate doxorubicin-induced cardiotoxicity. Five days after a single injection of doxorubicin (20 mg/kg; i.p.), left ventricular (LV) function was measured in fluvastatin-treated (DoxStatin; 100 mg/kg/day, p.o.) and saline-treated (doxorubicin) mice ($n = 8$ per group) by a micro conductance catheter. Untreated mice served as controls (placebo; $n = 8$ per group). After measurement of cardiac function, LV tissues were analyzed by molecular biological and immunohistologic methods. Injection resulted in significantly impaired LV function (LV pressure, -29% ; dp/dtmax, -45% ; cardiac output, -68% ; $P < 0.05$) when compared with placebo. This was associated with a significant increase in cardiac oxidative stress, inflammation and apoptotic mechanisms, as indicated by significant increased cardiac lipid peroxidation activity, protein expression of nitrotyrosine, tumor necrosis factor α and Bax ($P < 0.05$). In contrast, DoxStatin mice showed improved LV function (LV pressure, $+24\%$; dp/dtmax, $+87\%$; cardiac output, $+87\%$; $P < 0.05$) when compared with untreated doxorubicin mice. This was associated with reduced cardiac expression of nitrotyrosine, enhanced expression of the mitochondrial located antioxidative SOD 2, attenuated mitochondrial apoptotic pathways, and reduced cardiac inflammatory response. Statin pretreatment attenuates doxorubicin-induced cardiotoxicity via antioxidative and anti-inflammatory effects. [Cancer Res 2009;69(2):695–9]

Introduction

Doxorubicin is one of the most widely used anticancer drugs (1–4). The main limiting factor of this drug is the development of cardiotoxicity. For example, in a study with 399 patients, the incidence of congestive heart failure from patients who had received doxorubicin in a high-dose manner was $>18\%$ (5). Consequently, with respect to the dose-dependent cardiotoxicity of doxorubicin, an empirical dose limitation of up to 500 mg/m^2 is currently used in clinical practice to minimize the risk of

cardiomyopathy. Even at this dose limit, the incidence of doxorubicin-induced heart failure is still high (6). A subpopulation of children who received anthracycline therapy in a dose of $>300 \text{ mg/m}^2$, the risk for developing heart failure was 10% (7).

The mechanisms of doxorubicin-induced cardiomyopathy are not fully understood, but a solid body of evidence indicates that oxidative stress and cardiac inflammation are involved (6). We and others showed previously that both attenuated cardiac cytokine activation and lipid peroxidation activity might contribute to improved left ventricular (LV) function in a mouse model of doxorubicin-induced cardiotoxicity (8, 9).

3-Hydroxy-3-methylglutaryl coenzyme (HMG-CoA-reductase) inhibitors, also known as statins, are very effective in patients with lipid disorders and atherosclerosis (10). However, in addition, it is their anti-inflammatory and antioxidative pleiotropic effects which, independent of their lipid lowering potential, are relevant beneficial mechanisms of this drug class (11). For example, we previously showed in a diabetic animal model, a chronic treatment with statin using a dose too low to alter the lipid profile, led to attenuation of vascular damage, which was contributed by a reduction of inflammation and oxidative stress (12). Because doxorubicin-induced cardiotoxicity has been shown to be sufficiently triggered by cardiac oxidative stress and inflammation, we investigated whether or not statin pretreatment can produce cardioprotective effects in a mouse model of doxorubicin-induced cardiomyopathy (10).

Materials and Methods

Surgical procedures. Eight- to 10-wk-old C57BL/10 mice were randomly divided into three groups ($n = 6$ per group). Two groups received doxorubicin (Doxo Cell; Cell Pharm; 20 mg/kg ; i.p.) at a dose that had been shown to be cardiotoxic (13), and 1 group received saline (i.p.). Four days before doxorubicin application, in one doxorubicin group, a treatment with fluvastatin (100 mg/kg/day , p.o.; DoxStatin) was started. The other doxorubicin group received saline (doxorubicin). The third group without doxorubicin application received no further treatment (placebo). Five days after doxorubicin injection, mice were hemodynamically characterized. Finally, hearts were excised and prepared for molecular biological and immunohistochemical analyses as described below. This investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US NIH (NIH Publication No. 85-23, revised 1996).

Surgical procedures and hemodynamic measurements. Animals were anaesthetized (thiopental $125 \text{ }\mu\text{g/g}$; i.p.), intubated, and artificially ventilated. As described previously (14, 15), a 1.4 F micro conductance pressure catheter (ARIA SPR-719; Millar-Instruments, Inc.) was positioned in the LV for continuous registration of LV pressure–volume loops in a closed-chest model.

Systolic function was quantified by LV end-systolic pressure (LVP; mmHg), and dp/dt max (mmHg/s) as an index of LV contractility. Diastolic function was measured by dp/dtmin (mmHg/s), and the end-diastolic-pressure-volume relationship (stiffness constant β ; mmHg/ μL), an indicator

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for LV chamber stiffness, was determined from an exponential fit with the end-diastolic pressure-volume points (16). Global cardiac function was quantified by stroke volume (SV; μL), heart rate (HR; beat/min), and cardiac output (CO; mL/min; ref. 14).

Tissue preparation. For immunohistochemical analyses, cardiac tissues were embedded in optimal cutting temperature compound (Tissue Tec; Sakura Finetek). For molecular biological analyses, the isolated heart tissues were snap frozen immediately in liquid nitrogen and stored at -80°C .

Cardiac activity of lipid peroxidation. LV lipid peroxidation was measured using the commercially available colorimetric assay kit Bioxytech LPO-586 (Oxis International; ref. 17). Briefly, 150 μL of protein extracts were used for measurement of malondialdehyde, and 4-hydroxyalkenals were used as indicators of lipid peroxidation as described in the manufacturer's directions for use.

Immunostaining of nitrotyrosine. Tissue samples of quadriceps muscles were embedded in optimal cutting temperature compound (Tissue Tek; Sakura Finetek), frozen in liquid nitrogen, and stored at -80°C . Serial 5- μm -thick cryosections were placed on 10% poly-L-lysine precoated slides and fixed in cold acetone. After blocking endogenous peroxidase activity, sections were incubated with an avidin/biotin blocking kit (Vector Laboratories, Inc.). Staining was performed with the following primary antibody at the dilutions given as follows: antinitrotyrosine (Serotec; 1:100). This antibody was detected using a biotinylated goat-anti-mouse IgG (Dianova; 1:400). Sections were incubated with peroxidase-conjugated streptavidin (Vectastain ABC Elite; Vector Laboratories, Inc.). Antigen-antibody complexes were visualized by 3-amino-9-ethylcarbazole (Merck). The slides were counterstained (Mayer's hematoxylin), mounted in Kaiser's gelatin (both from Merck), and evaluated in a blinded fashion using the digital image analysis system Lucia G Version 3.52b (Nikon Deutschland GmbH) as described previously (18).

Western blot analysis. As previously described (19), Western blot analyses were performed using primary antibodies raised against Bax (Santa Cruz; diluted 1:500) and Bcl-2 (Santa Cruz; diluted 1:500; Santa Cruz; 1:1,000). Glyceraldehyde-3-phosphate dehydrogenase (Biodesign International; diluted 1:1,500) served as loading control. Detection of the signals was performed using the *LumiPhos* reagent (Pierce) and chemiluminescence was detected by X-ray films. For the analysis of superoxide dismutase (SOD) protein expression, the primary antibodies used were as follows: Anti-Mn SOD (Calbiochem; diluted 1:1,000), Anti-Cu/Zn SOD (Calbiochem; diluted 1:1,000), and Anti-EC SOD (R&D Systems; diluted 1:1,000). Detection of signals was performed using the *Western Lightning* reagents (PerkinElmer Lifesciences, Inc.).

Statistical analysis. Statistical analysis was performed using SPSS Version 12.0. Data are expressed as the mean \pm SE. Statistical differences were assessed by using the Kruskal-Wallis test in conjunction with the Mann-Whitney *U* post hoc test. Differences were considered to be statistically significant at a *P* value of <0.05 .

Results

LV function in doxorubicin-induced cardiomyopathy. Five days after doxorubicin injection, untreated mice displayed significantly impaired systolic (LVP, -29% ; dP/dt_{max} , -45% ; $P < 0.05$), diastolic (dP/dt_{min} , -44% ; stiffness, $+275\%$; $P < 0.05$), and global (SV, -61% ; HR, -18% ; CO, -68% ; $P < 0.05$) LV function when compared with the placebo group (Table 1). In contrast, among DoxStatin mice, variables of systolic (LVP, $+24\%$; dP/dt_{max} , $+59\%$; $P < 0.05$), diastolic (dP/dt_{min} , $+40\%$; stiffness constant β , -66% ; $P < 0.05$), and global (SV, $+75\%$; CO, $+87\%$; $P < 0.05$) LV function were significantly improved compared with untreated doxorubicin mice (Table 1).

Oxidative stress in doxorubicin-induced cardiomyopathy. As shown in Fig. 1, in untreated doxorubicin mice, both cardiac lipid peroxidation activity ($+37\%$; $P < 0.05$) and cardiac nitrotyrosine protein expression ($+204\%$; $P < 0.05$) were increased when compared with placebo mice. In DoxStatin mice, nitrotyrosine (-56% ; $P < 0.05$), but not lipid peroxidation, was significantly attenuated when compared with doxorubicin mice values.

Cardiac regulation of SODs in doxorubicin-induced cardiomyopathy. As shown in Fig. 2, 5 days after doxorubicin injection, cardiac protein expression of SOD1, SOD2, and SOD3 was not significantly regulated when compared with placebo. In contrast, SOD2 expression was significantly increased ($+18\%$; $P < 0.05$) in DoxStatin mice when compared with doxorubicin mice, whereas SOD1 and SOD3 were not significantly regulated.

Cardiac tumor necrosis factor α expression in doxorubicin-induced cardiomyopathy. As shown in Fig. 3, cardiac protein expression of tumor necrosis factor (TNF)- α was significantly increased in doxorubicin mice when compared with placebo ($+40\%$; $P < 0.05$). Statin treatment led to a significantly decreased TNF- α expression 5 days after doxorubicin injection (-26% versus doxorubicin; $P < 0.05$).

Apoptotic mechanisms in doxorubicin-induced cardiomyopathy. As shown in Fig. 4, the cardiac protein expression of the proapoptotic protein Bax was significantly increased in doxorubicin mice when compared with healthy placebo mice ($+100\%$; $P < 0.05$). Statin treatment led to a suppression of doxorubicin-induced Bax over expression, approximately down to the basal level of placebo mice. Whereas the antiapoptotic protein Bcl-2 was not regulated when compared with placebo and doxorubicin mice, statin treatment led to a 2.3-fold ($P < 0.05$) enhanced cardiac expression of this protein.

Table 1. LV function

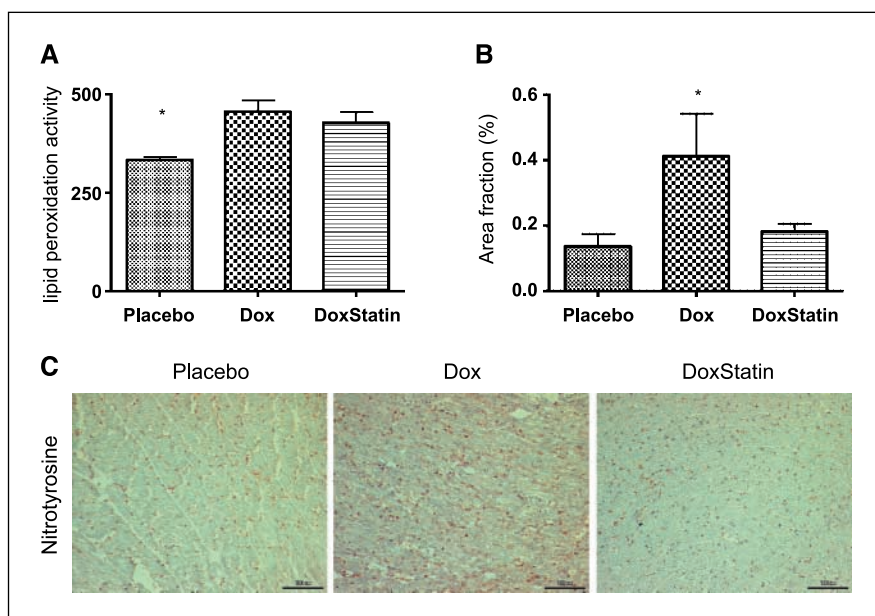
	Placebo	Dox	DoxStatin	<i>P</i> *	<i>P</i> †
HR (bpm)	294 \pm 28	241 \pm 38	261 \pm 30	<0.05	>0.05
LV pressure (mm Hg)	93 \pm 6	66 \pm 8	82 \pm 9	<0.05	<0.05
dP/dt_{max} (mm Hg/s)	5,711 \pm 571	3,126 \pm 571	4,973 \pm 462	<0.05	<0.05
SV (μL)	41 \pm 5	16 \pm 4	28 \pm 7	<0.05	<0.05
CO unit/mL/min)	12.0 \pm 1.9	3.9 \pm 0.5	7.3 \pm 1.1	<0.05	<0.05
LV end-diastolic pressure (mm Hg)	6 \pm 2	5 \pm 2	7 \pm 1	>0.05	>0.05
dP/dt_{min} (mm Hg/s)	$-4,925 \pm 705$	$-2,743 \pm 371$	$-3,846 \pm 499$	<0.05	<0.05
Stiffness constant β (in mmHg/ μL)	0.04 \pm 0.004	0.15 \pm 0.002	0.051 \pm 0.006	<0.05	<0.05

NOTE: Data from *in vivo* assessment of LV function are expressed as mean \pm SE.

**P*: statistical differences between Co and Dox.

†*P*: statistical differences between Dox and DoxStatin ($n = 8$ per group).

Figure 1. Cardiac lipid peroxidation activity and nitrotyrosine expression. Lipid peroxidation activity from heart tissue (A) were quantified using a commercial kit ($n = 6$ per group). Cardiac nitrotyrosine expression (B) was quantified by immunostaining and digital image analysis. Columns, mean; bars, SE. Representative pictures of nitrotyrosine staining (red) are depicted here (C; $n = 6$ per group). Dox, doxorubicin.



Discussion

Here, we show that pretreatment with the HMG-CoA-reductase inhibitor fluvastatin leads to cardioprotection against doxorubicin in mice. We show that a statin-mediated decrease of cardiac nitrotyrosine production and activated mitochondrial-located antioxidative and antiapoptotic mechanisms—which were associated with attenuated inflammatory response—contributed to improved LV function.

In agreement with previous studies and in line with the findings derived from human studies, doxorubicin in our model led to severely reduced LV systolic and diastolic function, which consequently resulted to decreased global heart function as indexed by suppressed CO (8, 20). The inhibition of HMG-CoA-reductase by pretreatment with fluvastatin improved LV function under these conditions. A number of pharmacologic trials investigating common cardiovascular drugs as well has been investigated to reduce cardiac toxicity of doxorubicin. Both antiadrenergic therapy using β -blockers and inhibition of the

renin-angiotensin system can reduce the progression of this disease at the early stage of cardiotoxicity (21–23). Regarding these drugs, anti-inflammatory effects and changes in LV loading conditions contributed to their ability to attenuate cardiac damage after doxorubicin treatment. However, the ability of these treatments to prevent short- and long-term doxorubicin-induced cardiac damage has remained limited (24). In the present study, we show that pretreatment with a statin improved LV function independently of direct effects on loading conditions because statins influence neither preload nor after load, and they do not exert any inotropic effect.

Although it was initially thought that the general reduction of cardiovascular risk by this drug class was related solely to their lipid-lowering capacity, it is currently accepted that statins do exert protective cardiovascular effects, and this in spite of any lipid lowering (25–27). In this regard, antioxidative properties are one of the main factors by which statins exert so-called pleiotropic effects. Because the production of oxidative stress is

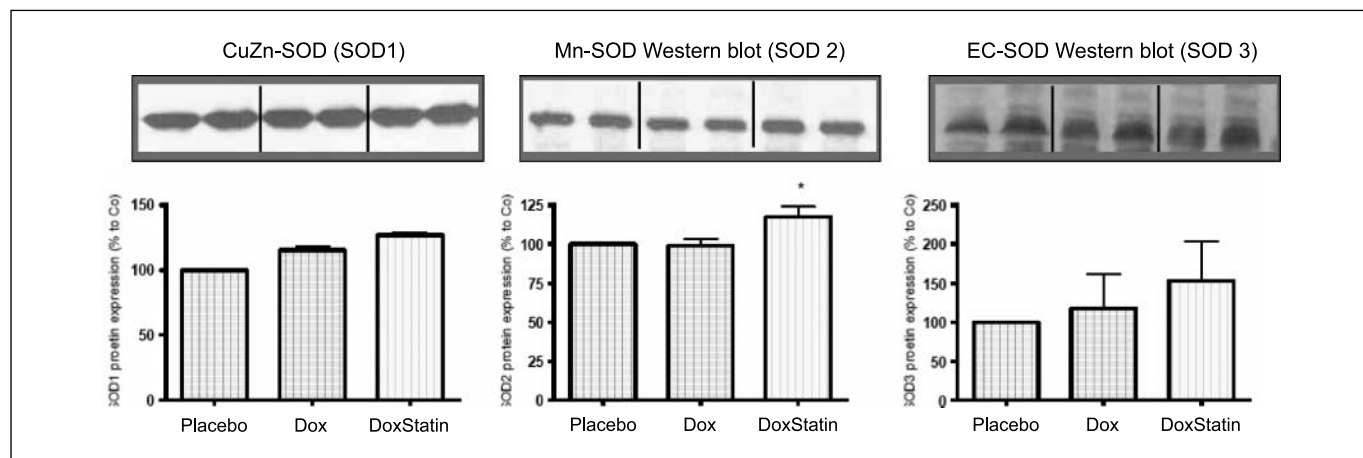


Figure 2. Cardiac regulation of SODs. Cardiac protein expression on SODs 1, 2, and 3 was measured by Western blot analyses. Representative bands are depicted above the quantification graph ($n = 6$ per group). Columns, mean; bars, SE. *, $P < 0.05$ versus doxorubicin.

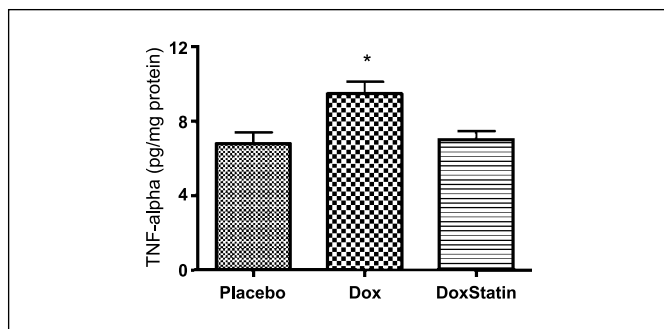


Figure 3. Cardiac protein expression of TNF- α in doxorubicin-induced cardiomyopathy. Cardiac protein expression was measured using ELISA ($n = 6$ per group). Columns, mean; bars, SE. * = $P < 0.05$ versus DoxStatin.

believed to be a hallmark in doxorubicin-induced cardiotoxicity, we analyzed two different sources of cardiac oxidative stress, namely nitrotyrosine expression and lipid peroxidation activity. Both have been shown previously to contribute to doxorubicin-induced cardiotoxicity, a finding which we note too in the present study (8, 19). Whereas nitrotyrosine expression has been significantly attenuated by statin treatment, lipid peroxidation was not affected by this therapy, suggesting that the latter is not a cardiac target for fluvastatin, at least in our model. This is however in accord with previous findings of others showing a suppression of nitrotyrosine expression by statin treatment under diabetic conditions (28). In our case, we found this drug potency in doxorubicin-induced cardiotoxicity.

Another cause of enhanced oxidative stress is, despite any activated sources, an insufficient activation of antioxidative mechanisms. In this regard, doxorubicin itself can exert oxidative effects by the direct production of free radicals (5). For example, peroxynitrite, a reactive oxidant produced by the rapid reaction of nitric oxide and superoxide, is a relevant pathophysiologic mechanism by which doxorubicin can induce cardiac damage (29). In view of this, antioxidant enzymes play a critical role in the detoxification of these radicals. Thus, we found it to be of interest to analyze the regulation of SODs, which are known to play a major role in preventing the production of reactive oxygen species (30). There are three isoforms, namely SOD1 (CuZn-SOD), SOD2 (Mn-SOD), and SOD3 (EC-SOD, where EC stands for extracellular), and these are all present in different locations of the heart. In the present study, none of the isoforms were regulated due to doxorubicin because cardiac protein expression did not differ significantly between Co and doxorubicin mice. In addition, statin pretreatment did not affect the regulation of SOD1 and SOD3. In contrast, SOD2, which is located in the

mitochondria, was significantly increased after statin treatment. This finding suggests that our treatment not only attenuates the production of oxidative stress, but that it also leads to the activation of mitochondrial elimination of reactive oxygen species as well. This is in agreement with previous findings showing that SOD2 can indeed exert cardioprotection against doxorubicin-induced toxicity and other cardiac stress conditions such as ischemia-reperfusion (31, 32).

To further investigate the effects of statin treatment on mitochondrial protection, we analyzed two components of the mitochondrial apoptotic pathway. In agreement with previous studies from two other groups (33, 34), we, too, found an activation of the mitochondrial apoptotic pathway in the heart as indexed by a marked up-regulation of the proapoptotic protein Bax. In the present model, this was 5 days after doxorubicin administration, at which time the activation was blunted after statin treatment. To further investigate mitochondrial mechanisms potentially involved, which may modulate apoptosis, we measured the antiapoptotic protein Bcl-2 (35), which constitutes a pivotal regulator of mitochondrial apoptosis (36). A recent study strengthens the emergent role for Bcl-2 in protecting cardiac cells against death, including apoptosis (37). Although others have found a reduction in the antiapoptotic protein Bcl-2 in chronic doxorubicin-induced cardiomyopathy (34), we found in the acute situation of our model no regulation of this protein at all. Interestingly, despite the nonregulation of Bcl-2 due to doxorubicin, the Bcl-2 content after statin treatment was markedly increased, thereby suggesting a mitochondrial protection against apoptosis due statin treatment.

We showed in a previous study a strong association between oxidative stress and cardiac inflammatory response including cytokine release after doxorubicin treatment (8). One of the proinflammatory cytokines involved, which mediate cardiac damage, is TNF- α . doxorubicin led to an increase in cardiac TNF- α expression in our model, which is in agreement with other findings (13). Because statin treatment reduced cardiac doxorubicin-induced TNF- α expression in our study, it is intriguing to speculate that alongside antioxidative mechanisms, it may also be antiinflammatory mechanisms derived from statin treatment, which contribute to cardioprotection against doxorubicin-induced cardiotoxicity.

In summary, inhibition of the HMG-CoA-reductase by pretreatment with fluvastatin led to improved LV function in a mouse model of doxorubicin-induced cardiomyopathy.

Our data suggest that attenuation of cardiac nitrotyrosine expression, mitochondrial protection, and cardiac inflammatory response due to statin treatment may well contribute to improved heart function. Thus, pretreatment with statin might constitute a potentially new therapeutic option to prevent doxorubicin-induced

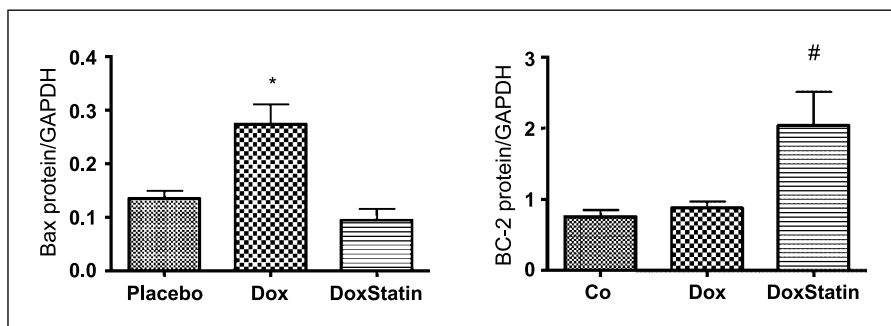


Figure 4. Cardiac protein expression of Bax and Bcl-2. Cardiac protein expression of Bax (A) and Bcl-2 (B) as measured by Western blot analyses ($n = 6$ per group). Columns, mean; bars, SE. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

cardiotoxicity. However, further clinical studies will have to verify if this concept is valid in patients as well and further, whether or not statin treatment counteracts with the oncological effect of doxorubicin.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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