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Original Article

BN52021 protects rat cardiomyocyte from doxorubicin induced cardiotoxicity

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Abstract: The aim of this study was to assess the role of platelet activating factor (PAF) antagonist BN52021 in doxorubicin induced cardiotoxicity and to explore the mechanisms. H9c2 cardiomyocytes were employed to investigate the effect of BN52021 on doxorubicin induced cell viability and cell apoptosis. Signaling pathway of caspase 3, cytochrome c, calcium and p38 mitogen-activated protein (MAPK) was determined during the doxorubicin induced apoptosis. Our results showed BN52021 pretreatment could protected cell death induced by doxorubicin in H9c2 cardiomyocytes. Decrease concentration of $[Ca^{2+}]$ and expression of phosphorylated P38 MAPK were accounted for the protection effect. Inhibition of signaling pathway of calcium and p38 MAPK showed similar effect exerted by BN52021 in doxorubicin induced cell apoptosis. Our results demonstrated BN52021 protected against doxorubicin induced cell death in H9c2 cardiomyocytes by calcium and p38 MAPK signaling *in vitro*. These finding may give insight on the treatment of doxorubicin induced cardiomyopathy.

Keywords: BN52021, doxorubicin, cardiotoxicity, calcium signaling, MAPK signaling

Introduction

Doxrubicin, a secondary metabolite of *Streptomyces peucetius* var. *Caesius*, is a well-established and highly effetive agent in cancer treatment. It is used to treat several different type of cancers, including solid tumors, leukemia, lymphomas and breast cancer, and is applied in both adult and pediatric patients [1]. The successful use of doxorubicin has been hindered by toxicities such as hematopoietic suppression, nausea, extravasation and alopecia, yet the most severe side-effect is cardiotoxicity [2, 3]. Therefore, it is necessary to investigate cardioprotective adjuvants to minimize the adverse reactions exerted by doxorubicin.

BN52021, which are present in the whole extract of Ginkgo biloba leavies, is one of the four Ginkgolide constituents (Ginkgolide A, B, C and J). Studies have shown that BN52021 possesses activity as antagonists of the action of platelet activating factor (PAF). It can inhibit PAF-induced cascade effect in inflammatory reactions, including decreasing the portal vein pressure of liver cirrhosis, exhibiting an anti-shock effect, and exerting protective functions on experimental asthma [4-6]. However, the role of BN52021 in doxorubicin induced cardiomyocytes death has not elucidated yet. Therefore, we employ it here and hypothesized that BN52021 actions in doxorubicin induced cardiomyocytes.

In present study, we employed BN52021 pretreatment in doxorubicin induced cardiomyocytes apoptosis model to see whether a protection effect could be found and our results suggested BN52021 protected against doxorubicin induced cell death in H9c2 cardiomyocytes.

Materials and methods

Cell culture and drug treatment

H9c2 cells were purchased from Institute of Shanghai Biochemistry and Cell biology, Chinese Academy of Science, Shanghai, China. The

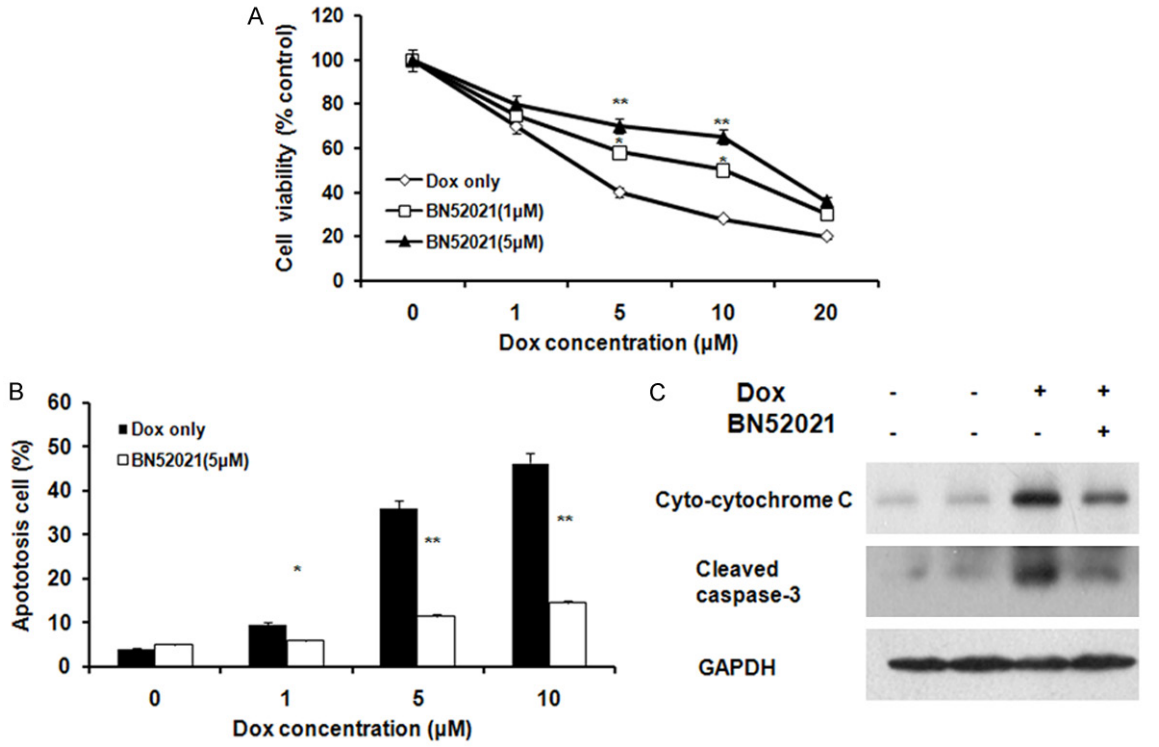

Figure 1. BN52021 protected against doxorubicin induced cell death in H9c2 cardiomyocytes. A. H9c2 cells were treated with doxorubicin (0-20 μM) for 48 h with or without BN52021 (1 μM and 5 μM) and the cell viability was measured using MTT method. B. H9c2 cells were treated with doxorubicin (1, 5 and 10 μM) for 24 h with or without BN52021 pretreatment (5 μM) and apoptotic cells were analyzed TUNEL staining. C. Cytosol cytochrome c and cleaved caspase-3 expression measured using immunoblot analysis. Data represent the mean ± SEM (n = 3). *P < 0.05, **P < 0.01 comparing with Dox only group.

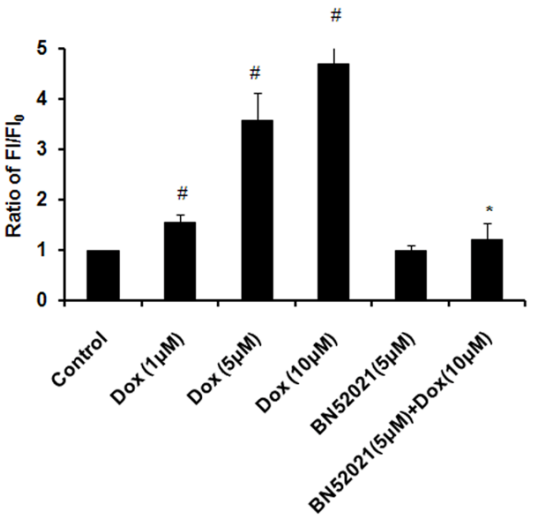

Figure 2. BN52021 protected against doxorubicin induced cell death in H9c2 cardiomyocytes through Ca^{2+} signaling. The concentration of $[Ca^{2+}]_i$ was determined using fluo-3/AM probe assay. Summarized results of FI/F0 obtained from experiments were shown. *Represents P < 0.05 comparing with doxorubicin (5 μM) treated group while #represents P < 0.05 comparing with blank control group.

H9c2 cardiomyotes were maintained at 37°C and 5% CO_2 incubator in DMEM media with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were passaged when 90% confluence was reached and the culture media was replaced with DMEM media containing 1% fetal bovine serum before exposed to treatment.

Cell viability assay

After preparing the single cell suspension, 4 × 10^3 cells in 100 μL culture media were seeded in 96-well plate in quadruplicate overnight and incubated doxorubicin (0-20 μM) for 48 h with or without BN52021 (1 μM and 5 μM) or SB203580 (10 μM) or BATPA/AM (20 μM). 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT: 0.5 mg/ml; Sigma, MO, USA) was added for 4 hr, and formazan dye was dissolved with DMSO and read at 570 nm in a microplate reader (Molecular Device, US). Cell viability expressed as relative viability compared with control in each experiment. All the experiments were performed for three times.

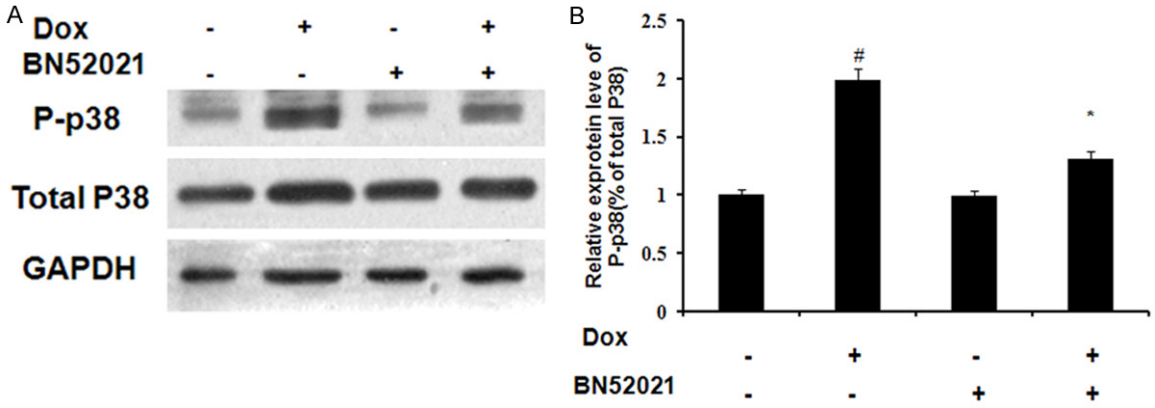

Figure 3. BN52021 protected against doxorubicin induced cell death in H9c2 cardiomyocytes through attenuating the phosphorylation of P-38 MAPK. A. The phosphorylated p38 MAPK was measured by immunoblot. B. Quantification of relative expression of phosphorylated p38 MAPK. *P < 0.05 represents comparing with doxorubicin (5 μM) treated group while #represents P < 0.05 comparing with blank control group.

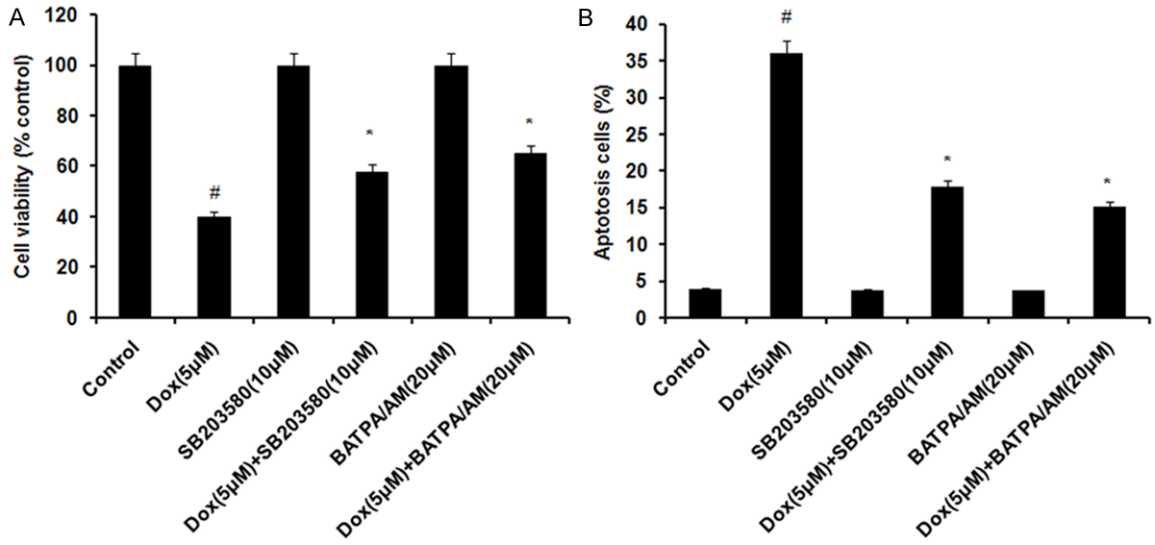

Figure 4. Inhibition Ca^{2+} signaling and P38 MAPK signaling showed similar protection effect as BN52021 in doxorubicin induced cell death in H9c2 cardiomyocytes. A. H9c2 cells were treated with doxorubicin (5 μM) for 48 h with or without SB203580 (10 μM) or BATPA/AM (20 μM) and the cell viability was measured using MTT method. B. H9c2 cells were treated with doxorubicin (5 μM) for 24 h with or without SB203580 (10 μM) or BATPA/AM (20 μM) and apoptotic cells were analyzed TUNEL staining. *P < 0.05 represents comparing with doxorubicin (5 μM) treated group while #represents P < 0.05 comparing with blank control group.

TUNEL staining

The H9c2 cardiomyocytes were processed with TUNEL staining according to the manufacturer's instructions (Roche, Switzerland). The samples were analyzed under light microscope with a magnification of 400 ×. Five fields were randomly picked and the cells with green particles presented in the nucleus were recognized as positive. The following equation was used for Apoptotic index (AI) calculation: AI = (Positive cell numbers in one field/Total cell numbers in one field) × 100%.

Western-blot analysis

The cardiomyocytes protein was prepared according to previous description [7]. Cellular or cytosol protein was separated by electrophoresis on SDS-PAGE gel and then transferred onto PVDF membrane. After blocking, the blots were incubated with the antibodies to Caspase-3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), cytochrome c (Santa Cruz), phosporylated or non-phosporylated p38 (Cell signaling Technology). GAPDH (Santa Cruz) was used as loading control. The appropriate HPR conjugat-

ed secondary antibodies were applied. The protein bands detected with SuperSignal Ultra Chemiluminescent Substrate (Pierce, Rockford, IL USA) on X-ray films (Koda, Lexington, MA, USA). Relative quantification of the protein was determined by Image J software (NIH, Bethesda, MD, USA).

Assessment of intracellular Ca^{2+}

After the cultured H9c2 cardiac myocytes were adhered to the cover-slips of the chamber, cells were rinsed once with the standard Tyrode's solution (126 mM NaCl, 5.4 mM KCl, 10 mM HEPES, 0.33 mM $NaH_2PO_4{\cdot}2H_2O$, 1.0 mM $MgCl_2{\cdot}6H_2O$, 1.8 mM $CaCl_2$ and 10 mM Glucose; pH = 7.40) and then incubated with a working solution containing fluo-3/AM (20 mM) and Pluronic F-127 (0.03%) at 37°C for 45 min. After loading, cells were washed with the standard Tyrode's solution to remove the extracellular fluo-3/AM. Concentration of $[Ca^{2+}]_i$ was measured using Ca^{2+} indicator fluo-3/AM as probe. The images were captured using a 20 × objective (488 nm excitation, 530 nm emission). Scanning time lasts for 30 min. Reagents were added between the 3rd and 4th scans (10 s interval) and the images were saved. Fluorescent intensities before (Fl_0) and after (Fl) the drug administration were recorded. Fold change in $[Ca^{2+}]_i$ was represented by the ratio of Fl/Fl_0.

Statistical analysis

All the statistical analyses were performed by SPSS11.5 software. The data was presented as Mean ± S.E.M. The differences on cell viability in **Figure 1**, concentration of $[Ca^{2+}]_i$ in **Figure 2** and the expression of p-p38 in **Figure 3** among different treatment were calculated by using one way-ANOVA methods. The cell apoptosis in **Figures 1** and **4**, cell viability in **Figure 4** were calculated by using Student t test. $P < 0.05$ was recognized as significant difference.

Results

BN52021 protected against doxorubicin induced cell death in H9c2 cardiomyocytes

A dose depedent manner was found on cell viability after doxorubicin treatment for 48 h (**Figure 1A**). Moreover, pre-treatment with 1 or 5 μM BN52021 prevent the effect of doxorubicin induced cell death (**Figure 1A**). The peak Dox concentration during anti-cancer therapy is 5 μM in human plasma [8]. BN52021 alone (< 20 μM) did not affect the cell numbers (data not shown). Thus, for all *in vitro* studies, the H9c2 cells were incubated for 24 h with 5 μM Dox, with or without 5 μM BN52021 pre-treatment, unless otherwise specified.

Furthermore, TUNEL staining showed that the number of cell apoptosis was significantly increased to 9.6%, 36.0% and 46.3% under 1, 5, 10 μM doxorubicin treatment (**Figure 1B**). BN52021 pre-treatment clearly decreased doxorubicin induced apoptosis and reduced the cell number of apoptosis to 5.9%, 11.4%, 14.4% under 1, 5, 10 μM doxorubicin treatment (**Figure 1B**), respectively. In addition, the expression level of cleaved caspase-3 and cytosol cytochrome c was significantly decreased after BN52021 pretreatment (**Figure 1C**).

BN52021 protected against doxorubicin induced cell death in H9c2 cardiomyocytes through Ca^{2+} signaling

According to previous study, calcium signaling play an critical role in cell apoptosis [9]. Therefore, we emply fluo-3/AM probe to detect the change of calcium signaling. As shown in **Figure 2**, Fl/Fl_0 increased dramatically after the treatment doxorubicin and a dose-dependent manner was found on the $[Ca^{2+}]_i$. On average, 1, 5, and 10 μM doxorubicin evoked a 1.55 ± 0.16, 3.56 ± 0.56, 4.69 ± 0.77 fold increase in $[Ca^{2+}]_i$, respectively ($P < 0.05$ when compared with control). Moreover, 5 μM BN52021 pretreatment for 2 h can significantly decreased the effect of 10 μM doxorubicin. In addition, we did not found an change of Fl/Fl_0 When 5 μM BN52021 was applied alone (**Figure 2**).

BN52021 protected against doxorubicin induced cell death in H9c2 cardiomyocytes through attenuating the phosphorylation of P-38 mitogen-activated protein (MAPK)

The p-38 MAPK signaling have been proposed in the regulation of doxorubicin induced cell death in H9c2 cardiomyocytes [10]. We examined the expression of phosphorylated p38 in doxorubicin treated H9c2 cells. A significant increased phosphorylated p38 was found after the treatment of doxorubicin ($P < 0.05$ compared with blank control group) (**Figure 3**). And the relative expression was significantly decreased when application with BN52021 pre-

treatment (**Figure 3**). In addition, we did not found a significant effect on other MAPK signaling, such as Erk and JNK (data not shown).

Inhibition Ca^{2+} signaling and P38 MAPK signaling protect cells from doxorubicin induced cell death in H9c2 cardiomyocytes

To further verify the effect of Ca^{2+} signaling and P38 MAPK signaling in a population level, we employed Calcium chelator BATPA/AM and p38 MAPK inhibitor SB203580. The results showed that BATPA/AM and SB203580 pretreatment can significantly increased the cell viability ($P < 0.05$ when comparing with doxorubicin treated group) and decreased the cell apoptosis ($P < 0.05$ when comparing with doxorubicin treated group), which showed a similar effect as BN52021 pretreatment.

Discussion

Cardiac toxicity induced by doxorubicin is manifested by decreased cell viability and increased cell apoptosis in cell culture system [11]. In present study, we first demonstrated that BN52021 can exert protective effect on doxorubicin induced cell death in H9c2 cardiomyocytes. Moreover, we found calcium signaling and p38 MAPK signaling might account for the effect exerted by BN52021. Since cardiotoxicity is common problem in clinical pratice, our results suggested a novel cardioprotective adjuvants to prevent cardiotoxicity induced by doxorubicin treatment.

BN52021, extract of Ginkgo biloba leavies and an antagonist of PAF, can inhibit inflammatory reactions during multiple disease condition [12]. Previous study conducted by Zhao *et al* has shown that BN52021 possesses activities in PAF induced cardiomyocytes death [13]. There are some similarities were shared by PAF induced and doxorubicin cardiomyocytes death, such as increased level of cleaved Caspase-3 and cytosol cytochrome c. We supposed BN52021 could also exert protective effect in doxorubicin induced cell death and we did see a protective effect after pretreatment with BN52021 here.

Studies have indicated that Dox- mediated alteration of Ca^{2+} homeostasis maybe one of the possible mechanisms of cardiotoxicity. Dox-induced Ca^{2+} overload of cardiac cells render mitochondrial calcium overloading, resulting in alteration of energy metabolism, promotion of ROS generation and finally cell death [14]. We employ fluo-3/AM probe to conduct the calcium level in doxorubicin induced cardiomyocytes death and found that BN52021 pretreatment abolished the elevated $[Ca^{2+}]_i$ caused by doxorubicin. Moreover, the calcium chelator BATPA/AM did rescue the effect exerted by doxorubicin and showed similar protective effect as BN52021.

The MAPK signalling that converges on JNK and p38 plays an important role in doxorubicin-induced senescence and apoptosis in many cell types [15, 16]. A number of studies have also established the involvement of MAPKs in cell death in H9c2 cardiomyocytes. Thandavarayan *et al* confirmed that p38 MAPK may play a role in the regulation of cardiac function, oxidative stress, and inflammatory and apoptotic mediators in the heart after doxorubicin administration by using dominat-negative p38 MAPK mice [17]. Wold *et al* employed P38 MAPK inhibitor SB203580 to confirm that doxorubicin induces cardiomyocyte dysfunction via a p38 MAP kinase-dependent oxidative stress mechanism [18]. We here detected increased level of phoshorylated p38 in doxorubicin treated cardiomyocytes and found that BN52021 pretreatment can effectively abolish the phosphorylation of p38. Furthermore, we tested the effect of p38 MAPK inhibitor SB203580 in cardiomyocytes and found a protective effect on cell death which was similar as what exerted by BN52021 pretreatment.

In conclusion, we found BN52021 pretreatment could effectively decrease the cell toxocity exerted by doxorubicin. BN52021 in the future may be employed by physician as cardioprotective adjuvants to minimize the adverse reactions exerted by doxorubicin.

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Disclosure of conflict of interest

None.

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