European Journal of Immunology

Immunomodulation and immune therapies

Basic

Research Article

Nitrosonisoldipine is a selective inhibitor of inflammatory caspases and protects against pyroptosis and related septic shock

Qianyue Chen[*1], *Jiashuo Zheng*[*2], *Dingyu Wang*[*3], *Qiyao Liu*[1], *Lulu Kang*[1], *Xiang Gao*[**1] and *Zhaoyu Lin*[**1]

[1] MOE Key Laboratory of Model Animals for Disease Study, Model Animal Research Center, State Key Laboratory of Pharmaceutical Biotechnology, Nanjing Drum Tower Hospital, The Affiliated Hospital of Nanjing University Medical School, Nanjing University, Nanjing, China
[2] Helmholtz Zentrum München, Institute of Metabolism and Cell Death, Neuherberg, Germany
[3] Jiangsu GemPharmatech Co., Ltd., Nanjing, China

Pyroptosis is a type of acute cell death that mainly occurs in immune cells. It is characterized with robust release of inflammatory cytokines and has emerged to play a critical role in the pathogenesis of sepsis-associated immune disorders. In this study, we screened for pyroptotic inhibitors with the ultimate goal to benefit sepsis treatments. Accidentally, we identified that nitrosonisoldipine (NTS), a photodegradation product of calcium channel inhibitor nisoldipine, inhibits noncanonical pyroptosis. Using murine immortalized BM-derived macrophage and human THP-1 cell line, we further discovered that NTS not only inhibits noncanonical pyroptosis mediated by caspase-11 or caspase-4 but also canonical pyroptosis mediated by caspase-1. Mechanistically, NTS directly inhibits the enzyme activities of these inflammatory caspases, and these inhibitory effects persist despite extensive washout of the drug. By contrast, apoptosis mediated by caspase-3/-7 was not affected by NTS. Mice pretreated with NTS intraperitoneally displayed improved survival rate and extended survival time in LPS- and polymicrobe-induced septic models, respectively. In conclusion, NTS is a selective inhibitor of inflammatory caspases that blocks both the noncanonical and canonical pyroptotic pathways. It is safe for intraperitoneal administration and might be used as a prototype to develop drugs for sepsis treatments.

Keywords: caspases · inflammation · nitrosonisoldipine · pyroptosis · septic shock

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

Pyroptosis is a type of programmed lytic cell death that occurs in immune cells upon infection [1]. It is a key response of the innate immune system to pathogens and it conduces to the elimination of the infected cells and the initiation of inflammatory response [2]. However, overactivation of pyroptosis may be detrimental to the host and leads to septic shock and multiple organ failure, thus inhibition of pyroptosis is potentially therapeutic for septic patients [3].

Correspondence: Dr. Zhaoyu Lin
e-mail: Linzy@nju.edu.cn; Gaoxiang@nju.edu.cn

*These authors contributed equally to this work.
**Both the authors jointly supervised this work.

It has been well established that two pathways are involved in the activation of pyroptosis [4]. For the canonical inflammasome pathway, the activation of caspase-1 by various inflammasomes containing cytoplasmic sensors (e.g., NOD-like receptors) elicits the cleavage of gasdermin D (GSDMD), which subsequently executes pyroptosis [5–9]. Simultaneously, caspase-1 mediates the maturation of pro-inflammatory cytokines such as interleukin (IL)-1β and IL-18 [8]. For the noncanonical inflammasome pathway, murine caspase-11 and its human orthologs caspase-4 and -5 are responsible for the detection of intracellular LPS with the help of guanylate-binding proteins [10, 11]; activation of these caspases also leads to the cleavage of GSDMD [5, 12-16]. Furthermore, potassium influx during the noncanonical pyroptosis may trigger the canonical Nod-like receptor protein 3 (NLRP3) inflammasome and thereby the activation of caspase-1 and pro-inflammatory cytokines [13, 17, 18].

GSDMD is the key executor of pyroptosis [19, 20]. Structurally, GSDMD amino-terminal (GSDMD-NT) end and carboxy-terminal end are connected by a 43-AA linker, and GSDMD-NT cytotoxicity is autoinhibited by GSDMD carboxy-terminal end [5, 9, 20–22]. When GSDMD is cleaved, released GSDMD-NT translocates to the plasma membrane and oligomerizes, forming a pore with an inner diameter of 10–14 nm that causes cell swelling and the release of cytoplasmic proteins [20, 23-25].

Calcium influx is an early-stage pyroptotic event preceding plasma membrane rupture [26]. We previously showed that blocking this process with magnesium affects the function of GSDMD-NT [27]. When we screened for calcium channel inhibitors that can prevent pyroptosis and thereby be used for sepsis treatment, we accidentally found that a degradation product of nisoldipine, namely nitrosonisoldipine (NTS), exerts anti-pyroptotic effect. We further revealed that NTS inhibits pyroptosis by impeding the activation of caspase-1/-4/-11. However, NTS does not affect apoptosis mediated by caspase-3 and -7, indicating that NTS is a specific inhibitor of inflammatory caspases, which may be safe for sepsis therapy.

Results

Decomposed nisoldipine inhibits noncanonical pyroptosis in iBMDMs

A library containing 47 types of calcium channel inhibitors (Supporting Information Table S1) was used to screen for inhibitors of noncanonical pyroptosis in immortalized BM-derived macrophage (iBMDM). Cell survival rate was determined by lactate dehydrogenase (LDH) measurements. To our surprise, among all 47 drugs only one, namely nisoldipine (#) (it is labeled with (#) because later it was found decomposed), conferred fully protection against pyroptosis. Nisoldipine is a calcium channel blocker of the 1,4-dihydropyridine (1,4-DHP) class, thus we rechecked all the 1,4-DHP drugs in the library and confirmed the special effect of nisoldipine (#) on pyroptosis (Fig. 1A). Nonetheless, when we tried to further investigate the underlying mechanisms, we found that nisoldipine purchased from another company (APExBIO) had no effect on pyroptosis (Fig. 1B). Morphological observation indicated a consistent result with the LDH measurements (Fig. 1C).

Nisoldipine is characterized with high photosensitivity and is known as the most unstable drug among 1,4-DHP derivatives [28, 29]. Under daylight illumination, nisoldipine can rapidly decompose into its nitrosophenylpyridine analog (i.e., NTS) (Fig. 1D) [30]. Hence, we speculated that the nisoldipine (#) from the library might be decomposed. To test our hypothesis, nisoldipine purchased from APExBIO was illuminated under artificial daylight for hours. As the light illuminates, the color of the solution fades (Supporting Information Fig. S1), which suggests photodegradation of nisoldipine. The products were then used to treat iBMDMs electroporated with LPS. Importantly, nisoldipine illuminated for only 1 h displayed significant inhibitory effect on pyroptosis, and the effect became stronger as nisoldipine was illuminated for longer hours (Fig. 1E). In addition, our mass spectrometry analysis confirmed that the main component of nisoldipine (#) is bona fide NTS (Fig. 1F).

NTS inhibits noncanonical pyroptosis in iBMDMs

We next performed MS analysis on the photodegradation products of nisoldipine. As the total ion chromatogram showed, the peak of nisoldipine decreased while the peak of NTS increased along with the illumination (Fig. 2A). Nevertheless, extracted ion chromatograms indicated that other degradation product including nitrophenylpyridine (NTR) was also generated, albeit its intensity was much weaker relative to NTS (10^4 vs. 10^6) (Fig. 2B and C). In order to clarify which degradation product inhibits pyroptosis, commercial pure NTS and NTR were used for treatment. It turned out that NTS, but not NTR, prevented noncanonical pyroptosis in iBMDMs, as indicated by LDH measurement and propidium iodide (PI) staining (Fig. 2D and E).

NTS blocks caspase-4/-11 activation in noncanonical pyroptosis

We next analyzed the underlying mechanism of NTS. First, we explored whether NTS affects the protein expression of pro-caspase-11 and the cleavage of GSDMD in iBMDMs (Fig. 3A). Intriguingly, cells treated with NTS showed a higher level of pro-caspase-11 than cells treated with DMSO. However, it is unlikely that NTS boosts the transcription of pro-Casp-11 in the process of pyroptosis, since NTS exerts a protective effect against pyroptosis. Instead, it is more likely a feedback of that NTS blocks the activation of caspase-11. Furthermore, GSDMD cleavage was inhibited by NTS. These results suggested that NTS acts upstream of GSDMD cleavage.

Since noncanonical pyroptosis is mediated by caspase-4 in human cell lines, we also examined the effect of NTS in human THP-1 cells. Similar to iBMDMs, THP-1 cells electroporated with LPS were largely rescued by NTS but not NTR treatment (Fig. 3B,

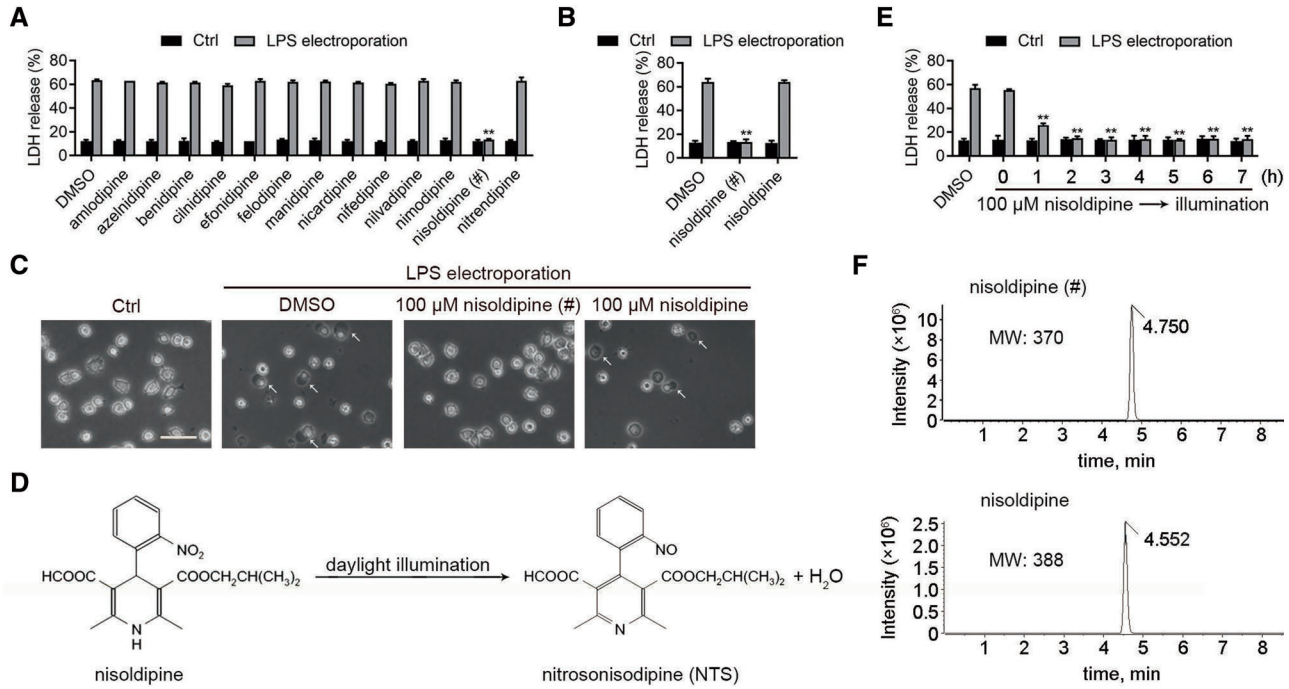

Figure 1. Decomposed nisoldipine inhibits noncanonical pyroptosis in iBMDMs. **(A)** LPS-primed iBMDMs were electroporated with (or without) 50 ng·mL^{-1} LPS for 1 h and LDH release was measured using a nonradioactive cytotoxicity assay kit. Cells electroporated without LPS were used as controls (Ctrl). DMSO and 0.1 mM calcium channel inhibitors were added 30 min before the electroporation. Nisoldipine labeled with "(#)" was decomposed. **(B)** LDH release from iBMDMs treated as in (A), as measured with a nonradioactive cytotoxicity assay kit. Nisoldipine without the label "(#)" was in undecomposed form. **(C)** Representative brightfield cell images of (B). Pyroptotic cells were indicated with white arrows. Bar = 20 μm. **(D)** Scheme of NTS formation in the process of nisoldipine photodegradation under daylight exposure. **(E)** LDH release from iBMDMs treated as in (A), as measured with a nonradioactive cytotoxicity assay kit. For artificial daylight illumination, nisoldipine was exposed under a 200 W Philips tungsten lamp for indicated hours. **(F)** Representative extracted ion chromatograms of nisoldipine (#) and nisoldipine as measured by MS. Data are representative of at least three independent experiments ($n = 3$). For panels A, B, and E, data are presented as mean ± SD; $^{**}p < 0.01$, as compared to iBMDMs electroporated with LPS and treated with DMSO by Student's *t* test.

Supporting Information Fig. S2). We further confirmed that caspase-4 enzyme activity was inhibited and GSDMD cleavage was blocked by NTS treatment (Fig. 3C). To clarify whether NTS targets caspase-4 or LPS, caspase-4 and GSDMD were overexpressed in 293T cells whereby pyroptosis could be induced in the absence of LPS [31]. In this case, NTS still conferred protection against pyroptosis and blocked GSDMD cleavage (Fig. 3D and E), suggesting that NTS targets caspase-4 rather than LPS. Taken together, these results demonstrate that NTS blocks noncanonical pyroptosis via inhibition of caspase-4/-11 activation.

NTS blocks caspase-1 activation in canonical pyroptosis

We next asked whether NTS could inhibit canonical pyroptosis. Surprisingly, NTS exhibited similar inhibitory effect on canonical pyroptosis in iBMDMs, no matter it was triggered by nigericin or ATP (Fig. 4A, Supporting Information Fig. S3A). However, the detection of unaffected ASC specks in the presence of NTS ruled out the possibility that NTS impeded the formation of NLRP3 inflammasome (Fig. 4B). Instead, caspase-1 activation and GSDMD cleavage (Fig. 4C), along with IL-1β release (Supporting Information Fig. S3B), were blocked by NTS. Furthermore, NTS also inhibited nigericin-induced canonical pyroptosis in THP-1 cells (Supporting Information Fig. S4A and 4B). Collectively, these data indicated that NTS blocks caspase-1 activation in canonical pyroptosis.

NTS does not affect apoptosis

The above data suggested that NTS might be a pan-caspase inhibitor, thus we used NTS to treat HeLa cells stimulated with TNF-α and cycloheximide (CHX). Morphological observation and flow cytometry analysis suggested that NTS has no effect on apoptosis (Fig. 5A-C, Supporting Information Fig. S5), and neither does it affect caspase-3/-7 enzyme activity (Fig. 5D). These data excluded the effect of NTS on apoptosis.

NTS directly and irreversibly inhibits the enzyme activities of inflammatory caspases

To clarify whether the inhibitory effect of NTS on inflammatory caspases is direct or indirect, recombinant human

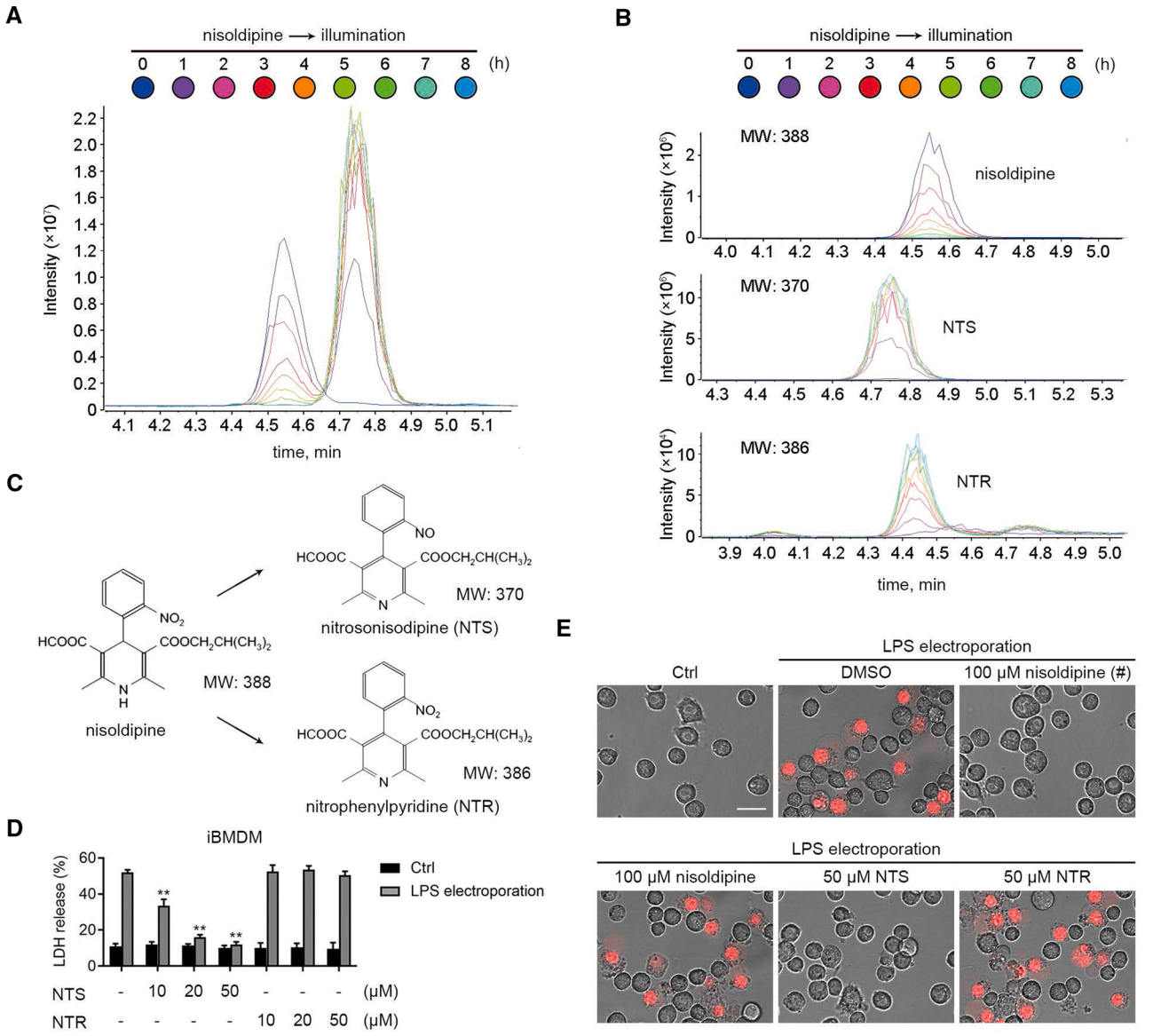

Figure 2. NTS inhibits noncanonical pyroptosis in iBMDMs. **(A)** Representative total ion chromatograms of nisoldipine illuminated under a 200 W Philips tungsten lamp for indicated hours, as measured by MS. **(B)** Representative extracted ion chromatograms from (A). MW, molecular weight. **(C)** Scheme of the photodegradation products of nisoldipine. **(D)** LPS-primed iBMDMs were electroporated with (or without) 50 ng·mL^{-1} LPS for 1 h and LDH release was measured with a nonradioactive cytotoxicity assay kit. Cells electroporated without LPS were used as controls (Ctrl). Drugs were added 30 min before the electroporation. **(E)** Representative images of PI (red) uptake of iBMDMs treated as in (D). Nisoldipine with the label "(#)" was decomposed. Bar = 20 μm. Data are representative of at least three independent experiments ($n = 3$). For panel D, data are presented as mean ± SD; $^{**}p < 0.01$, as compared to iBMDMs electroporated with LPS and treated with DMSO by Student's *t*-test.

caspase-1 and -4 were incubated with their respective substrates in the presence of 10 μM NTS (a higher concentration of NTS was avoided due to the significant impact on absorbance), and recombinant human caspase-3 was used as a control (Fig. 6A). Importantly, the enzyme activities of caspase-1, -4, but not -3 were markedly reduced by NTS treatment. Furthermore, washout experiments performed in iBMDMs triggered with canonical or noncanonical pyroptosis clearly showed that the inhibitory effect of NTS on inflammatory caspases was irreversible (Fig. 6B), suggesting NTS as a covalent inhibitor.

NTS is protective in LPS- and polymicrobe-induced sepsis

We then studied the safety of NTS in vivo. Fifteen male C57BL/6 mice at 6–8 weeks of age were divided into three groups with five mice each. Mice in each group were injected with 4.44, 14.8, and 44.4 mg/kg NTS, respectively, and their body weight and viability were monitored every day. All the mice were healthy after the injection and there were no significant changes in their body weight during the 1-week observation (Supporting Information Fig. S6).

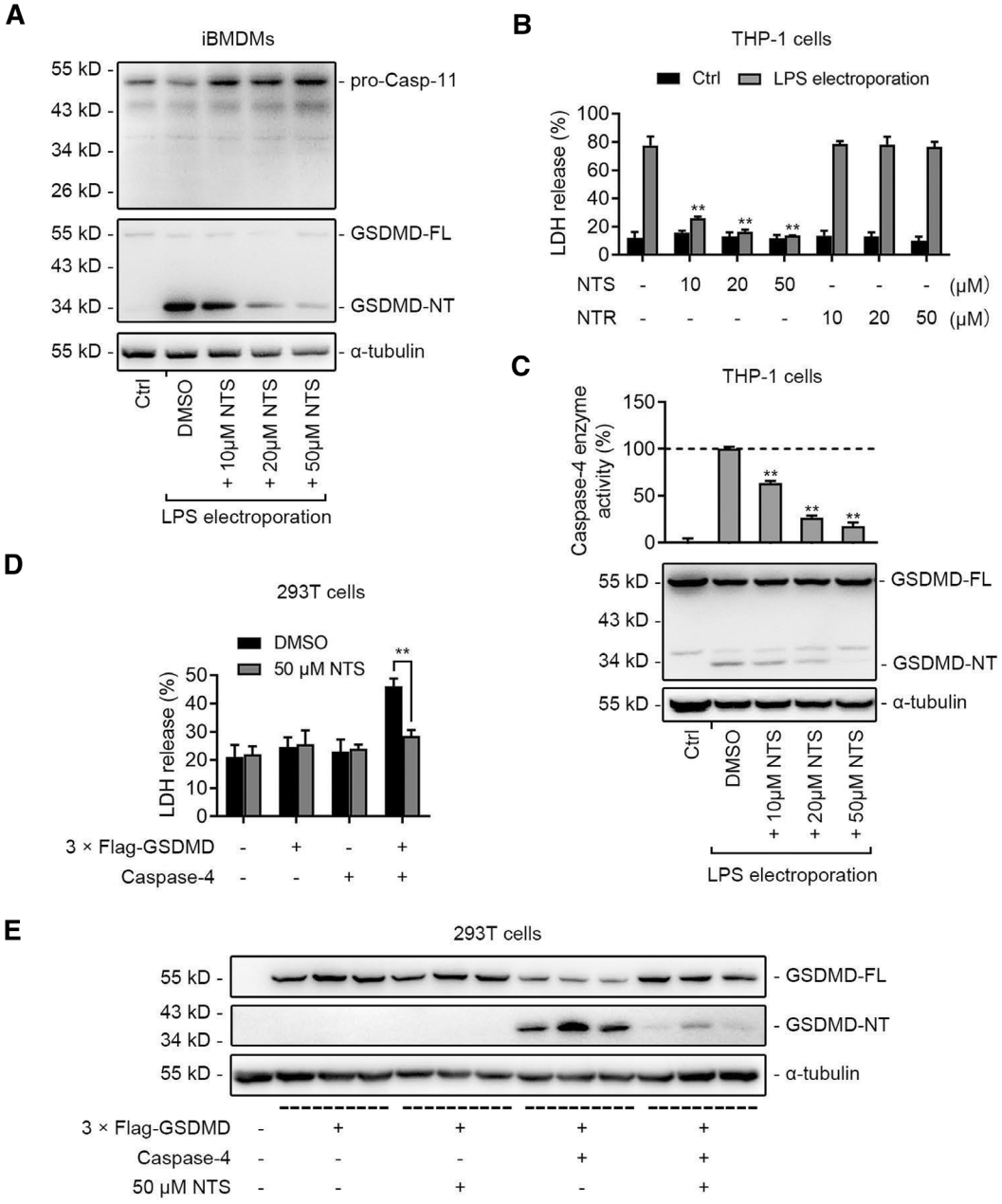

Figure 3. NTS blocks caspase-4/-11 activation in noncanonical pyroptosis. **(A)** LPS-primed iBMDMs were electroporated with (or without) 50 ng·mL^{-1} LPS for 1 h. Cells electroporated without LPS were used as controls (Ctrl). Drugs were added 30 min before the electroporation. Immunoblots for pro-caspase-11 (pro-Casp-11) and full-length GSDMD (GSDMD-FL) and its N-terminal (GSDMD-NT) are shown. α-Tubulin is a loading control. Raw data are showed in Supporting Information Fig. S7A. **(B)** LDH release from non-primed THP-1 cells treated as in (A) as measured with a nonradioactive cytotoxicity assay kit. **(C)** Caspase-4 enzyme activity and immunoblots for GSDMD-FL and GSDMD-NT in nonprimed THP-1 cells treated as in (A). α-Tubulin is a loading control. Raw data are showed in Supporting Information Fig. S7B. **(D)** LDH release from 293T cells transfected with indicated cDNA and cultured in the absence or presence of 50 μM NTS for 16 h, as measured with a nonradioactive cytotoxicity assay kit. $^{**}p < 0.01$, by Student's *t* test. **(E)** Immunoblots for Flag in 293T cells treated as in (D). α-Tubulin is a loading control. Raw data are shown in Supporting Information Fig. S7C. Data are representative of at least three independent experiments ($n = 3$). For panels B, C, and D, data are presented as mean ± SD. $^{**}p < 0.01$, as analyzed by Student's *t* test. For panels B and C, THP-1 cells electroporated with LPS and treated with DMSO were used to compare.

We next examined whether NTS protects against LPS-induced septic shock. While mice treated with 0.9% NaCl (LPS group) or low-dose NTS all succumbed within 36 h, 20–30% mice survived over 72 h in medium- and high-dose NTS groups (Fig. 7A). Consistent with the improved survival, mice treated with NTS showed markedly reduced amount of plasma IL-1β, IL-6, and TNF-α at 12 h after LPS challenge (Fig. 7B), associated with alleviated lung injury (Fig. 7C).

To mimic the human septic condition, mice were challenged with 1.5 g/kg cecal contents collected from the healthy

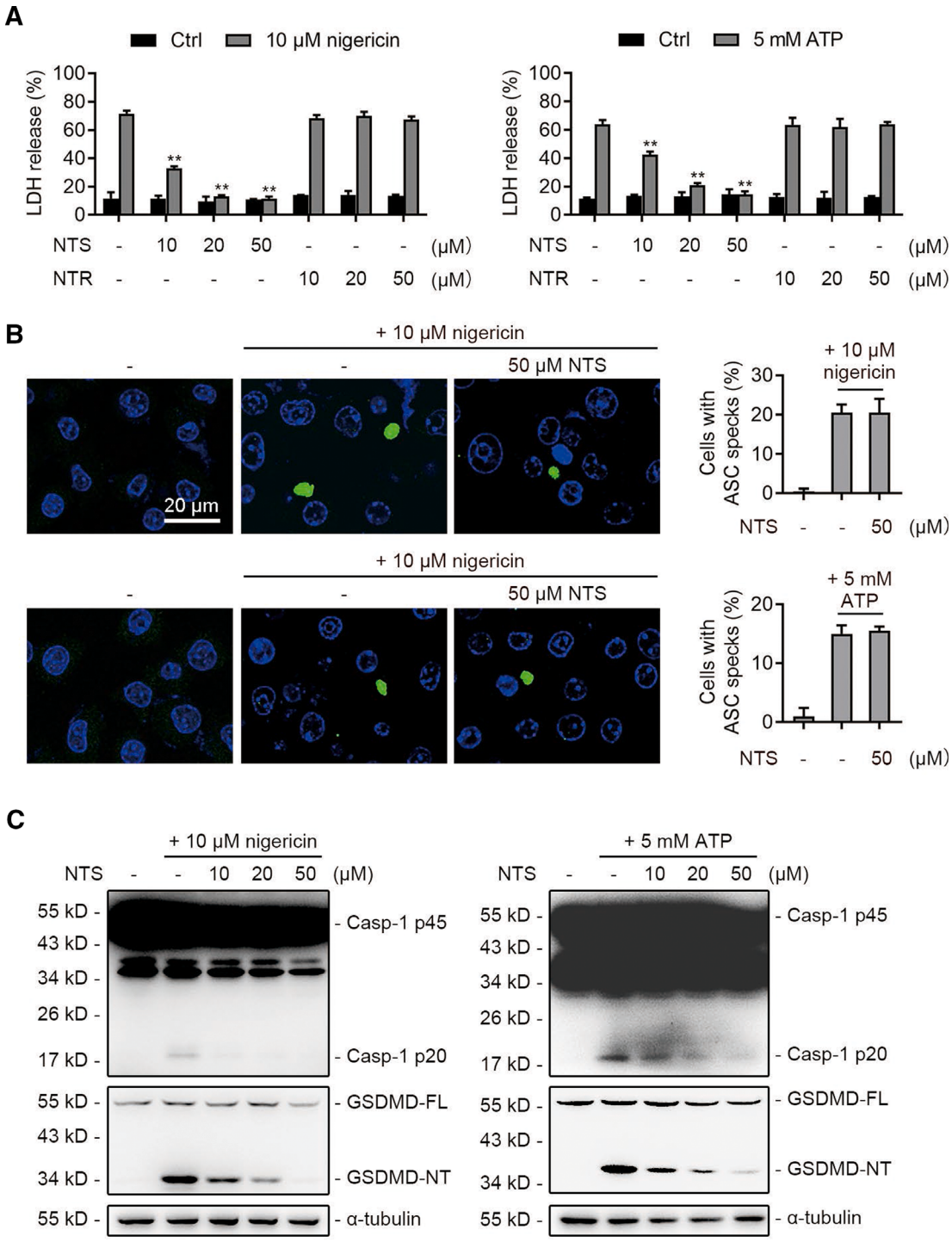

Figure 4. NTS blocks caspase-1 activation in canonical pyroptosis. **(A)** LPS-primed iBMDMs were treated with (or without) 10 μM nigericin or 5 mM ATP for 1 h and LDH release was measured using a nonradioactive cytotoxicity assay kit. Cells treated without nigericin and ATP were used as controls (Ctrl). Drugs were added 30 min before the treatment. **(B)** Representative confocal immunofluorescence images of endogenous ASC specks (green) and DAPI (blue) in cells treated as in (A). Bar = 20 μm. Quantification of ASC specks is also presented. **(C)** Immunoblots for pro-caspase-1 (Casp-1 p45) and its cleavage product (Casp-1 p20), GSDMD-FL, and GSDMD-NT in cells treated as in (A). α-Tubulin is a loading control. Raw data are shown in Supporting Information Fig. S7D and E. Data are representative of at least three independent experiments ($n = 3$). Data are presented as mean ± SD. For panel A, $^{**}p < 0.01$, as compared to cells treated with nigericin or ATP but without NTS or NTR by Student's *t* test.

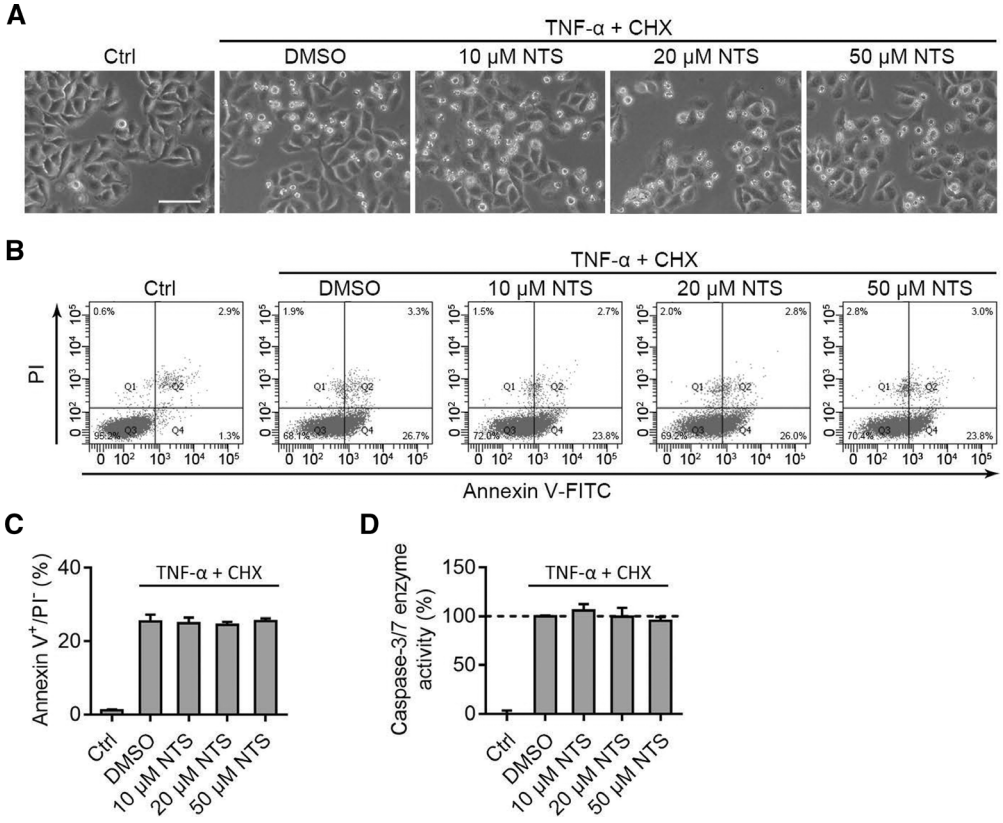

Figure 5. NTS does not affect caspase-3/-7-mediated apoptosis in HeLa cells. **(A)** Representative brightfield cell images of HeLa cells stimulated with TNF-α plus CHX for 8 h. Cells treated without TNF-α and CHX were used as controls (Ctrl). Drugs were added 30 min before the stimulation. Bar = 20 μm. **(B)** Representative flow cytometry plots of cells treated as in (A). **(C)** Quantification of apoptotic HeLa cells (annexin V^+PI^-) in (B). **(D)** Caspase-3/-7 enzyme activity in cells treated as in (A) measured by a caspase-3/-7 assay kit. Data are representative of at least three independent experiments ($n = 3$).

mice. Although all mice in this study succumbed within 36 h, mice treated with high-dose NTS exhibited longer survival time (Fig. 7D). The amount of plasma IL-1β at 12 h after challenge was also markedly reduced by NTS treatment, albeit IL-6 and TNF-α levels were largely unaffected (Fig. 7E). In summary, NTS is a safe drug that displays certain therapeutic effects on septic mouse models.

Discussion

In this study, we identified NTS, a photodegradation product of nisoldipine, as an inflammatory caspase inhibitor through the screening of 47 types of calcium channel inhibitor. Although this discovery seems to be quite fortuitous, it is associated with the high photosensitivity of nisoldipine. It has been reported that among 11 types of 1,4-DHPs, nisoldipine is the most unstable one under light exposure [28]. Another study on the thermal stability of seven types of 1,4-DHPs also indicated that the decomposition of nisoldipine is the fastest [29]. As such, it appears that our identification of the photodegradation product of nisoldipine among 47 types of calcium channel inhibitor was not purely accidental. In addition to NTS, NTR is also a photodegradation product of nisoldipine, which is mainly generated upon UV exposure [30, 32, 33]. The only structural difference between NTS and NTR is that the aromatic nitro group in NTR is replaced by the nitroso group in NTS. The fact that NTS but not NTR inhibits pyroptosis indicated that the aromatic nitroso group might be essential for NTS to act as an inflammatory caspase inhibitor. Of note, nifedipine shares similar structure with nisoldipine and both of them belong to 4-(2-nitrophenyl)-1,4-DHPs [29]. Like nisoldipine, nifedipine is thermally unstable and light sensitive [28, 29], and it generates nitrosonifedipine upon light exposure [34]. It has been reported that nitrosonifedipine had antioxidant effects and conferred protection against aortic aneurysm formation [35–37], thus nitrosonifedipine might be a potential inflammatory caspase inhibitor worth further study.

Our data revealed that NTS inhibits pyroptosis by specifically blocking the enzyme activities of inflammatory caspases (i.e., caspase-1/-4/-11). Although caspase inhibition has long been viewed as a promising strategy to mitigate inflammation and some caspases inhibitors did show some therapeutic efficiency in animal models of human diseases, no drug based on caspase inhibition has been approved on the market so far [38]. This is because most synthetic caspase inhibitors, especially the peptide inhibitors, are metabolically unstable and might cause nonspecific

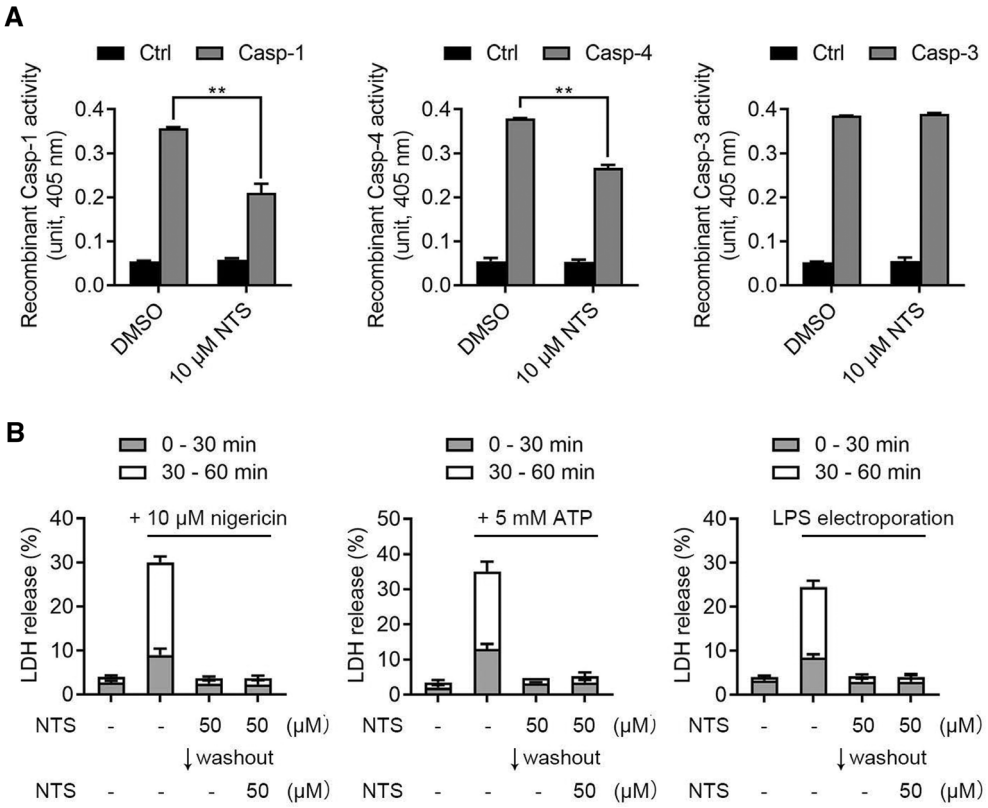

Figure 6. NTS directly and irreversibly inhibits the enzyme activities of inflammatory caspases. **(A)** Enzyme activities of recombinant human caspase (Casp)-1, -4, and -3 in the absence or presence of 10 μM NTS as determined by absorbance at 405 nm after incubation with their respective substrates, namely Ac-YVAD-*p*NA, Ac-LEVD-*p*NA, and Ac-DEVD-*p*NA. Substrates treated without caspases were used as control (Ctrl). **(B)** LPS-primed iBMDMs were treated with (or without) 10 μM nigericin or 5 mM ATP, or electroporated with (or without) 50 ng·mL^{-1} LPS. After 30 min, cells were washed twice with PBS and NTS was removed as indicated. LDH release was measured at 30 min and 1 h, respectively, using a nonradioactive cytotoxicity assay kit. Drugs were added 30 min before the stimulation. Data are representative of three independent experiments ($n = 3$). Data are presented as mean ± SD. For panel A, $^{**}p < 0.01$, as analyzed by Student's *t* test.

toxic effects [39]. By contrast, NTS is one of the metabolites of nisoldipine, an FDA-approved drug that has been widely used in clinic, which suggests that NTS is relatively safer than those inhibitors directly screened out from compound libraries. Furthermore, the characteristic that NTS only targets inflammatory caspases makes it a much more desirable drug for inflammation inhibition. Indeed, pan-caspase inhibitors like zVAD-fmk have repeatedly been shown to trigger alternative cell death pathways like necroptosis and autophagic cell death, which may function as backup cell death programs for apoptosis [40]. Notably, a recent study reported disulfiram as a potentially therapeutic drug to counteract inflammation by abrogating the pore formation of GSDMD-NT [41]. Compared with disulfiram, NTS not only inhibits pyroptosis but also IL-1β processing, thus is expected to exert a better anti-inflammatory effect.

The anti-inflammatory effect of NTS was revealed in LPS and polymicrobial septic mice models. Of note, mice treated with medium- and high-dose NTS did not display significant difference in the level of inflammatory cytokines after LPS challenge, and the survival in the former group was even higher than the latter (30% vs. 20%), suggesting that treatment with a higher dose of NTS does not necessarily lead to a better outcome. In the polymicrobial septic model, NTS treatment only extends the survival time but not survival rate, probably due to the following factors. First, the polymicrobe model was much more severe than the LPS model as indicated by higher levels of plasma IL-1β, IL-6, and TNF-α. Second, NTS is not known as a bactericidal drug, thus bacterial reproduction in the polymicrobe model might be out of control. Third, noncanonical pyroptosis plays a pivotal role in LPS model and it has been reported that caspase-11 KO mice were resistant to LPS challenge [14, 15]. By contrast, the polymicrobe model is more complicated and may involve various pro-inflammatory pathways. Nevertheless, the reduced plasma IL-1β level suggested that NTS still had some anti-inflammatory effects on polymicrobial septic model, presumably by blocking caspase-1 activation.

In conclusion, we identified NTS, a photodegradation product of nisoldipine, as a selective inhibitor of inflammatory caspases (i.e., caspase-1/-4/-11), which directly and irreversibly inhibits the enzyme activities of these caspases. NTS confers protection against pyroptosis induced by either the canonical or noncanonical inflammasome pathway, but it does not affect apoptosis mediated by caspase-3/-7. Furthermore, NTS is safe for intraperitoneally injection and pretreatment with NTS confers protection against sepsis in mice.

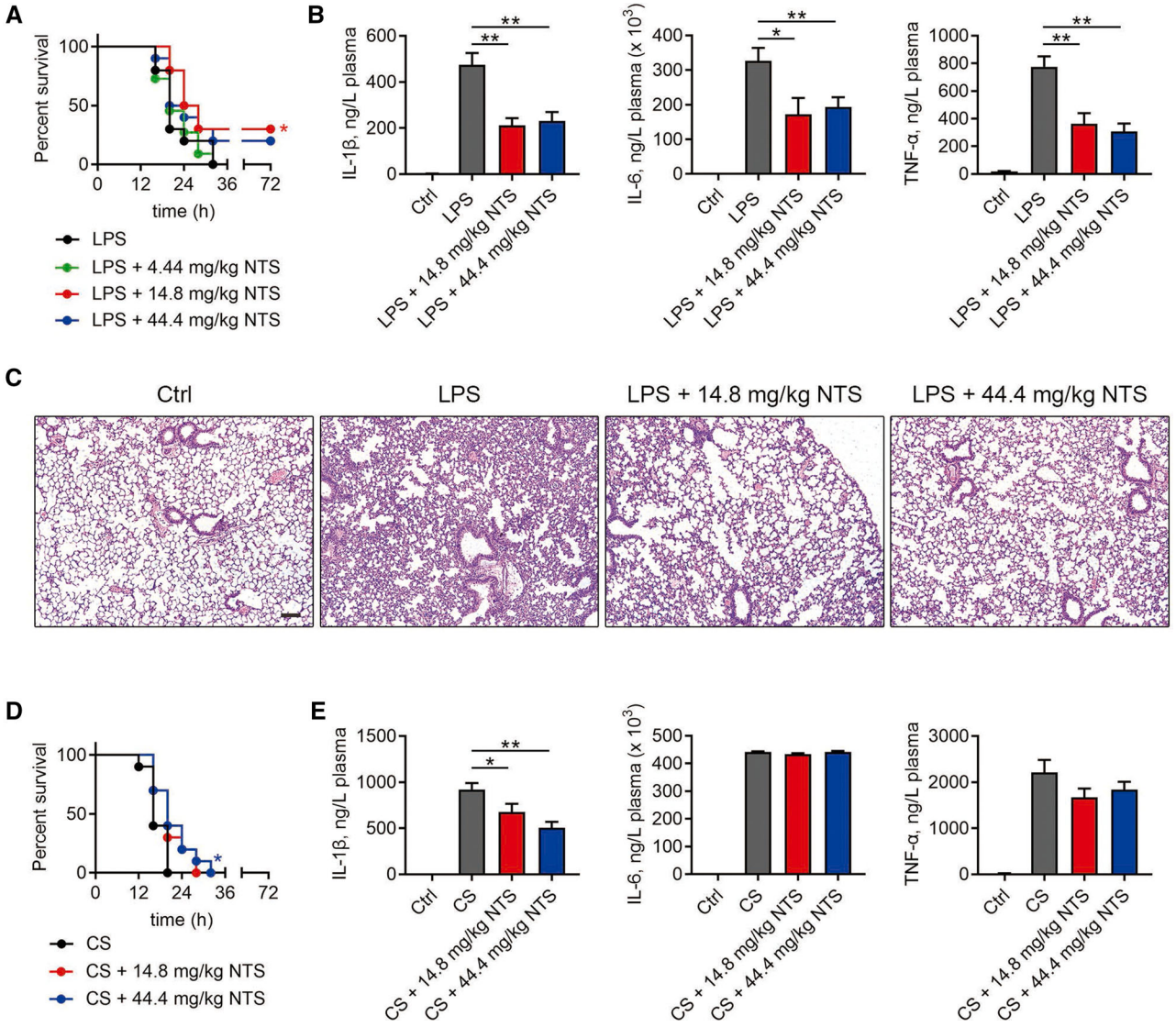

Figure 7. NTS is protective in LPS- and polymicrobe-induced sepsis. **(A)** Survival of mice challenged with 20 mg/kg *E. coli* (Escherichia) O111:B4 LPS intraperitoneally. NTS of indicated doses (or DMSO) was given 0.5 h before the challenge. N = 10 mice per group. **(B)** IL-1β, IL-6, and TNF-α levels in mouse plasma measured by ELISA at 12 h after challenge as in (A). Mice challenged with 0.9% NaCl were used as controls (Ctrl). N = 6 mice per group. **(C)** Representative H&E-stained lung sections obtained from mice at 12 h after challenge as in (B). Bar = 100 μm. N = 6 mice per group. **(D)** Survival of mice challenged with 1.5 g/kg cecal slurry intraperitoneally. NTS of indicated doses (or DMSO) was given 0.5 h before the challenge. N = 10 mice per group. **(E)** IL-1β, IL-6, and TNF-α levels in mouse plasma measured by ELISA at 12 h after challenge as in (D). Mice challenged with 0.9% NaCl were used as controls (Ctrl). N = 6 mice per group. Data are representative of two independent experiments. For panels A and D, $^{*}p < 0.05$, as compared to LPS or CS group by log-rank (Mantel–Cox) test. For panels B and E, data are presented as mean ± SD; $^{*}p < 0.05$, $^{**}p < 0.01$, as analyzed by Student's *t* test. CS, cecal slurry.

Materials and methods

Cell culture

Murine iBMDMs kindly provided by Dr. F. Shao (National Institute of Biological Sciences, China) and human THP-1 cells obtained from Shanghai Institute of Cell Biology (Shanghai, China) were grown in RPMI 1640 medium. HeLa cells and 293T cells obtained from ATCC were grown in DMEM. Media were supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells of passages fewer than 15 were used for experiments. All cells were cultured at 37°C and 5% CO_2.

Reagents

The calcium channel inhibitor library containing 47 types of drugs was purchased from MedChemExpress (Compound Library Layout: Rack SA00503815). The "nisoldipine" in the library was demonstrated decomposed, thus was labeled with "(#)". Nisoldipine (without label) was purchased from APExBIO (Cat No.

B1989). NTS (CAS No. 87375-91-5) and NTR (CAS No. 103026-83-1) were purchased from MedChemExpress. All reagents were dissolved in DMSO.

MS analysis

MS in positive ion mode was carried out from *m/z* 100–1250 on the 4600 LC-MS/MS system (AB SCIEX TripleTOF™) in State Key Laboratory of Pharmaceutical Biotechnology affiliated to Nanjing University.

Plasmid and transfection

GSDMD and caspase-4 cDNAs generated from THP-1 cells were inserted into the *Not*I and *Xba*I sites of pCS2 vectors with or without an N-terminal 3×Flag epitope tag, respectively. Plasmids were verified by DNA sequencing. Transfection was performed in Opti-MEM using Lipofectamine 2000 Transfection Reagent (Cat No. 11668027; Invitrogen).

Stimulation of noncanonical pyroptosis, canonical pyroptosis, and apoptosis

To stimulate noncanonical pyroptosis, iBMDMs primed with LPS overnight or nonprimed THP-1 cells were electroporated with 50 ng·mL^{-1} LPS using the Neon Transfection System (Invitrogen). To stimulate canonical pyroptosis, iBMDMs or THP-1 cells were primed with 1 μg·mL^{-1} LPS for 4 h followed by 5 mM ATP or 10 μM nigericin treatment for 1 h. To stimulate apoptosis, HeLa cells were treated with 10 ng·mL^{-1} TNF-α plus 10 μg·mL^{-1} CHX for 8 h.

Measurement of LDH release, caspase enzyme activities, and IL-1β

A CytoTox 96 Non-Radioactive Cytotoxicity Assay Kit (Cat No. G178; Promega) was used for measurement of LDH release. Caspase-4 (Cat No. ab65659; Abcam) and caspase-3/-7 (Cat No. ab270771; Abcam) Assay kits were used for measurement of cytosolic caspase enzyme activities according to the manufacturer's protocol. The enzyme activities of recombinant human caspase-1 (Cat No. CC126; Sigma-Aldrich), caspase-3 (Cat No. CC119; Sigma-Aldrich), and caspase-4 (Cat No. ab51994; Abcam) were measured after incubation with their respective substrates Ac-YVAD-*p*NA, Ac-DEVD-*p*NA, and Ac-LEVD-*p*NA (Cat No. P9701, P9710, and P9714; Beyotime Biotechnology) at 37°C for 1 h in a reaction buffer containing 50 mM Hepes, pH 7.2, 50 mM NaCl, 0.1% Chaps, 10 mM EDTA, 5% glycerol, and 10 mM DTT. The amount of IL-1β was measured by ELISA (Cat No. MLB00C; R&D Systems) according to the manufacturer's protocol. Data were collected using microtiter plate readers. LDH values represent the percentage of LDH release by a maximum lysis control (1% Triton X-100-lysed cells).

Live-cell imaging

To observe pyroptotic or apoptotic morphology, cells were treated as indicated in the six-well plates. Static bright field images were captured using an Olympus IX71 microscope. For PI staining, cells electroporated with or without LPS were treated as indicated with 1 μg·mL^{-1} PI dye addition. Images were captured 1 h later using a confocal microscope (FV-1000; Olympus).

Immunostaining

Cells grown on coverslips were first fixed in PBS with 4% paraformaldehyde for 15 min, and then permeabilized in 0.1% Triton X-100 in PBS for 15 min. After blocking with 5% BSA for 1 h, cells were stained with ASC antibody (ab175449; Abcam) and then Alexa Fluor 488-conjugated secondary antibody. Nuclei were counterstained with DAPI (Cell Signaling). Images were captured at room temperature using a confocal microscope (FV-1000; Olympus). For each sample, more than four images were taken and the percentage of ASC specks formation was counted manually.

Flow cytometry

Cells treated with TNF-α and CHX for 8 h were washed with ice-cold PBS twice and then stained with PI on ice for 5 min. Cellular debris was excluded with forward scatter and side scatter gating and PI signals were detected using flow cytometry (BD LSRFortessa; BD Biosciences). Data were analyzed using FlowJo software (FlowJo, LLC). PI dye was obtained from an Annexin V-FITC/PI Apoptosis Detection Kit (Cat No. A211-01; Vazyme).

Immunoblot

Total cell protein was extracted using RIPA lysis buffer (50 mmol·L^{-1} Tris-HCl [pH 7.4], 150 mmol·L^{-1} NaCl, 1% NP-40, 0.1 mmol·L^{-1} EDTA, 1 mmol·L^{-1} DTT with 0.4 mmol·L^{-1} PMSF, 0.1 mmol·L^{-1} Na_3VO_4, 0.1 mmol·L^{-1} NaF, and protease inhibitor cocktail). Protein concentrations were examined using a Bradford Protein Assay Kit (Cat No. C503031; Sangon Biotech). Proteins were separated by ExpressPlus PAGE gels (Cat No. M42015C; GenScript) and then transferred to polyvinylidene difluoride membranes (Cat No. 29022566; GE Health Life Sciences). After blocking with 5% fat-free milk at room temperature for 1 h, blots were incubated with primary antibody overnight at 4°C. The next day, the blots were washed with TBST (0.1% Tween-20 in Tris buffered saline) and then incubated with secondary antibody for 1 h at room temperature. Proteins were visualized using a

High-sig ECL Western Blotting Substrate (Cat No. 180–501; Tanon). Primary antibodies used in this work included anti-caspase-11 (Cat No. NB120-10454; Novus Biologicals, 1:5000), anti-caspase-1 (Cat No. SC-515; Santa Cruz, 1:5000), anti-GSDMD (Cat No. ab209845; Abcam, 1:2000), anti-Flag (Cat No. F1804; Sigma-Aldrich, 1:5000), and anti-tubulin (Cat No. BS1699; Bioworld, 1:5000). Secondary antibodies anti-mouse IgG and anti-rabbit IgG were purchased from Sigma-Aldrich.

Animal studies

C57BL/6 mice were provided by the Model Animal Research Center (MARC) of Nanjing University (Nanjing, Jiangsu Province, China) and were housed in a specific pathogen-free facility accredited by Association for Assessment and Accreditation of Laboratory Animal Care International. Six- to 8-week-old male mice were challenged with 20 mg/kg *E. coli* O111:B4 LPS or 1.5 g/kg cecal contents intraperitoneally [42]. NTS of indicated doses was given 0.5 h before the challenge. For cytokine measurements, mice were euthanized at 12 h after the challenge, and whole blood collected by cardiac puncture using the EDTA-2K tubes was centrifuged to provide plasma. The amount of IL-1β, IL-6, and TNF-α was measured by ELISA (Cat No. MLB00C, M6000B, and MTA00B; R&D Systems) according to the manufacturer's protocol. For H&E staining, lung tissue collected at 12 h after the challenge was embedded in paraffin and sectioned, and staining was performed according to the manufacturer's protocol (Cat#G1005; Servicebio).

Statistics

Data are shown as mean ± SD. Statistical analysis was performed using two-tailed Student's *t*-test or log-rank (Mantel–Cox) test for survival with GraphPad Prism 7 Software (GraphPad Software, Inc); $*p < 0.05$, $**p < 0.01$.

Acknowledgements: This work was supported by the Ministry of Science and Technology of China (2019YFC1711000 and 2018YFA0801100), the National Natural Science Foundation of China (31772550 and 31971056), the Natural Science Foundation of Jiangsu Province (BK20181260), the Fundamental Research Funds for the Central Universities (14380034), and the Collaborative Innovation of Industry, University, and Research Institute Major Program of Guangzhou (201604046020).

Author contributions: QC, JZ, and DW designed the research, performed the experiments, and analyzed the data; QC and JZ prepared the figures and wrote the manuscript; QL and LK assisted in performing experiments; ZL and XG supervised the research and edited the manuscript; and all authors read and approved the final manuscript.

Conflict of Interest: The authors declare no commercial or financial potential conflict of interest concerning the research, authorship, and/or publication of this article, except a patent that we have (Number: 201911293108.3).

Peer review: The peer review history for this article is available at https://publons.com/publon/10.1002/eji.202048937

Data availability statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

References

1 **Wallach, D.**, **Kang, T. B.**, **Dillon, C. P.** and **Green, D. R.**, Programmed necrosis in inflammation: toward identification of the effector molecules. *Science* 2016. **352**: aaf2154.

2 **Jorgensen, I.**, **Rayamajhi, M.** and **Miao, E. A.**, Programmed cell death as a defence against infection. *Nat. Rev. Immunol.* 2017. **17**: 151–164.

3 **Pfalzgraff, A.** and **Weindl, G.**, Intracellular lipopolysaccharide sensing as a potential therapeutic target for sepsis. *Trends Pharmacol. Sci.* 2019. **40**: 187–197.

4 **Broz, P.**, Immunology: caspase target drives pyroptosis. *Nature* 2015. **526**: 642–643.

5 **Shi, J.**, **Zhao, Y.**, **Wang, K.**, **Shi, X.**, **Wang, Y.**, **Huang, H.**, **Zhuang, Y.** et al., Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death. *Nature* 2015. **526**: 660–665.

6 **Miao, E. A.**, **Rajan, J. V.** and **Aderem, A.**, Caspase-1-induced pyroptotic cell death. *Immunol. Rev.* 2011. **243**: 206–214.

7 **Man, S. M.**, **Karki, R.** and **Kanneganti, T. D.**, Molecular mechanisms and functions of pyroptosis, inflammatory caspases and inflammasomes in infectious diseases. *Immunol. Rev.* 2017. **277**: 61–75.

8 **Lamkanfi, M.** and **Dixit, V. M.**, Mechanisms and functions of inflammasomes. *Cell* 2014. **157**: 1013–1022.

9 **Kayagaki, N.**, **Stowe, I. B.**, **Lee, B. L.**, **O'Rourke, K.**, **Anderson, K.**, **Warming, S.**, **Cuellar, T.** et al., Caspase-11 cleaves gasdermin D for non-canonical inflammasome signalling. *Nature* 2015. **526**: 666–671.

10 **Wandel, M. P.**, **Kim, B. H.**, **Park, E. S.**, **Boyle, K. B.**, **Nayak, K.**, **Lagrange, B.**, **Herod, A.** et al., Guanylate-binding proteins convert cytosolic bacteria into caspase-4 signaling platforms. *Nat. Immunol.* 2020. **21**: 880–891.

11 **Santos, J. C.**, **Boucher, D.**, **Schneider, L. K.**, **Demarco, B.**, **Dilucca, M.**, **Shkarina, K.**, **Heilig, R.** et al., Human GBP1 binds LPS to initiate assembly of a caspase-4 activating platform on cytosolic bacteria. *Nat. Commun.* 2020. **11**: 3276.

12 **Shi, J.**, **Zhao, Y.**, **Wang, Y.**, **Gao, W.**, **Ding, J.**, **Li, P.**, **Hu, L.** and **Shao, F.**, Inflammatory caspases are innate immune receptors for intracellular LPS. *Nature* 2014. **514**: 187–192.

13 **Kayagaki, N.**, **Warming, S.**, **Lamkanfi, M.**, **Vande Walle, L.**, **Louie, S.**, **Dong, J.**, **Newton, K.** et al., Non-canonical inflammasome activation targets caspase-11. *Nature* 2011. **479**: 117–121.

14 **Kayagaki, N.**, **Wong, M. T.**, **Stowe, I. B.**, **Ramani, S. R.**, **Gonzalez, L. C.**, **Akashi-Takamura, S.**, **Miyake, K.** et al., Noncanonical inflammasome

activation by intracellular LPS independent of TLR4. *Science* 2013. **341**: 1246–1249.

15 **Hagar, J. A.**, **Powell, D. A.**, **Aachoui, Y.**, **Ernst, R. K.** and **Miao, E. A.**, Cytoplasmic LPS activates caspase-11: implications in TLR4-independent endotoxic shock. *Science* 2013. **341**: 1250–1253.

16 **Baker, P. J.**, **Boucher, D.**, **Bierschenk, D.**, **Tebartz, C.**, **Whitney, P. G.**, **D'Silva, D. B.**, **Tanzer, M. C.** et al., NLRP3 inflammasome activation downstream of cytoplasmic LPS recognition by both caspase-4 and caspase-5. *Eur. J. Immunol.* 2015. **45**: 2918–2926.

17 **Ruhl, S.** and **Broz, P.**, Caspase-11 activates a canonical NLRP3 inflammasome by promoting K(+) efflux. *Eur. J. Immunol.* 2015. **45**: 2927–2936.

18 **Schmid-Burgk, J. L.**, **Gaidt, M. M.**, **Schmidt, T.**, **Ebert, T. S.**, **Bartok, E.** and **Hornung, V.**, Caspase-4 mediates non-canonical activation of the NLRP3 inflammasome in human myeloid cells. *Eur. J. Immunol.* 2015. **45**: 2911–2917.

19 **Aglietti, R. A.** and **Dueber, E. C.**, Recent insights into the molecular mechanisms underlying pyroptosis and gasdermin family functions. *Trends Immunol.* 2017. **38**: 261–271.

20 **Ding, J.**, **Wang, K.**, **Liu, W.**, **She, Y.**, **Sun, Q.**, **Shi, J.**, **Sun, H.** et al., Pore-forming activity and structural autoinhibition of the gasdermin family. *Nature* 2016. **535**: 111.

21 **Kuang, S.**, **Zheng, J.**, **Yang, H.**, **Li, S.**, **Duan, S.**, **Shen, Y.**, **Ji, C.** et al., Structure insight of GSDMD reveals the basis of GSDMD autoinhibition in cell pyroptosis. *Proc. Natl. Acad. Sci. USA* 2017. **114**: 10642–10647.

22 **Kovacs, S. B.** and **Miao, E. A.**, Gasdermins: effectors of pyroptosis. *Trends Cell Biol.* 2017. **27**: 673–684.

23 **Mulvihill, E.**, **Sborgi, L.**, **Mari, S. A.**, **Pfreundschuh, M.**, **Hiller, S.** and **Muller, D. J.**, Mechanism of membrane pore formation by human gasdermin-D. *EMBO J.* 2018. **37**:e98321.

24 **Liu, X.**, **Zhang, Z.**, **Ruan, J.**, **Pan, Y.**, **Magupalli, V. G.**, **Wu, H.** and **Lieberman, J.**, Inflammasome-activated gasdermin D causes pyroptosis by forming membrane pores. *Nature* 2016. **535**: 153–158.

25 **Aglietti, R. A.**, **Estevez, A.**, **Gupta, A.**, **Ramirez, M. G.**, **Liu, P. S.**, **Kayagaki, N.**, **Ciferri, C.** et al., GsdmD p30 elicited by caspase-11 during pyroptosis forms pores in membranes. *Proc. Natl. Acad. Sci. USA* 2016. **113**: 7858–7863.

26 **de Vasconcelos, N. M.**, **Van Opdenbosch, N.**, **Van Gorp, H.**, **Parthoens, E.** and **Lamkanfi, M.**, Single-cell analysis of pyroptosis dynamics reveals conserved GSDMD-mediated subcellular events that precede plasma membrane rupture. *Cell Death Differ.* 2019. **26**: 146–161.

27 **Wang, D.**, **Zheng, J.**, **Hu, Q.**, **Zhao, C.**, **Chen, Q.**, **Shi, P.**, **Chen, Q.** et al., Magnesium protects against sepsis by blocking gasdermin D N-terminal-induced pyroptosis. *Cell Death Differ.* 2020.**27**: 466–481.

28 **Baranda, A. B.**, **Alonso, R. M.**, **Jimenez, R. M.** and **Weinmann, W.**, Instability of calcium channel antagonists during sample preparation for LC-MS-MS analysis of serum samples. *Forensic Sci. Int.* 2006. **156**: 23–34.

29 **Marciniec, B.** and **Ogrodowczyk, M.**, Thermal stability of 1,4-dihydropyridine derivatives in solid state. *Acta Pol. Pharm.* 2006. **63**: 477–484.

30 **Marinkovic, V.**, **Agbaba, D.**, **Karljikovic-Rajic, K.**, **Comor, J.** and **Zivanov-Stakic, D.**, UV derivative spectrophotometric study of the photochemical degradation of nisoldipine. *Farmaco* 2000. **55**: 128–133.

31 **Chen, Q.**, **Shi, P.**, **Wang, Y.**, **Zou, D.**, **Wu, X.**, **Wang, D.**, **Hu, Q.** et al., GSDMB promotes non-canonical pyroptosis by enhancing caspase-4 activity. *J. Mol. Cell Biol.* 2019.**11**: 496–508.

32 **Alvarez-Lueje, A.**, **Naranjo, L.**, **Nunez-Vergara, L. J.** and **Squella, J. A.**, Electrochemical study of nisoldipine: analytical application in pharmaceutical forms and photodegradation. *J. Pharm. Biomed. Anal.* 1998. **16**: 853–862.

33 **Marinkovic, V. D.**, **Agbaba, D.**, **Karljikovic-Rajic, K.**, **Vladimirov, S.** and **Nedeljkovic, J. M.**, Photochemical degradation of solid-state nisoldipine monitored by HPLC. *J. Pharm. Biomed. Anal.* 2003. **32**: 929–935.

34 **Fasani, E.**, **Dondi, D.**, **Ricci, A.** and **Albini, A.**, Photochemistry of 4-(2-nitrophenyl)-1,4-dihydropyridines. Evidence for electron transfer and formation of an intermediate. *Photochem. Photobiol.* 2006. **82**: 225–230.

35 **Imanishi, M.**, **Izawa-Ishizawa, Y.**, **Sakurada, T.**, **Kohara, Y.**, **Horinouchi, Y.**, **Sairyo, E.**, **Zamami, Y.** et al., Nitrosonifedipine, a photodegradation product of nifedipine, suppresses pharmacologically induced aortic aneurysm formation. *Pharmacology* 2018. **102**: 287–299.

36 **Fukuhara, Y.**, **Tsuchiya, K.**, **Horinouchi, Y.**, **Tajima, S.**, **Kihira, Y.**, **Hamano, S.**, **Kawazoe, K.** et al., Protective effect of photodegradation product of nifedipine against tumor necrosis factor alpha-induced oxidative stress in human glomerular endothelial cells. *J. Med. Invest.* 2011. **58**: 118–126.

37 **Horinouchi, Y.**, **Tsuchiya, K.**, **Taoka, C.**, **Tajima, S.**, **Kihira, Y.**, **Matsuda, Y.**, **Shishido, K.** et al., Antioxidant effects of photodegradation product of nifedipine. *Chem. Pharm. Bull. (Tokyo)* 2011. **59**: 208–214.

38 **Kudelova, J.**, **Fleischmannova, J.**, **Adamova, E.** and **Matalova, E.**, Pharmacological caspase inhibitors: research towards therapeutic perspectives. *J. Physiol. Pharmacol.* 2015. **66**: 473–482.

39 **Callus, B. A.** and **Vaux, D. L.**, Caspase inhibitors: viral, cellular and chemical. *Cell Death Differ.* 2007. **14**: 73–78.

40 **Vandenabeele, P.**, **Vanden Berghe, T.** and **Festjens, N.**, Caspase inhibitors promote alternative cell death pathways. *Sci. STKE* 2006. **2006**: pe44.

41 **Hu, J. J.**, **Liu, X.**, **Xia, S.**, **Zhang, Z.**, **Zhang, Y.**, **Zhao, J.**, **Ruan, J.** et al., FDA-approved disulfiram inhibits pyroptosis by blocking gasdermin D pore formation. *Nat. Immunol.* 2020.**21**: 736–745.

42 **Wynn, J. L.**, **Scumpia, P. O.**, **Delano, M. J.**, **O'Malley, K. A.**, **Ungaro, R.**, **Abouhamze, A.** and **Moldawer, L. L.**, Increased mortality and altered immunity in neonatal sepsis produced by generalized peritonitis. *Shock* 2007. **28**: 675–683.

Abbreviations: **1,4-DHP**: 1,4-dihydropyridine · **GSDMD**: gasdermin D · **iBMDM**: immortalized BM-derived macrophage · **LDH**: lactate dehydrogenase · **NTR**: nitrophenylpyridine · **NTS**: nitrosonisoldipine

Full correspondence:: Dr. Xiang Gao and Dr. Zhaoyu Lin, MOE Key Laboratory of Model Animals for Disease Study, Model Animal Research Center, State Key Laboratory of Pharmaceutical Biotechnology, Nanjing Drum Tower Hospital, The Affiliated Hospital of Nanjing University Medical School, Nanjing University, Nanjing 210061, China.
e-mail: Linzy@nju.edu.cn; Gaoxiang@nju.edu.cn

Received: 21/8/2020
Revised: 2/12/2020
Accepted: 15/1/2021
Accepted article online: 17/1/2021

