Schisandrin B Prevents Doxorubicin-Induced Cardiotoxicity via Enhancing Glutathione Redox Cycling

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Abstract

Purpose: The dose-cumulative cardiotoxicities and the emerging cancerous apoptotic/drug resistance are two major obstacles limiting the efficacy of anthracycline antibiotics, notably doxorubicin. We attempted to prove if schisandrin B (Sch B), a dual inhibitor of P-glycoprotein and multidrug resistance – associated protein 1, could protect against doxorubicin-induced cardiotoxicity, on the premise that Sch B is an enhancer of glutathione redox cycling that may attenuate doxorubicin-induced oxidative stress in the cardiomyocytes.

Experimental Design: Mice or rat were dosed with a single injection of doxorubicin (25 mg/kg, i.p.) with or without pretreatment of Sch B. The protective roles of Sch B against doxorubicin-induced cardiac damage were evaluated on the aspects of the release of cardiac enzymes into serum, the formation of malondialdehyde, the activation of matrix metalloproteinase, the structural damage in the left ventricles, the mortality rates, and the cardiac functions.

Results: Pretreatment of Sch B significantly attenuated doxorubicin-induced cardiotoxicities on all the aspects listed above. The underlying mechanism was associated with the effect of Sch B on maintaining the cardiomyocytic glutathione and the activities of superoxide dismutase, and the key enzymes (glutathione peroxidase, glutathione reductase, and glutathione transferase) responsible for glutathione redox cycling, which neutralized doxorubicin-induced oxidative stress.

Conclusion: To the best of our knowledge, Sch B is the only molecule ever proved to function as a cardioprotective agent as well as a dual inhibitor of P-glycoprotein and multidrug resistance – associated protein 1, which is potentially applicable to treat cancers, especially the multidrug-resistant cancers involving doxorubicin or its kin.

Anthracycline antibiotics are a class of potent anticancer drugs against many types of hematologic and solid malignancies. Nevertheless, their efficacy is severely complicated by their cumulative dose-dependent cardiotoxicity (1–4) and the emerging cancerous apoptotic/drug resistance (5–8). Thus, reducing this side effect while maintaining their efficacy is critical for the success of chemotherapy.

The redox cycling on quinone moiety of doxorubicin is a critical event leading to cardiotoxic consequences. The quinone redox cycling produces excessive reactive oxygen species and reactive nitrogen species, which would disrupt the normal biochemical and cellular processes of cardiomyocytes and cause cell death (1–3). This side effect will be further significantly enhanced if combined with other anticancer drugs such as taxanes, trastuzumab, and cyclooxygenase-2 inhibitors (1, 9–12).

Cancer multidrug resistance and apoptotic defect significantly diminish the anticancer efficacy of anthracycline antibiotics. Cancer multidrug resistance is characterized by the cellular overexpression of drug transporters, such as P-glycoprotein and multidrug resistance–associated protein 1 (MRP1), which function as unilateral "drug pumps," blocking the entrance of anticancer drugs into cancer cells (5, 6). These drug transporters have a broad substrate specificities, covering many structurally and functionally unrelated anticancer drugs, including anthracyclines (5, 6). Apoptotic defect, which is very common in cancers (7, 8), also contributes significantly to cancer drug resistance. It should be noted that cancer drug resistance is often multifactorial.

There are two pharmacologic approaches to mitigate the side effect of doxorubicin while restoring its efficacy against drug-resistant cancers via either (a) using a combination of a cardiac protective agent, a P-glycoprotein, and a MRP1 inhibitor, and an apoptotic sensitizer; or (b) an agent with multiple functions. Whereas the first choice has the advantages of the drug being specific but disadvantages of the elevated chances of drug-drug interaction and the risk of cumulative toxicities, the second...
choice has the opposite advantages and disadvantages. To date, there are many agents with a specific function, but few with multiple functions.

We have previously reported that dibenzocyclooctadiene lignans, isolated from the traditional Chinese medicinal herb *Schisandra chinensis* (Turcz.) Baill, represent a novel class of dual inhibitors of P-glycoprotein and MRP1 (13–16). Recently, Fong et al. (17, 18) added the mechanism whereby the compounds in this chemical class inhibited P-glycoprotein. Schisandrin B (Sch B), the most abundant dibenzocyclooctadiene lignan in *S. chinensis* (Turcz.) Baill, was also able to enhance doxorubicin-induced apoptosis in HMMC7721, a human hepatic cancer cell line, and MCF-7, a human breast cancer cell line, through activation of mitochondrial apoptotic pathway, without obvious enhanced toxicities toward normal cells, such as primary rat cardiomyocytes and primary human fibroblasts (19).

Here, we hypothesize that Sch B may protect against doxorubicin-induced cardiotoxicity, on the basis that Sch B is an enhancer of glutathione (GSH) redox cycling (20–22). GSH is the major intracellular antioxidant and GSH redox cycle is a major defense system against oxidative stress. In cardiomyocytes, GSH redox cycle is particularly important because the catalase level is low (23). Unfortunately, after exposure to doxorubicin, glutathione peroxidase in cardiomyocytes, the key enzyme in GSH redox cycling, are inactivated to a significant extent (23, 24). This is probably why cardiomyocytes are more susceptible to doxorubicin than other types of cells. Thus, we speculate that Sch B may, at least partially, prevent doxorubicin-induced cardiac damage via enhancing GSH redox cycling. Our results showed that Sch B could significantly increase cardiac GSH redox cycling in mice and rats, with concomitant protection against doxorubicin-induced cardiotoxicities.

**Materials and Methods**

**Reagents.** Doxorubicin was from Sigma, and Sch B was from the National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China.

**Animals.** Male ICR mice (18-22 g) and male Sprague-Dawley rats (300-350 g) were obtained from Shanghai SLAC Laboratory Animal Co., Ltd, Shanghai, China. Animals were housed in a controlled conditions of temperature (23 ± 2°C), humidity (50 ± 5%), and light (10 and 14 h of light and dark, respectively). The animals had free access to sterile food and water, and were housed in a sterile polypropylene cage containing sterile paddy husk as bedding. The study was approved by the institutional animal ethical committee of the hospital.

**Preparation of samples, measurement of biochemical variables, and electron microscopy.** A total of 132 ICR mice were randomly assigned to six groups: 1, control; 2, Sch B control; 3, doxorubicin treatment; 4, 5, and 6, doxorubicin combined with Sch B (100, 50, and 25 mg/kg). Groups 1 and 3 were dosed intragastrically with the vehicle (0.5% paraxamer), and groups 2, 4, 5, and 6 were dosed intragastrically with Sch B (100, 100, 50, and 25 mg/kg, respectively) for 3 days. Within 2 h of last Sch B administration, mice in the groups 3 to 6 were injected with doxorubicin (25 mg/kg, i.p.), whereas the mice in groups 1 and 2 received saline.

Forty-eight hours after doxorubicin injection, 72 mice (12 mice for each group) were bled to collect the serum for the determination of serum cardiac enzymes (total creatine kinase and its MB isozyme, aspartate aminotransferase, and lactate dehydrogenase) by kits (Ausbio) using an OLYMPUS AU5400 automatic chemical analyzer. The left ventricle was excised and the myocardial homogenates were prepared for matrix metalloproteinase zymography as described (25, 26).

Ninety-six hours after doxorubicin injection, another 60 mice were killed; the left ventricle was excised; and myocardial homogenates were prepared for the detection of malondialdehyde, glutathione S-transferase, glutathione reductase, glutathione peroxidase, GSH, oxidized GSH, superoxide dismutase, and catalase, by corresponding kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), which
are based on the principles in the previous reports (27–33). Left ventricles from 18 mice were processed for ultrathin section preparation for ultrastructural morphologic examination by electron microscopy, as described previously (34).

Twenty-four rats were randomly assigned to four groups: 1, control; 2, Sch B control; 3, doxorubicin treatment; 4, doxorubicin combined with Sch B (50 mg/kg). Groups 1 and 3 were dosed intragastrically with the vehicle (0.5% paraxamer), and groups 2 and 4 were dosed intragastrically with Sch B (50 mg/kg) for 3 days. Within 2 h of the last Sch B administration, rats in groups 3 and 4 were injected with doxorubicin (25 mg/kg, i.p.), whereas the rats in groups 1 and 2 received saline. Ninety-six hours after doxorubicin injection, rats were killed; the left ventricle was excised; and myocardial homogenates were prepared for the detection of malondialdehyde, glutathione S-transferase, glutathione reductase, glutathione peroxidase, GSH, oxidized glutathione, superoxide dismutase, and catalase by corresponding kits, which are based on the principles in the previous reports (27–33).

**Mortality rate.** A total of 154 ICR mice were randomly assigned to four groups: 1, control; 2, Sch B control; 3, doxorubicin treatment; 4, doxorubicin combined with Sch B (100 mg/kg). The dose and schedule were the same as aforementioned. Mortality was monitored and recorded twice daily for 7 days as described (25).

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**Fig. 2.** SchB suppressed doxorubicin-induced cardiotoxicities. Mice were pretreated with Sch B, followed by a single dose of doxorubicin (25 mg/kg, i.p.) as described in Materials and Methods. **A,** matrix metalloproteinase zymograph. **B,** densitometric analysis of the zymograph. **C,** malondialdehyde (MDA) formation in the left ventricles (n = 7 for each group). **D,** mortality rates: control (n = 34), doxorubicin (n = 52), Sch B (n = 34), doxorubicin + Sch B (n = 34). **D,** electron microscopy graphs showing the ultrastructural changes after treatment with doxorubicin with or without pretreatment of Sch B. Columns and points, means from two independent experiments; bars, SE. *, P < 0.05, compared with doxorubicin group; **, P < 0.05, compared with control group.
Measurement of cardiac function. Sprague-Dawley rats were randomly assigned to four groups: 1, control (n = 18); 2, Sch B control (n = 6); 3, doxorubicin treatment (n = 18); 4, doxorubicin combined with Sch B (n = 18). Groups 1 and 3 were dosed intragastrically with the vehicle (0.5% paraxamer), and groups 2 and 4 were dosed intragastrically with Sch B at 100 mg/kg/d for 3 consecutive days. Within 2 h after the last Sch B administration, rats in groups 3 and 4 were administered with doxorubicin (25 mg/kg, i.p.), and saline was given to the rats in groups 1 and 2. Ninety-six hours after doxorubicin injection, the myocardial contractile function of the rats was recorded and analyzed using the MedLab-U/4c Biological Signal Collecting System (MedEase Science and Technology Co., Ltd.). Left ventricular performance was analyzed in rat anesthetized with injections of chloral hydrate (360 mg/kg, i.p.). A micromanometer-tipped catheter was inserted into the right carotid artery and advanced into the left ventricular under pressure control as described (35). After stabilization for 20 min, the signals were recorded continuously with a pressure-volume conductance system coupled with a Powerlab converter, stored, and displayed on a personal computer. The heart rate, maximal left ventricular systolic and end-diastolic pressures, maximal slope of systolic pressure increment, and diastolic pressure decrement were recorded and analyzed.

Statistical analysis. Data are expressed as mean ± SE. Statistical comparisons between different groups were done by using one-way ANOVA followed by Tukey-Kramer multiple comparisons test (SPSS 10.0). Significance was accepted at P < 0.05.

Results

Sch B attenuates the doxorubicin-induced acute cardiotoxicity in mice. Pretreatment of Sch B significantly attenuated doxorubicin-induced cardiotoxicities, including the increased serum cardiac enzyme (Fig. 1), the activated matrix metalloproteinase and the elevated malondialdehyde in the left ventricle (Figs. 2A and B), the mortality rates (Fig. 2C), and the myocardial ultrastructural damage (Fig. 2D). The effect is dose dependent.

Mice receiving Sch B alone did not show any obvious abnormalities compared with vehicle control (Figs. 1 and 2).

Sch B prevents doxorubicin-induced acute loss of cardiac function. Because mouse cardiac function could not be properly measured by MedLab-U/4c Biological Signal Collecting System, we used a rat model for this purpose. After doxorubicin administration, the mortality rates were recorded on the daily basis for the next 4 consecutive days. The remaining rats were subjected for the analysis of myocardial function. Sch B alone did not significantly affect the cardiac function. Doxorubicin caused a loss of the cardiac function, which was significantly attenuated by pretreatment of Sch B (Fig. 3). Consistently, there were 11 deaths of a total of 18 rats (61%) in the doxorubicin
group, whereas pretreatment with Sch B significantly reduced the death rate [4 of 18 (22%); Fig. 3].

**Sch B enhances antioxidant enzymes in cardiac tissues.** Doxorubicin treatment resulted in a significant decrease of enzymes in GSH redox cycling (Fig. 4). In mice treated with doxorubicin alone, the activities of cardiac glutathione peroxidase, glutathione reductase, and glutathione S-transferase were decreased ~38%, 42%, and 39% compared with control mice. Accordingly, reduced GSH only constituted 25% of the control, whereas oxidized GSH increased for 2.1-fold. In addition, total GSH were ~40% of the control. With the pretreatment of Sch B, doxorubicin-induced reduction of glutathione peroxidase, glutathione reductase, and glutathione S-transferase were attenuated in a dose-dependent manner. The reduced and total GSH in the group pretreated with Sch B (100 mg/kg) recovered to 63% and 68% of control, respectively, whereas the oxidized GSH was comparable with that of control. Nevertheless, although Sch B (100 mg/kg) blocked the effect of doxorubicin-reduced GSH redox-cycling enzymes, total GSH and reduced GSH levels were not recovered to that of the control, suggesting that doxorubicin may inhibit GSH synthesis.

Doxorubicin caused a marked reduction of cardiac superoxide dismutase (Fig. 4), which was prevented by Sch B, again in a dose-dependent manner. Neither doxorubicin nor Sch B significantly affected the catalase activities (Fig. 4).

In Fig. 4, mice treated with Sch B alone did not show an increase of GSH and its redox cycling enzymes, and superoxide dismutase, in the left ventricle, simply because the changes of

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**Fig. 4.** Sch B attenuated doxorubicin-induced suppression of the antioxidative components in the left ventricles. Mice were treated with a single dose of doxorubicin (25 mg/kg, i.p.) with or without pretreatment of Sch B, followed by analysis of the antioxidative components in the left ventricle as described in Materials and Methods. Columns, mean (n = 7 for each group) from two independent experiments; bars, SE. *, P < 0.05, compared with doxorubicin group; **, P < 0.05, compared with control group. GSSG, oxidized glutathione; GR, glutathione reductase; GST, glutathione S-transferase; GPX, glutathione peroxidase; SOD, superoxide dismutase; CAT, catalase.
these biochemical variables induced by Sch B were time dependent. The effect of Sch B reached maximum at 24 h, then declined and returned to the starting point at 72 h after administration (Fig. 5). This can explain why in Fig. 4, although there were no apparent changes of these biochemical variables in Sch B alone control, Sch B significantly suppressed doxorubicin-induced inhibition of superoxide dismutase and GSH redox cycling, given that the biochemical variables were measured 96 h after the last Sch B administration.

Similarly, in rats, Sch B significantly attenuated doxorubicin-induced effects on the formation of malondialdehyde, intracellular levels of GSH, activities of GSH redox cycling enzymes, and superoxide dismutase (Fig. 6).

Discussion

To mitigate the cardiotoxicity of anthracycline antibiotics without compromising their anticancer activities is still an issue to be solved. For example, dexrazoxane is the only Food and Drug Administration–approved drug for the prevention of cardiac dysfunction induced by anthracyclines. Although this drug is effective in preventing anthracycline cardiotoxicity, “there are some concerns on the potential unfavorable effect of dexrazoxane on antineoplastic activity and hematologic toxicity of anthracyclines as well as on the carcinogenic activity on the cells of bone marrow and skin (2)”.

In this study, we showed that Sch B significantly prevented doxorubicin-induced cardiotoxicities. The underlying mechanism is associated with its action on the defensive system against doxorubicin-induced oxidative stress, notably GSH redox cycling and superoxide dismutase. The obvious question is, would Sch B affect the anticancer efficacy of doxorubicin? First, Sch B, like cyclosporine A, is a dual inhibitor of P-glycoprotein and MRP1 (13–18). By inhibiting drug efflux mediated by P-glycoprotein or MRP1, Sch B restores intracellular accumulation of doxorubicin, whereby multidrug resistance cancer cells regain susceptibility toward doxorubicin. Thus, Sch B could greatly enhance the anticancer activities of doxorubicin against multidrug resistance cancers. Second, Sch B is able to enhance doxorubicin-induced apoptosis in cancer cell via reactive oxygen species–independent activation of caspase-9, but not in normal cells such as rat cardiomyocytes (19). Although the mechanism is not fully understood, it has been long thought that the mechanisms whereby doxorubicin induces apoptosis in cancer cells and cardiomyocytes are different (1). For example, although doxorubicin triggers cardiomyocytic...
apoptosis mainly via reactive oxygen species, it induces cancer cell death via p53 independent of reactive oxygen species (19, 36). The other lines of evidence supporting this notion include the opposite effects of some proteins critical for apoptotic signaling in cancer cells and cardiomyocytes induced by doxorubicin, such as proapoptotic effects of nuclear factor-κB activation in cardiomyocytes in contrast to its antiapoptotic effects in cancer cells, and the proapoptotic effects of c-Jun-NH2-kinase/activator protein 1 activation in ceramide-challenged cancer cells in contrast to the antiapoptotic effects on c-Jun-NH2-kinase/activating transcription factor 3 activation in some cardiac cell lines (37–39). The distinct mechanisms whereby doxorubicin induces apoptosis in cardiomyocytes and cancer cells are probably the fundamental basis for Sch B to prevent its cardiotoxicity with concomitant enhancement of its antimalignancy activity.

Compared with the antioxidant probucol (40, 41) and peroxynitrite decomposition catalyst FP15 (25), Sch B achieves...
a similar protection against doxorubicin-induced formation of malondialdehyde, activation of matrix metalloproteinase, loss of cardiac function, and reduction of enzymes against oxidative stress. In contrast to probucol and FP15, which could completely prevent doxorubicin-induced cardiomyopathy and acute death, respectively, Sch B could only partially prevent doxorubicin-induced acute death. It is still not completely clear why Sch B can show comparable cardioprotective activities to probucol or FP15 through the same mechanism against the oxidative stress but not achieve a complete prevention of doxorubicin-induced acute death. The dose schedule might play a role. For example, probucol offered partial protection when it was used concurrently with doxorubicin treatment (40). However, complete protection was seen when probucol was started 2 weeks before doxorubicin treatment (24).

On the basis that Sch B has multiple functions against cancer, such as reversal of cancer multidrug resistance by inhibiting P-glycoprotein and MRPI (13–18), enhancing anticancer activities of doxorubicin (19), and mitigating doxorubicin-induced cardiotoxicity, this compound has potential clinical application in chemotherapy against cancers, especially the multidrug resistance cancers involving doxorubicin or its analogues. In addition, this compound provides a core structure for the development of a class of drugs with multiple functions.
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