HSPB1 Promotes Doxorubicin-induced Cardiomyocyte Pyroptosis by Inhibiting AATF

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Research

Keywords: Cardiomyocyte, HSPB1, AATF, Pyroptosis, Doxorubicin

Posted Date: April 21st, 2021

DOI: https://doi.org/10.21203/rs.3.rs-434645/v1

Abstract

Background: Doxorubicin (DOX) has been widely used for the treatment of different kinds of cancers. However, the adverse effects, especially cardiotoxicity, which limit the long-term use of DOX. Although some signaling pathways have been investigated to be participated in DOX-induced cardiotoxicity, however, the mechanisms underlying DOX-induced cardiotoxicity need to be further investigated. Recent study suggested that heat shock proteins (HSPs) play a role in cell apoptosis and pyroptosis. However, we do not know yet whether heat shock protein beta-1 (HSPB1), a member of HSPs family, is involved in DOX-induced cardiomyocyte pyroptosis. This study aimed to evaluate the effects of HSPB1 and apoptosis antagonizing transcription factor (AATF) in DOX-induced pyroptosis in cardiomycytes.

Results: We found that DOX remarkably enhanced the expression of HSPB1 but reduces AATF expression in cardiomyocytes. We also found that either inhibition of HSPB1 by small interfering RNA (siRNA) or overexpression of AATF by transfection of AATF plasmid significantly attenuated DOX-induced cardiomyocyte pyroptosis. Moreover, HSPB1 interacts with AATF. Furthermore, inhibition of HSPB1 and AATF restores the DOX-induced cardiomyocyte pyroptosis.

Conclusion: Our findings reveal a novel pathway that cardiomyocyte pyroptosis is regulated through HSPB1-AATF-caspase-3-GSDME pathway following DOX treatment, suggesting that HSPB1-AATF-dependent pyroptosis may provide a novel therapeutic strategy to reduce cardiotoxicity induced by DOX.

Introduction

Doxorubicin (DOX), an anthracycline anticancer drug, however, has serious side effects on anti-cancer treatment such as cardiotoxicity, Encephalotoxicity and nephrotoxicity [1], resulting in several limitations of the clinical use [2]. Changes in electrocardiogram, irreversible degenerative cardiomyopathy, arrhythmia and congestive heart failure are the main manifestations of cardiotoxicity when DOX is used to treat cancer [3]. Accumulating pathogenic mechanisms of DOX-induced cardiotoxicity have been put forward, most of studies focusing on reactive oxygen species (ROS), ROS-related autophagy, peroxynitrite-related necrosis, lipid peroxidation-related ferroptosis, DNA damage, releases or regulations of cytokines, mitochondrial dysfunction, activation of apoptotic/necroptosis/pyroptotic signaling cascades and inflammatory reactions occurrence [4-5]. However, the underlying mechanisms of cardiotoxicity of DOX still needs to be further investigated.

As a new form of programmed cell death, pyroptosis causes the release of intracellular substances and pro-inflammatory molecules. It is characterized by cell swelling, large bubbles blowing from the plasma and cell lysis [6-7]. Gasdermin D (GSDMD) cleaved by inflammatory-activated caspase-1 and LPS-activated caspase-11, -4, -5 functions as a pyroptosis execution protein [8]. Moreover, pyroptosis can also be induced by the cleavage of Gasdermin E (GSDME) by activated caspase-3 (CASP3) [9]. CASP3 activates GSDME-mediated pyroptosis, contributing to the toxicity of chemotherapy in mice [10]. Furthermore, we recently reported that DOX induces pyroptosis through the Bnip3/CASP3/GSDME

pathway in HL-1 cardiomyocytes [11]. However, the molecular mechanisms by which DOX promotes pyroptosis in cardiomyocytes remains to be further elucidated.

Heat shock protein beta-1 (HSPB1), also known as HSP25 in rodents and HSP27 in humans, belongs to the mammalian small heat shock protein (sHSP) family [12]. HSPB1 is expressed in all normal human tissues, but mainly in skeletal, smooth and cardiac muscles [13]. Most studies have shown that HSPB1 contributes to anti-apoptosis and autophagy and can be induced by various stresses, including heat shock, oxidative stress cytokines, growth factors [12, 14], myocardial infarction [15] and heart failure [16]. In contrast, Guo et al found that HSPB1 plays a pro-apoptotic role through CASP3 [17]. Taken together, HSPB1 contributes to either anti-apoptotic or pro-apoptotic effect. Interestingly, inhibition of HSPB1 by a HSPB1 inhibitor combined with anticancer drugs, effect of tumor chemotherapy could be significantly improved [18], suggesting that HSPB1 may not always be a protector in chemotherapy drugs-induced cell death. However, we do not know yet whether HSPB1 plays a role in DOX-induced pyroptosis in cardiomyocytes.

Apoptosis antagonizing transcription factor (AATF), also termed Che-1, a partner of RNA polymerase II, is a multifunctional protein that is highly conserved in eukaryotes [19]. AATF acts as a pro-proliferative role or an anti-apoptotic role to promote cell survival by inducing cell cycle arrest, autophagy, DNA repair, and inhibition of apoptosis [19-20]. It has been shown that AATF is involved in the transcription of E2F target genes and the depletion of cells to anticancer agents [21]. HSPB1 suppresses expression of E2F-4 in human lung fibroblast cells [22]. This leads us to determine whether there is a functional relationship between HSPB1 and AATF in DOX-induced cardiomyocyte pyroptosis.

In the current study, we investigated the mechanisms by which pyroptosis contributed to DOX-induced cardiac injury [11]. We found that knockdown of HSPB1 attenuates cardiomyocyte pyroptosis induced by DOX. Moreover, overexpression of AATF alleviates DOX-induced pyroptosis in cardiomyocytes. Furthermore, we found that HSPB1 aggravates DOX-induced cardiomyocyte pyroptosis via inhibiting the function of AATF. Thus, our studies may provide a novel potential therapeutic target against DOX-induced cardiovascular injury.

Materials And Methods

Cell culture

HL-1 cardiomyocytes were purchased from the American Type Culture Collection (ATCC) and grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco), as previously described, incubated in a humidified atmosphere containing 5% CO_2 at 37°C [11].

Cell viability assay

HL-1 cells were seeded into 96-well culture plates with 5000 cells/well and treated

with DOX at 5 μM for 9 h. We utilized the Cell Counting Kit-8 assay kit (CCK8, Bimake,China) to measure cell viability according to the manufacturer's instructions. We examined the Optical density (OD) values at 450 nm by an Infinite™ M200 Microplate reader (Tecan, Mannedorf, Switzerland) [23].

LDH release assay

We used the LDH cytotoxicity assay kit (Beyotime, Shanghai, China) to determine the LDH release, according to the manufacturer's instructions. After DOX treatment, we first transferred 120 μl supernatants of HL-1 cell supernatant to the 96-well plate, and then added 60 μl reaction mixture to each well. After the plate was incubated in the dark for 30 min at room temperature, the absorbance at 490 nm was measured [24].

Microscopy imaging

To observe the morphology of pyroptotic cells, the cells were first incubated into a 6-well plate and then treated with DOX. Still bright-field images were taken with Nikon TE2000 microscope [11].

Cell transfection

HSPB1 siRNA (si-HSPB1), AATF siRNA (Si-AATF) and negative control siRNA (NC) were purchased from JTSbio (Wuhan, China) and transfected into HL-1 cells while the cell confluence is 30%-50%. AATF expression vector, pcDNA3.1-AATF was constructed by cloning full-length wild-type AATF coding sequence into pcDNA3.1. Empty vector was transfected as a control. Plasmids were transfected into HL-1 cells while the cell confluence is 60%-70%. HL-1 cells were seeded into 6-well plate the day before transfection as described previously [25]. Transfection in HL-1 cells was performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturers' protocols. After 48 h, we treat the transfected cells with DOX.

Co-immunoprecipitation

We used ice-cold IP lysis buffer (Thermo Fisher Scientific, USA) to collect HL-1 cells lysate. The lysate was transferred into a microcentrifuge tube and centrifuged at 2500 rpm for 10 minutes. Then, the supernatant was transferred into a new microcentrifuge tube to determine the protein concentration and perform further analysis. Briefly, the protein A/G PLUS-Agarose (Santacruz Biotechnology, CA, USA), the target antibody and the lysate were mixed together and then incubated at 4°C overnight [26]. Next day, we centrifuged the mixture at 2500 rpm for 10 minutes and washed the precipitated complex with phosphate buffer saline. Repeat this step at least three times. We used anti-AATF antibody as a bait antibody to capture HSPB1 protein, and normal rabbit IgG (Cell Signaling Technology, USA) as a negative control. The control was processed in the same way as the Co-IP sample. Lysates from both control and DOX treated cells without immunoprecipitation were used as the positive control (input). After co-immunoprecipitation, the proteins pulled down by AATF antibody were analyzed by Western blot.

Western blot analysis

HL-1 cardiomyocytes and mouse heart were harvested and then split using RIPA lysate containing protease inhibitor and phosphatase inhibitor. Protein concentration was estimated by Bradford. Then equal amounts (30 μg) of proteins were separated by 12% SDS-polyacrylamide gel electrophoresis and transferred onto Immobilon® PVDF Membranes (Merck KGaA, Darmstadt, Germany). Membranes were blocked with 5% skim milk for 1.5 h at room temperature prior to incubation with antibodies at 4°C overnight. Then, the corresponding HRP-conjugated secondary antibodies (Goat anti-rabbit IgG, Proteintech, China) are added and the membranes are maintained at room temperature for 2 h [28]. The signals were visualized with the enhanced chemiluminescence (ECL) substrate (Cwbio, Beijing, China) and captured or analyzed by Image Lab™ software. Tubulin acts as loading control. The antibodies used for Western blot are presented in Table 1.

Table 1. Antibodies used for Western blot

Name	Description	Manufacturer
Anti-Tubulin	Rabbit monoclonal, 55 kDa	Proteintech (11224-1-AP)
Anti-GSDME	Rabbit monoclonal, 55;34 kDa Rabbit monoclonal, 35 kDa	Abcam (ab215191)
Anti-CASP3		CST (#9662S)
Anti-Cleaved-CASP3	Rabbit monoclonal, 17 kDa	CST (#9664S)
Anti-HSPB1	Rabbit monoclonal, 25kDa	Abcam (ab155987)
Anti-AATF	Rabbit monoclonal, 70 kDa	Abcam (ab233546)

Statistical analysis

Statistical analysis was performed with GraphPad Prism 6. The results were presented as the mean ± SD of at least three independent experiments. Data was analyzed using one-way analysis of variance (ANOVA) and Student's t-test. The P value < 0.05 shows statistical significance.

Results

HSPB1 is up-regulated in cardiomyocyte pyroptosis induced by DOX

To determine whether HSPB1 contributes to DOX-induced pyroptosis, we first examined the expression of HSPB1 in HL-1 cardiomyocytes following DOX treatment. Western blot analysis showed that DOX dramatically increased the expression of HSPB1, GSDME-N and cleaved-CASP3 in HL-1 cardiomyocytes

(Fig. 1a). We also observed the pyroptotic cell morphology (Fig. 1b), decreased cell viability (Fig. 1c) and increased LDH release (Fig. 1d) in HL-1 cardiomyocytes. These results indicate that HSPB1 may play a role in DOX-induced cardiomyocyte pyroptosis.

HSPB1 is required for DOX-induced cardiomyocyte pyroptosis

To determine whether DOX-induced cardiomyocyte pyroptosis is through HSPB1, small interfering RNA of HSPB1 (Si-HSPB1) was utilized in HL-1 cardiomyocytes. We found that inhibition of HSPB1 by Si-HSPB1 significantly diminished the number of pyroptotic cells (Fig. 2a), enhanced cell viability (Fig. 2b) and decreased LDH release (Fig. 2c) in DOX-treated cells. Moreover, knockdown of HSPB1 by Si-HSPB1 dramatically attenuated the levels of GSDME-N and cleavage of caspase-3 induced by DOX (Fig. 2d). Taken together, these results suggest that HSPB1 is required for DOX-induced pyroptosis in cardiomyocytes.

HSPB1 interacts with AATF in cardiomyocytes after DOX treatment

It has been reported that there is an indirect connection between HSPB1 and AATF following DOX treatment [21-22]. Thus, we then investigated whether HSPB1 interacted with AATF following DOX treatment. As shown in Fig. 3a, the AATF expression was significantly decreased following DOX treatment. Inhibition of HSPB1 by Si-HSPB1 dramatically enhanced the expression of AATF compared with NC following DOX treatment (Fig. 3a). Moreover, co-immunoprecipitation showed that after DOX treatment, HSPB1 binds with AATF (Fig. 3b). These data provoked us to think that HSPB1 interacts with AATF in cardiomyocytes after DOX treatment.

DOX-induced pyroptosis is through AATF in cardiomyocytes

To clarify whether AATF engaged in regulation of cardiomyocyte pyroptosis induced by DOX, full length AATF plasmids were transfected into HL-1 cardiomyocytes. Our results showed that overexpression of AATF significantly reduced pyroptotic cells induced by DOX (Fig. 4a), increased cell viability (Fig. 4b) and decreased LDH release (Fig. 4c). Moreover, overexpression of AATF dramatically attenuated the level of GSDME-N and cleavage of caspase-3 (Fig. 4d). Thus, these data reveal that AATF plays a critical role cardiomyocyte pyroptosis induced by DOX.

AATF involves in DOX/HSPB1-induced cardiomyocyte pyroptosis

We have proved HSPB1 affects expression of AATF (Fig. 3a). What confuses us is if there exists a regulatory relationship between HSPB1 and AATF. To explore the function of AATF in DOX/HSPB1-induced HL-1 cardiomyocyte pyroptosis, we knocked down the expression of HSPB1 and AATF by transfecting respective siRNAs, followed by DOX treatment. We showed that the number of pyroptotic cells (Fig. 5a), cell viability (Fig. 5b) and LDH release (Fig. 5c) in the Si-HSPB1+Si-AATF+DOX group have no difference compared to the Si-Ctrl + DOX group. Interestingly, inhibition of HSPB1 increased expression of AATF, but inhibition of AATF could not alter the expression of HSPB1 (Fig. 5d), suggesting that HSPB1 negatively regulates AATF. Taken together, HSPB1 plays an inhibitory effect on AATF in DOX-

induced cardiomycyte pyroptosis. Therefore, HSPB1 promotes pyroptosis by inhibiting the expression of AATF.

Discussion

Previous studies have shown that eliminate or reduce the cardiotoxicity of DOX and improve its curative effect is still an unsolved and prominent problem [29-34]. Furthermore, among six cardiomyocyte death forms (apoptosis, autophagy, necrosis, necroptosis, pyroptosis, and ferroptosis), accumulating evidences suggest an emerging role of pyroptosis in DOX-induced cardiomyopathy [5]. Our previous studies have shown that DOX induces cardiomyocyte pyroptosis through CASP3/GSDME pathway. Therefore, we assumed that alleviating pyroptosis could make contributions to treat DOX-induced cardiac injury.

Increasing numbers of heat shock proteins, such as a member of HSPs family HSP90, have been shown to be relevant to pyroptosis in THP-1 cells, hepatoma cell apoptosis and osteosarcoma cell autophagy [35-38]. Also, as a member of the HSPs, various studies have demonstrated that HSPB1 participates in apoptosis and autophagy and expressed mainly in skeletal, smooth and cardiac muscles [14]. But its role in pyroptosis is still unknown yet. Interestingly, HSPB1 contributed to apoptosis that related to CASP3 and tumor chemotherapy [17-18], providing new insights into HSPB1's effects through activation of CASP3. In our study, the data displayed that inhibition of HSPB1 mitigates the cleavage of CASP3 and DOX-induced pyroptosis while HSPB1 is obviously up-regulated in cardiomyocytes.

AATF functions as protective molecular by inducing cell cycle arrest, autophagy, DNA repair, and inhibition of apoptosis [19-20]. Interestingly, the fact that HSPB1 or AATF respectively associates with members of the E2F family implies the possible connection between HSPB1 and AATF [21-22]. Notably, our studies reveal that AATF is decreased and it restrains cardiomyocyte pyroptosis following DOX treatment. Our results from Western blot and Co-IP analysis reveal that HSPB1 binds to AATF directly or indirectly, which needs to be further determined in in the future studies. To our best knowledge, our study is the first to reveal that HSPB1 promotes DOX-induced cardiomyocyte pyroptosis. We also reveal that AATF exerts anti-pyroptotic effects. We further found that after DOX treatment, HSPB1 inhibits AATF.

Conclusion

In summary, our findings of the present study demonstrate that HSPB1 inhibits AATF, promotes DOX-induced cardiomyocyte pyroptosis, and provides a potential target for therapeutic intervention.

Abbreviations

DOX, doxorubicin; HSPB1, Heat shock protein beta-1; AATF, Anti-apoptotic transcriptional faactor; GSDME, gasdermin E; Cleaved-CASP3, cleaved-caspase 3

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All relevant data are within this published paper.

Competing interests

The authors declare that they have no competing interests.

Funding

This research was supported by the National Natural Science Foundation of China (No. 81570454).

Authors' contributions

YT and DY conceived and designed the experiments in the manuscript. YT and XYL performed the experiments. YT analyzed data, plotted the graphs for figures. YT wrote the manuscript. DY made manuscript revisions. All authors read and approved the final manuscript.

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Acknowledgements

We would like to thank all the colleagues in our research team for technical support.

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Figures

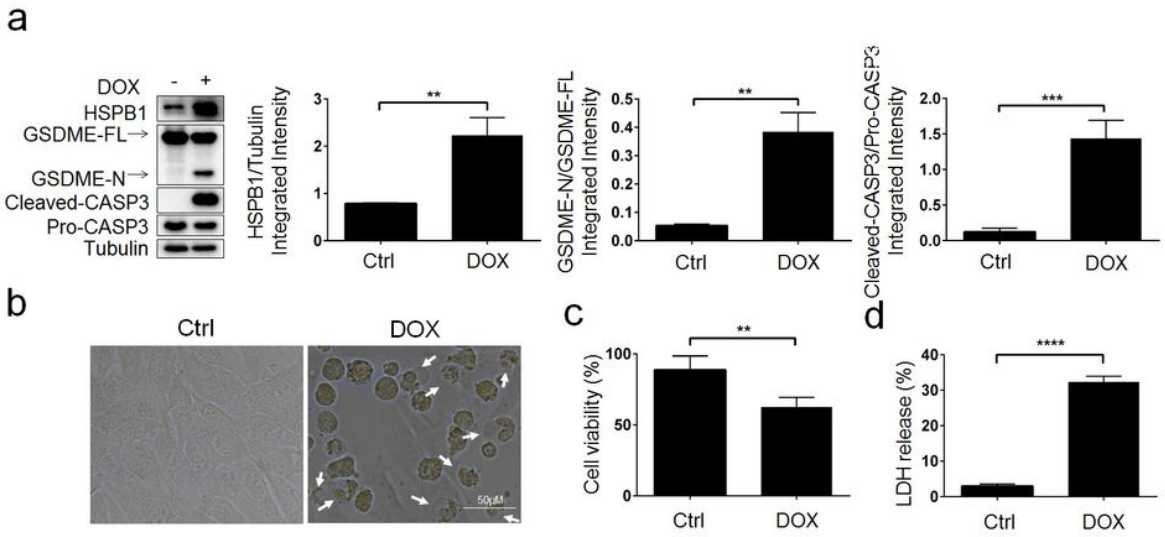

Figure 1

HSPB1 is up-regulated in cardiomyocyte pyroptosis induced by DOX. HL-1 cells were treated with doxorubicin (DOX) at 5 μM for 9 h to assess: a The expression of HSPB1, GSDME-N, and Cleaved-CASP3 was examined by Western blot analysis. B Representative microscopic images. White arrowheads indicate pyroptotic cells. c Cell viability. d The LDH content. N=3 per group; **P< 0.01, ***P< 0.001, ****P< 0.0001 compared with indicated groups.

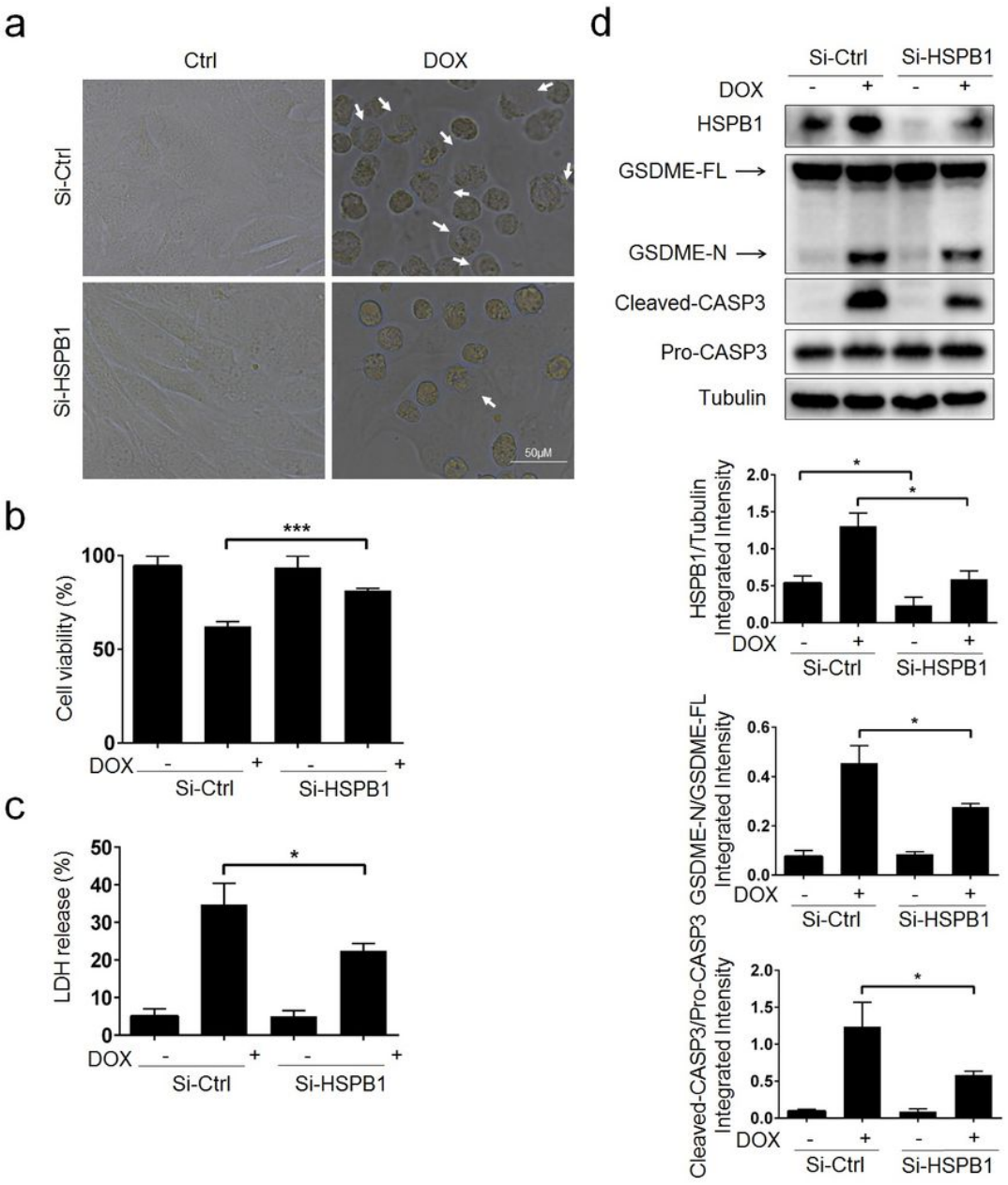

Figure 2

HSPB1 is required for DOX-induced pyroptosis in HL-1 cardiomyocytes. HL-1 cells were pretreated with Si-HSPB1 (HSPB1 siRNA) or NC (negative control) for 48 h, followed DOX (5 μM) treatment for 9 h to determine: a Representative microscopic images. White arrowheads indicate pyroptotic cells. b Cell viability. C The level of LDH. d The protein levels of HSPB1, GSDME-N, Cleaved-CASP3 were examined by Western blot analysis. N=3 per group; *P < 0.05, ***P< 0.001 compared with indicated groups.

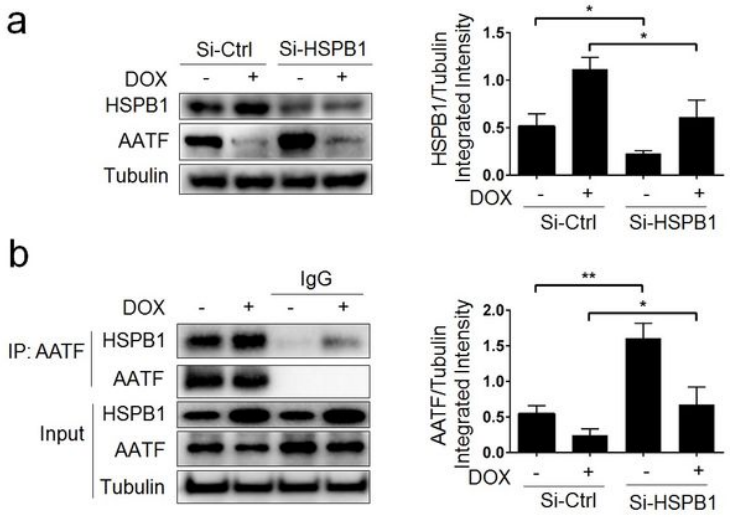

Figure 3

HSPB1 interacts with AATF in cardiomyocytes after DOX treatment. HL-1 cells were pretreated with Si-HSPB1 or NC for 48 h, followed DOX (5 μM) treatment for 9 h. a The protein levels of HSPB1 and AATF were examined by Western blot analysis. b The expression of HSPB1 and AATF were examined by Co-immunoprecipitation. N=3 per group; *P < 0.05, **P< 0.01 compared with indicated groups.

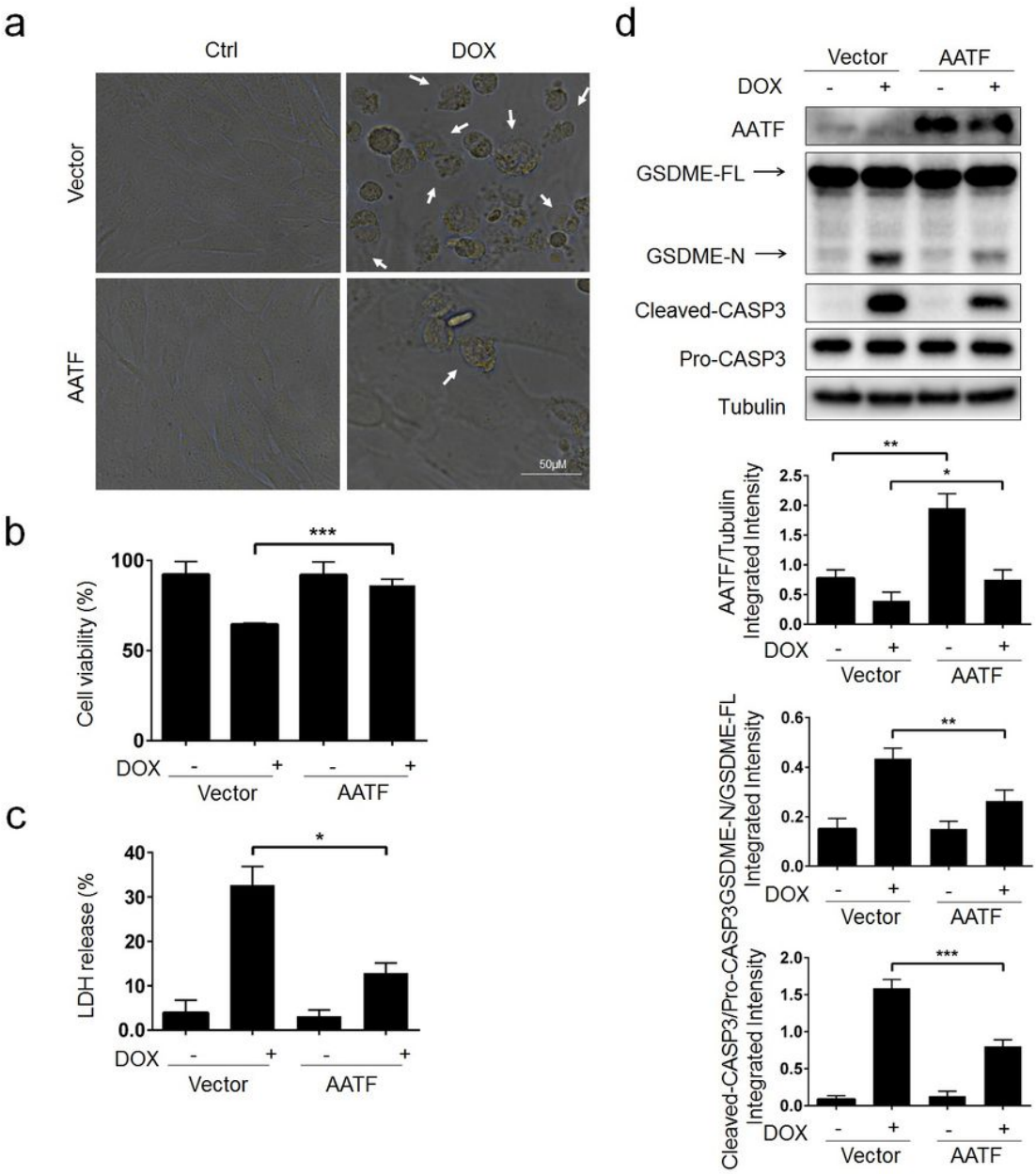

Figure 4

DOX-induced cardiomyocyte pyroptosis is through AATF. HL-1 cells were transfected with AATF plasmid (AATF) or empty vector for 48 h, followed DOX (5 μM) treatment for 9 h to assess: a Representative microscopic images. White arrowheads indicate pyroptotic cells. b Cell viability. c The LDH content. d The protein levels AATF, GSDME-N, Cleaved-CASP3 were examined by Western blot analysis. N=3 per group; *P < 0.05, **P< 0.01, ***P< 0.001 compared with indicated groups.

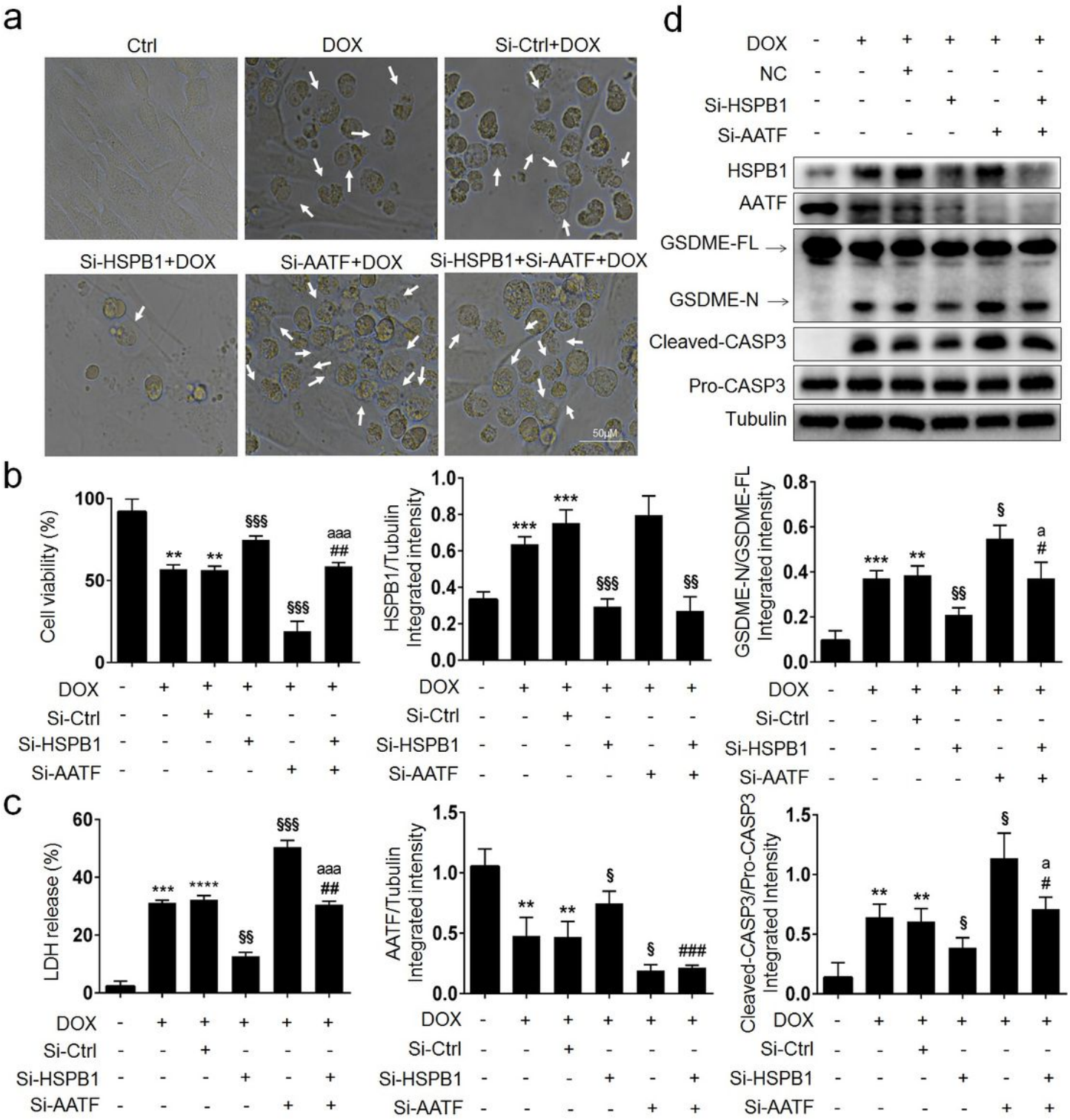

Figure 5

Inhibition of HSPB1 and AATF restores the DOX-induced cardiomyocyte pyroptosis. HL-1 cells were transfected with Si-HSPB1, Si-AATF (AATF siRNA) and NC for 48 h, followed DOX (5 μM) treatment for 9 h to examine: a Representative microscopic images. White arrowheads indicate pyroptotic cells. b Cell viability. c The level of LDH. D The expression of HSPB1, AATF, GSDME-N, Cleaved-CASP3 was examined by Western blot analysis. N=3 per group; **P< 0.01, ***P< 0.001, ****P< 0.0001 compared with control;

§P< 0.05, §§P< 0.01, §§§P< 0.001 compared with DOX+NC; ##P< 0.01, ###P< 0.001 compared with DOX+Si-HSPB1; aP < 0.05, aaaP < 0.001 compared with DOX+Si-AATF.