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# Sanguinarine Attenuates Lipopolysaccharide-induced Inflammation and Apoptosis by Inhibiting the TLR4/NF-κB Pathway in H9c2 Cardiomyocytes*

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**Summary**: The inflammatory response is involved in the pathogenesis of the most common types of heart disease. Sanguinarine (SAN) has various pharmacological properties such as anti-inflammatory, antioxidant, antibacterial, antitumor, and immune-enhancing properties. However, few studies have investigated the effects of SAN on lipopolysaccharide (LPS)-induced inflammatory and apoptotic responses in H9c2 cardiomyocytes. Therefore, in this study, H9c2 cells were co-treated with SAN and LPS, and the mRNA levels of pro-inflammation markers and the apoptosis rate were measured to clarify the effect of SAN on cardiac inflammation. The underlying mechanism was further investigated by detecting the activation of Toll-like receptor (TLR)4/nuclear factor-κB (NF-κB) signaling pathways. As a result, increased mRNA expression of interleukin (IL)-1β, IL-6, and TNFα induced by LPS was attenuated after SAN treatment; LPS-induced apoptosis of H9c2 cardiomyocytes and cleaved-caspase 8, 9, 3 were all significantly reduced by SAN. Further experiments showed that the beneficial effect of SAN on blocking the inflammation and apoptosis of H9c2 cardiomyocytes induced by LPS was associated with suppression of the TLR4/NF-κB signaling pathway. It was suggested that SAN suppressed the LPS-induced inflammation and apoptosis of H9c2 cardiomyocytes, which may be mediated by inhibition of the TLR4/NF-κB signaling pathway. Thus, SAN may be a feasible therapy to treat sepsis patients with cardiac dysfunction.
**Key words**: lipopolysaccharides; sanguinarine; inflammation; H9c2 cardiac cells; apoptosis

Sepsis is a systemic inflammatory response to severe inflammation characterized by bacterial infection, and is a major cause of death in intensive care units worldwide[1]. Previous studies have already indicated that endotoxin, such as lipopolysaccharide (LPS) that is a major constituent of the bacterial outer membrane, is a major inducer of septic cardiac dysfunction and contributes to cardiovascular collapse. Furthermore, sepsis patients with cardiac dysfunction are at a 50%–70% greater risk of sepsis-associated mortality than those without cardiac dysfunction[2, 3].

Other studies have demonstrated that LPS markedly induces pro-inflammatory cytokines in cardiomyocytes by binding to its specific receptor, Toll-like receptor-4 (TLR4), and triggers the downstream signaling transduction pathway of nuclear factor-κB (NF-κB), resulting in decreased cardiomyocyte contractility[4–6]. Administration of an NF-κB activation inhibitor has been shown to decrease LPS-induced pro-inflammatory cytokines and apoptosis, further ameliorating cardiac dysfunction[5]. Many studies have also shown that inflammation and

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apoptosis reduction is beneficial for cardiac dysfunction during sepsis and significantly improves sepsis patient care, leading to reduced mortality[3, 7].

Sanguinarine (SAN) derived from the root of *Sanguinaria canadensis* is a benzophenanthridine alkaloid[8]. As a traditional Chinese medicine, SAN has been proven to have anti-inflammatory, antioxidant, antibacterial, antitumor, and immune function-enhancing properties[9]. Some studies have demonstrated SAN is a potent inhibitor of NF-κB activation, which blocks the phosphorylation and degradation of IκBα, in different cell lines stimulated by TNFα or LPS[9]. Our recent studies have also demonstrated that SAN protects against pressure overload-induced cardiac remodeling by inhibiting NF-κB activation[10]. However, whether SAN has a protective effect on LPS-induced inflammation of H9c2 cardiomyocytes remains unknown. In our study, SAN was used to treat H9c2 cells reaching the logarithmic phase of growth, and its effect on LPS-induced inflammation and apoptosis in H9c2 cells was observed; herein, we discuss the underlying mechanism.

## 1 MATERIALS AND METHODS

### 1.1 Cell Culture

The rat cardiomyocyte-derived cell line H9c2 was obtained from the Cell Bank of the Chinese Academy of Sciences (China). Cells were cultured in DMEM basic culture medium (1×) (USA) supplemented with 10% fetal bovine serum (FBS, GIBCO, USA) and 1% penicillin-streptomycin (GIBCO) at 37°C in an incubator containing 5% $CO_2$ (SANYO 18M). When the cell density was carpeted 80% ($2\times10^5$ cells/mL) of dishes, the cells were digested using 1 mL of 0.25% Trypsin-EDTA (1316929, GIBCO) and passaged according to the ratio 1:2. Before treating the cells with a different irritant, they were cultured with serum-free DMEM basic culture medium (1×) (supplemented with 0.05% PS) for another 24 h in order to eliminate the influence of FBS and to synchronize the cells.

### 1.2 Chemicals

SAN ($C_2OH_{14}NO_4$) was obtained from Shanghai Winherb Medical S&T Development Co., Ltd. (China); its purity was 98%. It was dissolved in DMEM basic culture medium (1×) at the desired concentration. LPS was purchased from Sigma (USA).

### 1.3 Cell Viability Assay

Cell viability was measured by the CCK-8 assay according to the manufacturer's protocol. Briefly, after the indicated treatment, 10 μL of CCK-8 solution at a 1/10 dilution was added to each well, and then the plate was incubated in an incubator maintained at 37°C for 2.5 h. Absorbance (*A*) was measured at 450 nm by using a microplate reader (Synergy HT, BioTek Instruments, Inc. USA). The mean *A* value of the five wells was used to measure the percentage of cell viability according to the following formula: cell viability (%) = $A_{(treatment)}/A_{(control)}\times100\%$.

### 1.4 Quantitative Real-time PCR

Total RNA was isolated using TRIzol reagent (Invitrogen, USA). Its yield and purity were spectrophotometrically estimated using the $A_{260nm}/A_{280nm}$ and $A_{230nm}/A_{260nm}$ ratios by using NANODROP 2000c (Thermo Scientific, USA). cDNA synthesis was performed with the cDNA Synthesis Kit (Roche Diagnostics, Basel, Switzerland). SYBR Green PCR Master Mix (Roche) was used to quantify PCR amplification by using the Light Cycler 480 instrument (version 1.5; Roche Diagnostics), and the relative mRNA expression levels of interleukin-1β (IL-1β), IL-6, and tumor necrosis factor alpha (TNFα) were examined. All of the results were normalized to the expression of the GAPDH gene. The primer sequences used were as follows: IL-1β, forward 5′-GGGATGATGACGACCTGCTAG-3′ and reverse 5′-ACCACTTGTTGGCTTATGTTCTG-3′; IL-6, forward 5′-GTTGCCTTCTTGGGACTGATG-3′ and reverse 5′-ATACTGGTCTGTTGTGGGTGGT-3′; TNFα, forward 5′-AGCATGATCCGAGATGTGGAA-3′ and reverse 5′-TAGACAGAAGAGCGTGGTGGC-3′; GAPDH, forward 5′-GACATGCCGCCTGGAGAAAC-3′ and reverse 5′-AGCCCAGGATGCCCTTTAGT-3′.

### 1.5 Western Blotting Analysis

Protein was extracted from different groups, and protein concentration was measured using the BCA protein assay kit (Thermo Scientific) by Synergy HT. The cell lysates (50 μg) were fractionated by 10% SDS-PAGE (Invitrogen). After electrophoresis with a gel transfer device (Invitrogen), proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, China) and incubated with different primary antibodies including TLR4 (SANTA, USA), p-IκBα (Cell Signaling Technology, USA), IκBα (Cell Signaling Technology), p-p65 (BIOWORLD, USA), p65 (Cell Signaling Technology), Bcl-2 (Cell Signaling Technology), Bax (2722, Cell Signaling Technology), cleaved caspase 8 (c-caspase 8, 9429, Cell Signaling Technology), and cleaved caspase 9 (c-caspase 9, Cell Signaling Technology) for 12 h. Thereafter, the membranes were incubated with a secondary antibody (Goat anti-Rabbit IRDye 800CW or Goat anti-Mouse IRDye 800CW; Licor Biosciences, USA). The blots were scanned using a two-color infrared imaging system (Li-COR Biosciences, USA) to quantify protein expression. For total protein determination, the protein expression levels were normalized to those of GAPDH (Cell Signaling Technology).

### 1.6 Measurement of Mitochondrial Transmembrane Potential (△Ψm)

Changes in mitochondrial membrane potential (MMP) were assessed using a fluorescent dye, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide (JC-1, Beyotime Institute of Biotechnology, Shanghai, China).

After the indicated treatment, 5 μg/mL JC-1 was added to the medium and incubated in an incubator maintained at 37°C incubator for 20 min followed by washing with phosphate-buffered saline (PBS). Thereafter, a fluorescence microscope was also used to evaluate JC-1 in the cells on the coverslips.

### 1.7 Flow Cytometry Analysis for Apoptosis

Apoptosis was evaluated with Annexin V-FITC/PI Apoptosis kit (MultiSciences Biotech Co., Ltd., China). After the indicated treatment, the cells were harvested and washed with cold PBS, then resuspended with 500 μL of 1× binding Buffer and 5 μL of FITC, and then incubated in the dark at room temperature for 15 min. Then, 10 μL of propidium iodide (PI) was added, after which cellular fluorescence was measured by flow cytometry with a FACSCalibur Flow Cytometer (BD Biosciences, USA).

### 1.8 Statistical Analysis

All data were expressed as $\bar{x}\pm s_{\bar{x}}$ values. Differences among groups were determined by one-way ANOVA followed by a post-hoc Tukey test; $P<0.05$ was considered to indicate a statistically significant difference.

## 2 RESULTS

### 2.1 Effect of SAN on the Viability of H9c2 Cardiomyocytes

To determine whether SAN had cytotoxic effects on H9c2 cardiomyocytes, the cells were treated with different concentrations of SAN (0.125, 0.25, or 0.5 μmol/L) for 12 h. There were no significant changes in cell viability upon treatment with SAN at the indicated concentrations (fig. 1A), indicating that SAN alone showed no cytotoxicity against H9c2 cardiomyocytes. We further evaluated the cytotoxic effect of SAN on LPS-treated H9c2 cardiomyocytes, and found that LPS (10 μg/mL) treatment alone resulted in a decrease in cell viability compared with that of the control group, while SAN upregulated the viability of LPS-treated H9c2 cardiomyocytes in a dose-dependent manner (fig. 1B).

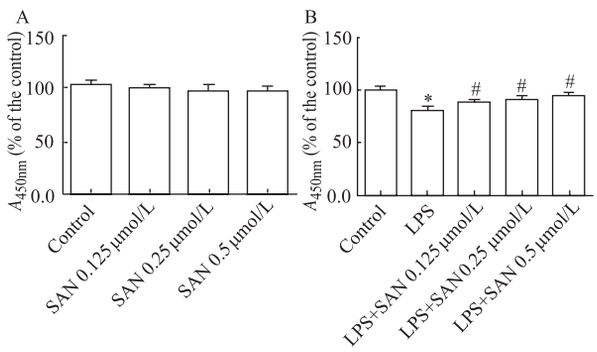


**Fig. 1** Effect of SAN on cell viability

After treatment with the indicated concentration of SAN (0.125, 0.25, 0.5 μmol/L) with (B) or without LPS (10 μg/mL) (A) for 12 h, cell viability was measured and expressed as the mean±SEM value for three independent experiments ($n$=6). $^{*}P<0.05$ *vs*. control group, $^{\#}P<0.05$ *vs*. the LPS group

### 2.2 SAN Inhibited mRNA Expression of IL-1β, IL-6, and TNFα

The effects of SAN on the expression of pro-inflammatory mediators (IL-1β, IL-6, and TNFα) in LPS-treated H9c2 cardiomyocytes were evaluated. In order to determine the optimum concentration of SAN, H9c2 cardiomyocytes were co-treated with LPS (10 μg/mL) and SAN at different concentrations (0.125, 0.25, and 0.5 μmol/L) for 12 h. It was found that mRNA expression levels of IL-1β, IL-6, and TNFα significantly increased in response to LPS treatment, which was markedly inhibited by SAN treatment in a dose-dependent manner ($P<0.05$), especially at the concentration of 0.5 μmol/L (fig. 2A). To determine whether the inhibition of inflammatory mediators was time-dependent, H9c2 cardiomyocytes were co-treated

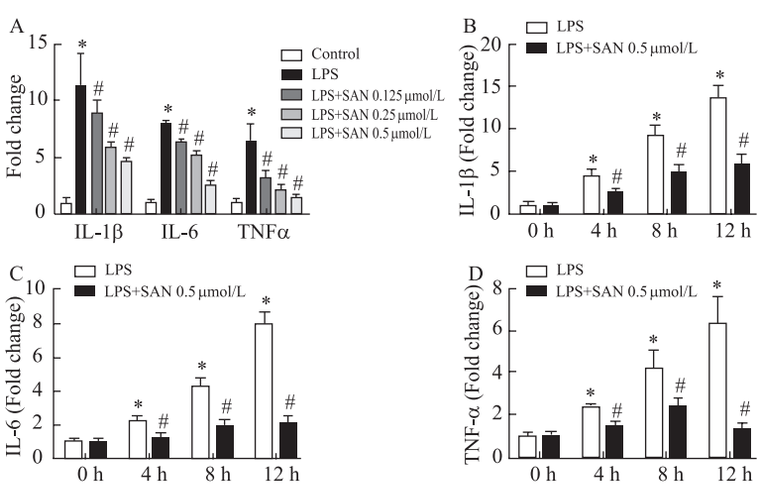


**Fig. 2** Effect of SAN on the mRNA expression of inflammatory mediators

A: Cells were pretreated with the indicated concentrations of SAN (0.125, 0.25, and 0.5 μmol/L) for 40 min, and later co-treated with LPS (10 μg/mL) for 12 h. $^{*}P<0.05$ *vs*. the control group, $^{\#}P<0.05$ *vs*. the LPS group ($n$=6). B–D: Cells were pre-treated with SAN (0.5 μmol/L) for 40 min, and later co-treated with LPS (10 μg/mL) for the indicated time (0, 4, 8, and 12 h) ($n$=6). $^{*}P<0.05$ *vs*. the control group, $^{\#}P<0.05$ *vs*. the LPS group at the same time point

with LPS and SAN (0.5 μmol/L) at different time points (0, 4, 8, and 12 h), and SAN was found to time-dependently inhibit mRNA expression levels of IL-1β, IL-6, and TNFα ($P$<0.05) (fig. 2B-2D). These findings indicated that SAN inhibited cardiac inflammation induced by LPS *in vitro*.

### 2.3 SAN Attenuated LPS-induced Apoptosis of H9c2 Cardiomyocytes

Previous studies have shown that SAN regulates cell apoptosis. Thus, we investigated the anti-apoptosis effect of SAN on LPS-treated H9c2 cardiomyocytes. Compared to the findings in the control cells, LPS significantly promoted cell apoptosis, as shown by FITC/PI staining, and both early and late apoptosis rates significantly increased (63.8% *versus* 0.057%; 15.8% *versus* 0.023%). SAN attenuated the increased apoptosis rate induced by LPS in a concentration-dependent manner, with the apoptosis rate decreasing to 25.8% (early) and 8.57% (late) at the concentration of 0.5 μmol/L (fig. 3).

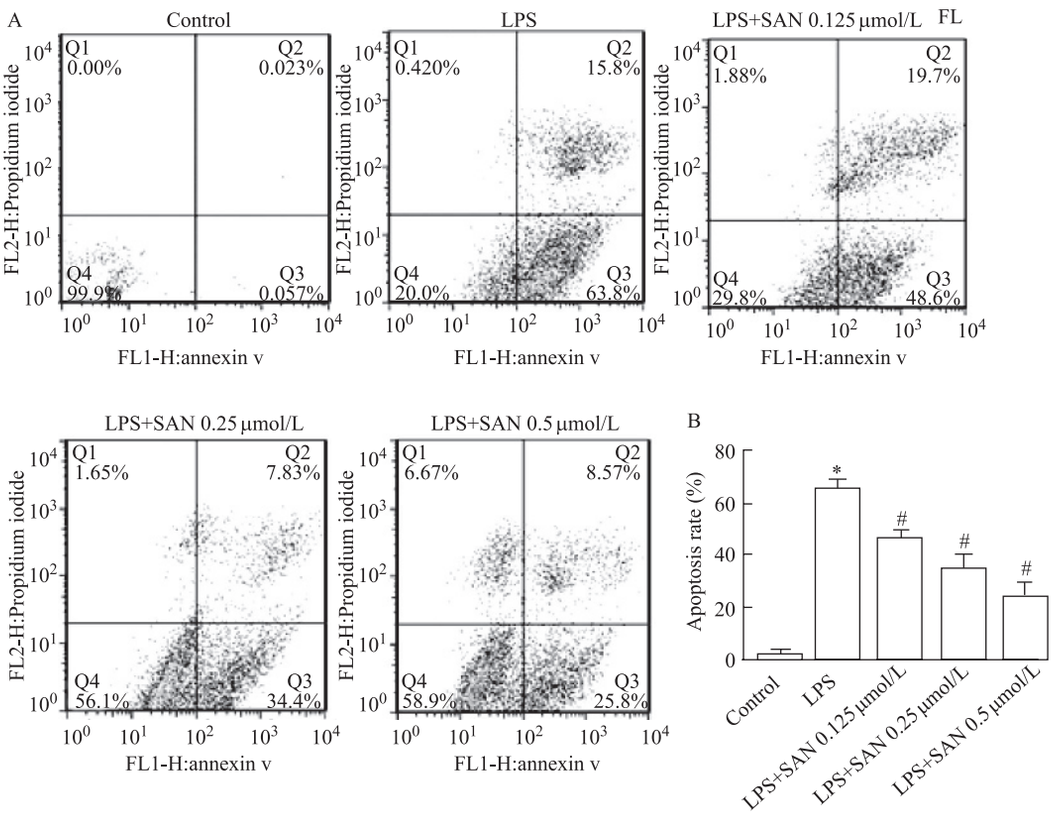


**Fig. 3** Inhibitory effect of SAN on LPS-induced apoptosis

Cells were pretreated with the indicated concentrations of SAN (0.125, 0.25, and 0.5 μmol/L) for 40 min, and later co-treated with LPS (10 μg/mL) for 12 h. The results were expressed as the $\bar{x}\pm s_{\bar{x}}$ value for three independent experiments ($n$=6). A: the representative flow cytometry image; B: the quantitative result. $^{*}P$<0.05 *vs*. the control group, $^{\#}P$<0.05 *vs*. the LPS group

### 2.4 SAN Attenuated the MMP Induced by LPS in H9c2 Cardiomyocytes

The stability of MMP was measured in H9c2 cardiomyocytes after LPS treatment. Dynamic changes in MMP were reflected by changes in the ratio of red (aggregated JC-1) fluorescence to green (monomeric form of JC-1) fluorescence. Our results revealed that the stability of MMP was significantly impaired by LPS (10 μg/mL) compared with that of the control group, while co-treatment with SAN (0.5 μmol/L) significantly improved the stability of MMP, indicating SAN attenuated functional abnormalities in the mitochondria after LPS treatment (fig. 4).

### 2.5 SAN Downregulated the Activity of Bax, Caspase 8, Caspase 9, and Caspase 3, Up-regulated Bcl-2 and Inhibited LPS-induced Apoptosis of H9c2 Cardiomyocytes

To further assess the effect of SAN on both the death-receptor-dependent pathway and mitochondrion-dependent pathway, we examined markers of the two pathways. The cells pretreated with SAN (0.125, 0.25, and 0.5 μmol/L/) showed dose-dependent reduction of caspase 8, caspase 9, and caspase 3 activity, and the resultant activity was significantly lower than that in the cells treated with LPS alone. In addition, in the SAN-pretreated cells, Bax expression was significantly lower while that of Bcl-2 was

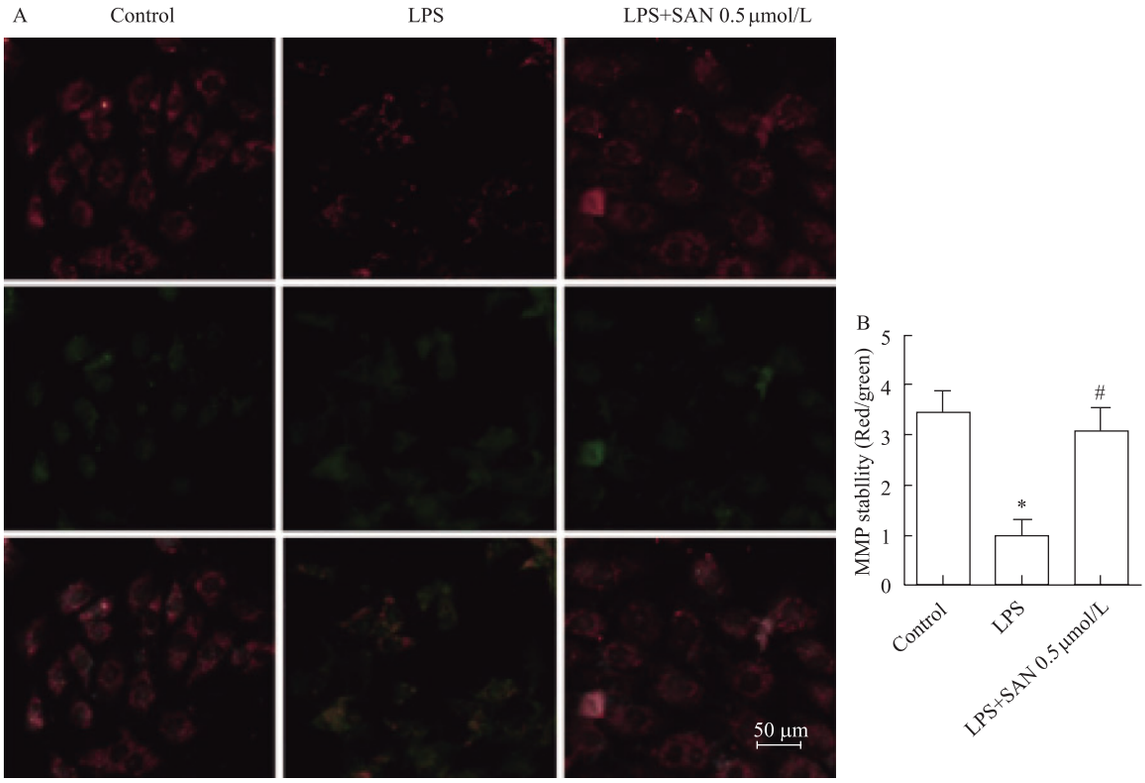


**Fig. 4** Effect of SAN on MMP stability in LPS-treated cells
Cells were pretreated with the indicated concentrations of SAN (0.125, 0.25, and 0.5 μmol/L) for 40 min, and later co-treated with LPS (10 μg/mL) for 12 h. The green image in the upper panel represents the unstable cells, the red image represents the stable cells, and the lower panel shows the merged images. The ratio of cell number in the red image to that in the green image shows the degree of healthiness of the cells ($n$=6). A: the representative image; B: the quantitative result. $^{*}P$<0.05 *vs*. the control group, $^{\#}P$<0.05 *vs*. the LPS group.

significantly greater than the corresponding expression in the cells treated with LPS alone (fig. 5A, B). All of the above results further suggested that SAN inhibited LPS-induced apoptosis via both the death-receptor-dependent pathway and mitochondrion-dependent pathway.

## 2.6 SAN Inhibited LPS-induced TLR4/NF-κB Activation

To explore the molecular mechanisms underlying the anti-inflammatory and anti-apoptotic effects of SAN, the TLR4/NF-κB signaling pathway was examined. TLR4,

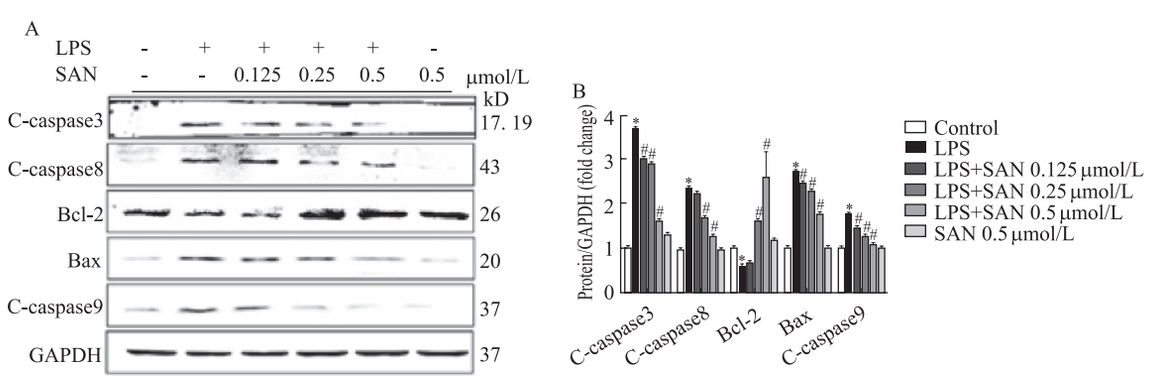


**Fig. 5** Effect of SAN on the activation of the apoptosis signaling pathways
Cells were pretreated with the indicated concentrations of SAN (0.125, 0.25, and 0.5 μmol/L) for 40 min, and later co-treated with LPS (10 μg/mL) for 12 h. A: representative blots; B: quantitative results ($n$=6). $^{*}P$<0.05 *vs*. the control group, $^{\#}P$<0.05 *vs*. the LPS group

p-IκBα, IκBα, p-p65, and p65 expression was measured by Western blotting. Treating H9c2 cardiomyocytes with LPS (10 μg/mL) rapidly up-regulated TLR4 protein and induced phosphorylation of IκBα and p65. However, the

degree of TLR4 protein upregulation and phosphorylation of IκBα and p65 was significantly suppressed by SAN (0.125, 0.25, and 0.5 μmol/L) in a dose-dependent manner ($P$<0.05) (fig. 6A, B).

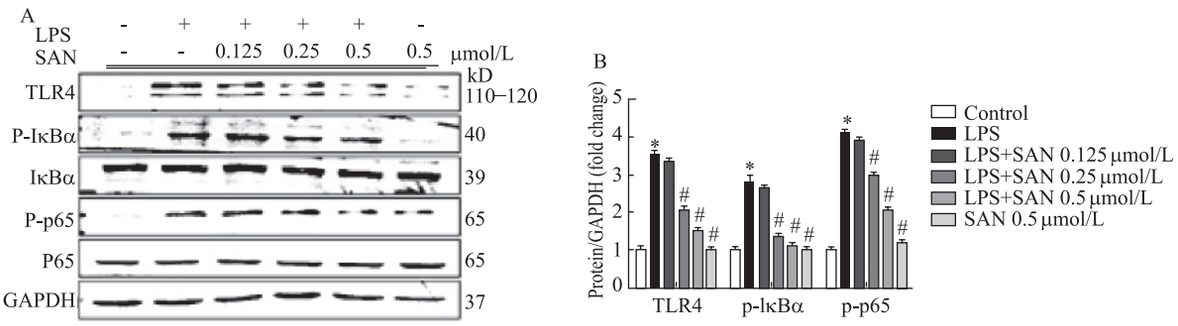


**Fig. 6** Effect of SAN on the activation of the TLR4/ NF-κB pathway
Cells were pretreated with the indicated concentrations of SAN (0.125, 0.25, and 0.5 μmol/L) for 40 min, and later co-treated with LPS (10 μg/mL) for 30 min. A: representative blots; B: quantitative results ($n$=6). $^{*}P$<0.05 *vs*. the control group, $^{\#}P$<0.05 *vs*. the LPS group

## 3 DISCUSSION

The benzophenanthridine alkaloid SAN, a structural homolog of chelerythrine, has been reported to show potential protective effects on the cardiovascular system[10, 11]. However, it is unknown whether SAN has a direct effect on LPS-induced cardiomyocyte injury. In this study, we found that SAN significantly protected H9c2 cardiomyocytes from LPS-induced cytotoxicity. We also found that the LPS-induced apoptosis of H9c2 cardiomyocytes via both the death-receptor-dependent pathway and mitochondrion-dependent pathway was inhibited by SAN in a dose-dependent manner. These beneficial effects of SAN on H9c2 cells were associated with the inhibition of the TLR4/NF-κB pathway (fig. 7).

H9c2 cardiomyocytes, an embryonic rat heart-derived cell line, have characteristics similar to those of primary cardiomyocytes, in terms of morphology, protein expression, inflammation, hypertrophic responses, and apoptosis[12, 13]. LPS functions as a key hormonal mediator in cardiac dysfunction, inducing

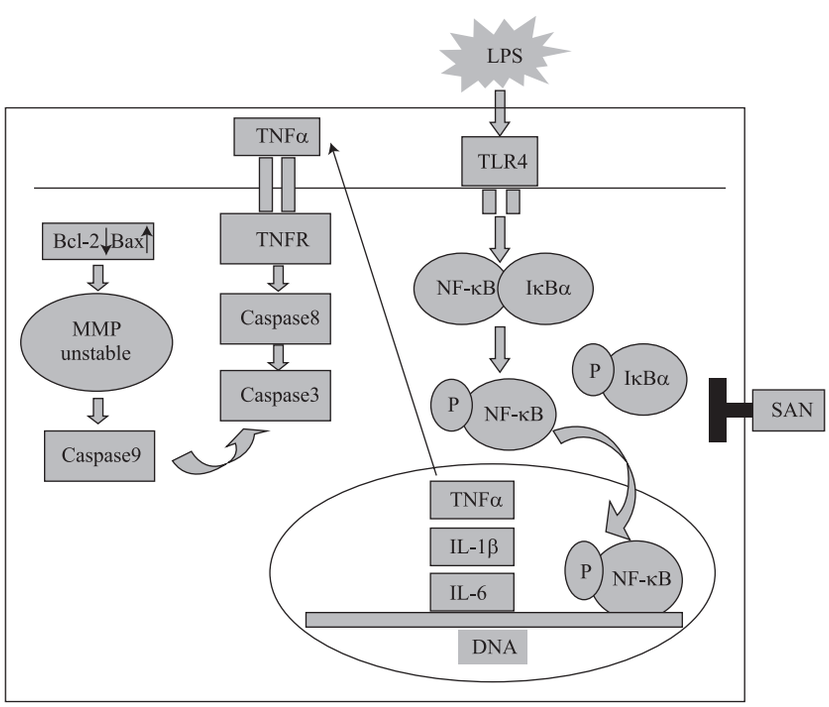


**Fig. 7** SAN prevents LPS-induced inflammation and apoptosis by inhibiting the TLR4/NF-κB pathway in H9c2 cardiomyocytes

inflammation and apoptosis in cardiomyocytes[14]. We first investigated the effect of SAN against LPS-induced cytotoxicity in H9c2 cardiomyocytes by using the CCK-8 assay. Our results showed that treating H9c2 cardiomyocytes with LPS alone resulted in a loss of cell viability, while treatment with SAN at different

concentrations markedly decreased the loss of cell viability. These results indicated that SAN protected H9c2 cardiomyocytes from LPS-induced cytotoxicity.

LPS from gram-negative bacteria is considered to be a strong inducer of the pathogenesis of cardiac dysfunction. Pro-inflammatory cytokines such as IL-1β, IL-6, and TNFα are produced in circulation in response to LPS, which are expanded for the activation of myeloid cells. It is now clear that cardiomyocytes represent the predominant source of pro-inflammatory cytokines in the myocardium[15,16]. LPS directly decreases cardiomyocyte contractility and induces pro-inflammatory cytokine expression in cardiomyocytes by binding to its specific membrane ligand TLR4, leading to the activation of intracellular signaling pathways. NF-κB is a transcription factor that exists in the cytosol. Under regular conditions, NF-κB dimers are inactivated by binding to IκBs, inhibiting the translocation of NF-κB to the nucleus[17]. IκBα is part of the IκB family. When stimulated by different activators, the IKK complex phosphorylates IκBα and then induces polyubiquitylation and proteasomal degradation of IκBα, allowing translocation of NF-κB dimers to the nucleus and induction of inflammatory mediators[18]. Our research showed that SAN treatment resulted in a significant decrease in inflammatory mediators including IL-1β, IL-6, and TNFα in a dose- and time-dependent manner. Further studies showed that SAN also downregulated the expression of TLR4 and decreased the levels of p-IκBα and p-p65, inhibiting the LPS-induced nuclear localization of NF-κB and p65.

A previous study showed that activation of the LPS-induced NF-κB signaling pathway also directly increased pro-apoptotic proteins and apoptosis in myocardial cells via the TNF death-receptor-dependent pathway and later activated t-Bid to further induce the mitochondrion-dependent pathway[19], which was confirmed in our study. Apoptosis is one of the factors that reduce cardiac contractility, which also affects heart failure through multiple aspects[20, 21]. Moreover, our study found that SAN inhibited the activation of the NF-κB signaling pathway. Thus, we investigated whether SAN participated in apoptosis inhibition. Our results showed that SAN attenuated the apoptosis of H9c2 cardiomyocytes induced by LPS via both the death-receptor-dependent pathway and mitochondrion-dependent pathway. Further study showed that SAN significantly inhibited LPS-induced mitochondrial membrane instability through upregulation of the anti-apoptosis protein Bcl-2 and downregulation of the pro-apoptotic protein Bax, both of which are upstream regulators of MMP.

In conclusion, the present study provides new insights into the molecular actions of SAN on LPS-induced inflammation and apoptosis in H9c2 cardiomyocytes. The results of our study provide experimental evidence for the potential application of SAN in treating sepsis patients with cardiac dysfunction.

**Conflict of Interest Statement**

The authors declare that there is no conflict of interest with any financial organization or corporation or individual that can inappropriately influence this work.

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