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WO 2009/062174 A1
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(54) **Title:** COMPOUNDS FOR INHIBITION OF INFLAMMATION

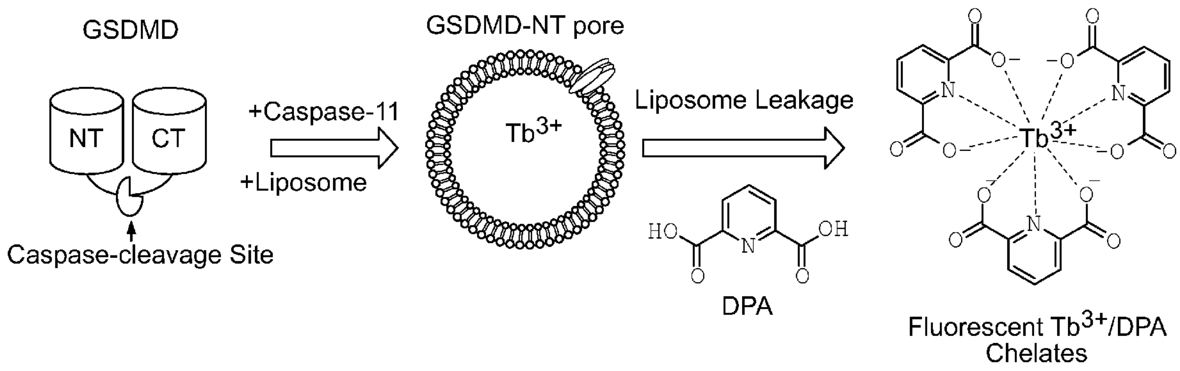

FIG. 1

(57) **Abstract:** The present application provides chemical compounds useful, for example, in inhibiting gasdermin pore formation in a cell, inhibiting inflammasome-mediated death of a cell (pyroptosis); inhibiting cytokine secretion from a cell, inhibiting an inflammatory caspase in a cell, and/or covalently reacting with a cysteine of a gasdermin protein in a cell. These compounds are also useful in treating or preventing diseases or conditions in which inflammasome activation is implicated in pathogenesis. One example of such disease or condition is sepsis.

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Compounds for inhibition of inflammation

CLAIM OF PRIORITY

This application claims priority to U.S. Provisional Patent Application Serial No. 62/690,788, filed on June 27, 2018, the entire contents of which are hereby incorporated by reference.

TECHNICAL FIELD

This invention relates to chemical compounds, in particular to compounds that inhibit inflammation and are useful in treating conditions associated with inflammation.

BACKGROUND

Inflammasomes are multi-protein signaling scaffolds that assemble in response to invasive pathogens and sterile danger signals to activate inflammatory caspases (1/4/5/11), which trigger inflammatory death (pyroptosis) and processing and release of pro-inflammatory cytokines. Inflammasome activation contributes to many human diseases, including inflammatory bowel disease, gout, type II diabetes, cardiovascular disease, Alzheimer's disease, and sepsis, the often fatal response to systemic infection.

SUMMARY

In a first general aspect, the present disclosure provides a method of:

- inhibiting gasdermin pore formation in a cell; and/or
- inhibiting inflammasome-mediated death of a cell (pyroptosis); and/or
- inhibiting cytokine secretion from a cell; and/or
- inhibiting an inflammatory caspase in a cell; and/or
- covalently reacting with a cysteine of a gasdermin protein in a cell; and/or
- covalently reacting with a cysteine of an inflammatory signaling molecule selected from: a sensor, an adaptor, and a transcription factor, or a regulator thereof;

the method comprising contacting the cell with an effective amount of any one of the compounds as described herein, or a pharmaceutically acceptable salt thereof.

In a second general aspect, the present disclosure provides a method of treating or preventing a disease or condition in which inflammasome activation and/or a gasdermin inflammatory cell death is implicated in pathogenesis, the method comprises administering to a subject in need thereof a therapeutically effective amount of any one of the compounds as described herein, or a pharmaceutically acceptable salt thereof.

In a third general aspect, the present disclosure provides a method of identifying a compound that:

- inhibits a gasdermin pore formation in a cell; and/or
- inhibits inflammasome-mediated death of a cell (pyroptosis); and/or
- inhibits cytokine secretion from a cell; and/or
- inhibits an inflammatory caspase in a cell; and/or
- covalently reacts with a cysteine of a gasdermin protein in a cell; and/or
- covalently reacts with a cysteine of an inflammatory signaling molecule selected from: a sensor, an adaptor, and a transcription factor, or a regulator thereof;

the method comprising:

a) providing a sample comprising a liposome comprising a metal cation capable of forming a complex with a chelating ligand, the chelating ligand, a test compound, and a gasdermin protein, or a fragment thereof;
b) contacting the gasdermin protein in the sample with a protease enzyme; and
c) determining whether the test compound inhibits leakage of the metal cation from the liposome, wherein said inhibition of the leakage of the metal cation from the liposome is an indication that the test compound:
   - inhibits a gasdermin pore formation in a cell; and/or
   - inhibits inflammasome-mediated death of a cell (pyroptosis); and/or
   - inhibits cytokine secretion from a cell; and/or
   - inhibits an inflammatory caspase in a cell; and/or
   - covalently reacts with a cysteine of a gasdermin protein in a cell; and/or

- covalently reacts with a cysteine of an inflammatory signaling molecule selected from: a sensor, an adaptor, and a transcription factor, or a regulator thereof.

In a fourth general aspect, the present disclosure provides a pharmaceutical composition comprising any one of the compounds described herein, or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable carrier.

Certain implementations of the first, the second, the third, and the fourth general aspects are described herein.

In some embodiments, the present disclosure provides a composition comprising any one of the compounds described herein, or a pharmaceutically acceptable salt thereof, for treating or preventing any one of the diseases or conditions described herein.

In some embodiments, the present disclosure provides any one of the compounds described herein, or a pharmaceutically acceptable salt thereof, for use as a medicament for treating or preventing any one of the diseases or conditions described herein.

In some embodiments, the present disclosure provides a use of any one of the compounds described herein, or a pharmaceutically acceptable salt thereof, in the manufacture of a medicament for the treatment or prevention of any one of the diseases or conditions described herein.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the present application belongs. Methods and materials are described herein for use in the present application; other, suitable methods and materials known in the art can also be used. The materials, methods, and examples are illustrative only and not intended to be limiting. All publications, patent applications, patents, sequences, database entries, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control.

Other features and advantages of the present application will be apparent from the following detailed description and figures, and from the claims.

DESCRIPTION OF DRAWINGS

FIG. 1 contains a pictorial representation of the terbium (Tb^{3+})/dipicolinic acid (DPA) fluorescence liposome leakage assay.

FIG. 2 contains a line plot showing a dose response curve of disulfiram in liposome leakage assay.

FIG. 3 contains line plot showing MST measurement of the binding of Alexa 488-labeled His-MBP-GSDMD (80 nM) with C-22, C-23 or C-24.

FIG. 4 contains a bar graph showing cell viability after treatment with compounds C-22, C-23, and C-24 in the presence of nigericin or medium.

FIG. 5 contains a bar graph showing cell viability after pretreatment with each test compound (before electroporation with PBS or LPS).

FIG. 6 contains a line plot showing IC_{50} of inhibition by compound C-23 of canonical inflammasome activation.

FIG. 7 contains a line plot showing IC_{50} of inhibition by compound C-23 of non-canonical inflammasome activation.

FIG. 8 contains a bar graph showing levels of IL-1β in culture supernatants treated by compound C-23 as assessed by ELISA (cells treated with LPS, or LPS and nigericin).

FIG. 9 contains a bar graph showing levels of IL-1β in culture supernatants treated by compound C-23 as assessed by ELISA (cells treated with PBS, or LPS transfection).

FIG. 10 contains a bar graph showing cell viability after pretreatment with C-23 before transfection with PBS or poly(dA:dT).

FIG. 11 contain chemical structures of compounds C-5, C-7, C-8, C-22, C-23, C-24, and C-25.

FIG. 12 contains dose response curves of inhibition of liposome leakage by disulfiram (C-23) or its metabolite DTC in the presence or absence of Cu(II).

FIG. 13 contains line plots showing that LPS-primed THP-1 were pretreated with C-23 or DTC in the presence or absence of Cu(II) for 1 hr before adding nigericin or medium for 2 hrs.

FIG. 14 contains line plots showing % mice survival after challenge with 15 mg/kg of LPS and treatment with C-23.

FIG. 15 contains a bar graph showing serum IL-1β measured by ELISA in mice pretreated with C-23 and challenged with 15 mg/kg LPS.

FIG. 16 contains line plots showing % mice survival after challenge with 25 mg/kg of LPS and treatment with C-23.

FIG. 17 contains line plots showing % mice survival after challenge with 50 mg/kg of LPS and treatment with C-23.

FIG. 18 contains line plots showing % mice survival after mice were treated with C-23 (50 mg/kg), C-23 (50 mg/kg) plus copper gluconate (0.15 mg/kg) or vehicle (Ctrl) by intraperitoneal injection 0 and 12 hours post intraperitoneal LPS challenge (25 mg/kg).

FIG. 19 contains a chemical scheme showing chemical reaction between DTC and Cu^{2+}.

FIG. 20 contains an MS/MS spectrum of the Cys191-containing human GSDMD peptide.

FIG. 21 contains an MS/MS spectrum of GSDMD peptide after incubation with C-23, having a covalent modification on Cys191 by the diethyldithiocarbamate moiety of C-23.

FIG. 22 contains images showing models of full-length human GSDMD in its auto-inhibited form and of the pore form of GSDMD N-terminal fragment (GSDMD-NT) based on the corresponding structures of GSDMA3.

FIG. 23 contains dose response curve of C-23 inhibition of liposome leakage induced by wild-type, C38A or C191A GSDMD (0.3 μM) plus caspase-11 (0.15 μM).

FIG. 24 contains a bar graph showing C-23 inhibition of pyroptosis of LPS + nigericin treated THP-1 cells after C-23 preincubation for 1 hour with N-acetylcysteine (NAC, 500 μM) or medium.

FIG. 25 contains a dose response curve of compound C-23 in liposome leakage induced by human GSDMD-3C (0.3 μM) plus 3C protease (0.15 μM).

FIG. 26 contains a dose response curve of compound C-23 in liposome leakage induced by human GSDMD-3C (0.3 μM) plus 3C protease (0.15 μM).

FIG. 27 contains a MS/MS spectrum for peptide FSLPGATCLQGEGQGHLSQK modified on cysteine 191 by carbamidomethyl.

FIG. 28 contains MS/MS spectrum for peptide FSLPGATCLQGEGQGHLSQK modified on cysteine 191 by C-23.

FIG. 29 contains sequence alignment of GSDMA3, hGSDMA, mGSDMD and hGSDMD showing Cys residues.

FIG. 30 contains line plots showing Tb^{3+}/DPA fluorescence of GSDMD (0.3 μM) pre-incubated with the indicated concentrations of C-23 (0–50 μM) for different durations (2–90 min) before caspase-11 (0.15 μM) in liposome (50 μM) was added.

FIG. 31 contains a line plots showing time course of caspase-1 activity in the presence of indicated concentrations of compound C-23.

FIG. 32 contains a line plots showing time course of caspase-11 activity in the presence of indicated concentrations of compound C-23.

FIG. 33 contains a dose response curve of compound C-23 in the caspase-1 activity assay.

FIG. 34 contains a dose response curve of compound C-23 in the caspase-11 activity assay.

FIG. 35 contains line plots showing time course of caspase-1 activity in the presence of indicated concentrations of compound C-23 + Cu(II).

FIG. 36 contains line plots showing time course of caspase-11 activity in the presence of indicated concentrations of compound C-23 + Cu(II).

FIG. 37 contains a dose response curve of compound C-23 + Cu(II) in the caspase-1 activity assay.

FIG. 38 contains a dose response curve of compound C-23 + Cu(II) in the caspase-11 activity assay.

FIG. 39 contains chemical structures of test compounds presented in Table 2.

FIG. 40 contains a bar graph showing results of cell viability assay for the compounds presented in Table 2 and Figure 39.

FIG. 41 contains a bar graph showing results of cell viability assay for the compounds of Table 2 and Figure 39, with or without nigericin.

FIG. 42 contains a bar graph showing results of cell viability assay for the compounds C-23A1, C-23A2 C-23A9, and C-23A10, after adding nigericin.

FIG. 43 contains a bar graph showing results of cell viability assay for the compounds C-23, Bay 11-7082, and C-23 + Bay 11-7082.

FIG. 44 contains a bar graph showing results of cell viability assay for the compounds C-23 and Bay 11-7082 after LPS transfection.

FIG. 45 contains images of immunoblots of THP-1 cells pretreated with C-23 and Bay 11-7082.

FIG. 46 contains images of LPS-primed THP-1 cells pretreated with C-23, Bay 11-7082 or z-VADfmk.

FIG. 47 contains a bar graph showing % of cell with APS aggregates after treatment with C-23, Bay 11-7082 or z-VADfmk.

FIG. 48 contains images of LPS-primed THP-1 cells pretreated with C-23, alone or with Cu(II).

FIG. 49 contains a bar graph showing % of cell with APS aggregates after treatment with C-23, alone or with Cu(II).

FIG. 50 contains images of immunoblots showing lysates of cells pretreated with C-23, Bay 11-7082 or z-VADfmk and visualized with indicated antibodies.

FIG. 51 contains images of immunoblots showing lysates of cells pretreated with C-23, alone or with Cu(II), and visualized with indicated antibodies.

FIG. 52 contains a bar graph showing caspase-1 activity of C-23, Bay 11-7082 and z-VADfmk.

FIG. 53 contains images of LPS-primed THP-1 cells that were pretreated with C-23, Bay 11-7082 or z-VAD-fmk, and stained with a mouse anti-GSDMD monoclonal antibody.

FIG. 54 contains a bar graph showing quantification of proportion of cells with GSDMD membrane staining and pyroptotic bubbles.

FIG. 55 contains response curve of Bay 11-7082 inhibition of liposome leakage by wild-type, C38A or C191A human GSDMD.

FIG. 56 contains a line plot showing thermophoresis measurement of the direct binding of Alexa 488-labeled His-MBP-GSDMD with Bay 11-7082.

FIG. 57 contains a dose response curve of the effect of Bay 11-7082 on caspase-1 activity.

FIG. 58 contains a dose response curve of the effect of Bay 11-7082 on caspase-11 activity.

FIG. 59 contains MS spectrum of GSDMD peptide modified on Cys191 by carbamidomethyl.

FIG. 60 contains MS spectrum of GSDMD peptide after GSDMD incubation with Bay 11-7082, which was modified at Cys191.

FIG. 61 contains a dose response curve of the effect of Bay 11-7082 on liposome leakage induced by human GSDMD-3C.

FIG. 62 contains a dose response curve of the effect of Bay 11-7082 on liposome leakage induced by mouse GSDMD-3C.

FIG. 63 contains a bar graph showing effect of preincubation of Bay 11-7082 with N-acetylcysteine (NAC) on inhibition of pyroptosis.

FIG. 64 contains images of immunoblots of HEK293T cells that were transfected with the indicated plasmids, gels were probed with the indicated antibodies.

FIG. 65 contains images of immunoblots of HCT116, 293T and THP-1 cells that were transfected with the indicated plasmids, gels were probed with the indicated antibodies.

FIG. 66 contains images of 293T and THP-1 cells that were immunostained with the anti-GSDMD monoclonal antibody and co-stained with DAPI

FIG. 67 contains a scheme showing biochemical processes leading to the formation of gasdermin D pore and subsequent release of inflammatory mediators.

FIG. 68 contains negative stain EM images of PS-containing nanodiscs with or without incubation with GSDMD-3C plus 3C protease. In the 3rd image from the left, C-23 was added to the GSDMD-3C plus 3C protease mixture before it was added to the nanodiscs; in the 4th image C-23 was added after the mixture was incubated with nanodiscs when pores had formed. Scale bar, 100 nm. Arrows point to empty nanodiscs and pores.

FIG. 69 contains a bar graph showing experimental results for the HT-29 cells that were pretreated (10 μM and 50 μM) or not with disulfiram (C-23) or 2 μM necrosulfonamide (NSA) or 10 μM Necrostatin-1 (Nec) for 1 h before adding 20 ng/ml TNFα (T), 100 nM SMAC mimetic (S), and 20 μM z-VAD-fmk (Z) and analyzed for cell viability by CellTiter-Glo assay 24 h later. Graphs show mean ± s.d; data are representative of three independent experiments. $^{**}P < 0.01$.

FIG. 70 contains a line graph showing results of pyroptosis as measured by SYTOX Green uptake in the presence of no inhibitor or 30 μM C-23 or z-VAD-fmk.

FIG. 71 contains a bar graph showing results of an experiment when full-length (FL) human GSDMD and GSDMD C191S were co-expressed with Caspase-11 in HEK293T cells. Cell death was determined by CytoTox96 cytotoxicity assay 20 hrs after transfection.

FIG. 72 contains a bar graph showing results of an experiment when FL human WT or C191S GSDMD were co-expressed with caspase-11 in HEK293T cells. 8 h post transfection, the indicated amount of disulfiram was added and cell death was determined by LDH release 12 h later. The bar graph shows the mean ± s.d. of 1 representative experiment of three independent experiments performed. $^{*}P < 0.05$, $^{**}P < 0.01$, n.s., not significant.

FIG. 73 contains a line plot showing dose response curve of disulfiram in liposome leakage induced by pre-cleaved human GSDMD (0.3 μM).

FIG. 74 contains a line plot showing dose response curve of disulfiram in liposome leakage induced by pre-cleaved mouse GSDMA3-3C (0.3 μM).

FIG. 75 contains images showing LPS-primed THP-1 cells, pretreated or not with 30 μM disulfiram or z-VAD-fmk for 1 hr, and stimulated with nigericin or medium.

FIG. 76 contains a bar graph showing results of analysis of LPS-primed THP-1 cells for ASC specks.

FIG. 77 contains an image showing results of analysis of LPS-primed THP-1 cells for NLRP3.

FIG. 78 contains an image showing results of analysis of LPS-primed THP-1 cells for caspase-1, GSDMD, and pro-IL-1β cleavage and IL-1 release by immunoblot of whole cell lysate (WCL) or culture supernatants.

FIG. 79 contains an image and a bar graph showing redistribution of GSDMD to the plasma membrane. Cells were fixed 30 min after adding nigericin and stained for GSDMD using a previously unreported monoclonal antibody generated in house Shown are representative confocal microscopy images and quantification of the proportion of cells with GSDMD membrane staining and pyroptotic bubbles. Arrows indicate GSDMD staining of pyroptotic bubbles. Graphs show the mean ± s.d; data are representative of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

FIG. 80 contains an image showing a model of inflammasome pathway steps and their inhibition by disulfiram, with a main effect on GSDMD.

FIG. 81 contains a plot showing results of an experiment where mice were pretreated with disulfiram (50 mg/kg) or vehicle (Ctrl) by intraperitoneal injection 24 and 4 h before intraperitoneal challenge with 15 mg/kg LPS and followed for survival. TNFa was measured by ELISA (n= 5/group) 12 hr post LPS challenge. Shown are mean ± s.d.

FIG. 82 contains a plot showing results of an experiment where mice were pretreated with disulfiram (50 mg/kg) or vehicle (Ctrl) by intraperitoneal injection 24 and 4 h before intraperitoneal challenge with 15 mg/kg LPS and followed for survival. Serum IL-6 were measured by ELISA (n= 5/group) 12 hr post LPS challenge. Shown are mean ± s.d.

FIG. 83 contains a line plot showing results of an experiment where mice were pretreated with disulfiram (50 mg/kg) or vehicle (Ctrl) by intraperitoneal injection 4 h before and daily after intraperitoneal LPS challenge (25 mg/kg) and followed for survival.

FIG. 84 contains an image showing results of an experiments where peritoneal macrophages from four indicated groups of mice were analyzed for NLRP3, GSDMD and HMGB1 by immunoblot.

FIG. 85 contains a line plot showing results of a liposome leakage assay. GSDMD (2.5 μM) and caspase-11 (2.5 μM) were incubated in liposome solutions at various concentrations in 20 mM HEPES buffer (150 mM NaCl) for 1 h. The concentration of liposome lipids for the screen was set at 50 μM.

FIG. 86 contains a line plot showing results of liposome leakage assay. Different concentrations of GSDMD and caspase-11 (1:1 ratio) were incubated in liposome (50 μM) solutions for 1 h. The concentration of GSDMD used in the screen was set at 0.3 μM.

FIG. 87 contains a line plot showing results of liposome leakage assay. Different concentrations of caspase-11 and GSDMD (0.3 μM) were incubated in liposome (50 μM) solutions for 1 h. The concentration of caspase-11 used in the screen was set at 0.15 μM. The fluorescence intensity at 545 nm was measured after excitation at 276 nm.

FIG. 88 contains a bar graph showing results of an experiment where mouse iBMDMs were pretreated or not with disulfiram (C-23) ranging from 5-40 μM for 1 h before transfection with PBS or poly(dA:dT) and analyzed for cell viability by CellTiter-Glo assay 4 h later. $^{**}P < 0.01$.

FIG. 89 contains an image showing sequence alignment of GSDMA3, hGSDMA, mGSDMD and hGSDMD showing Cys residues.

FIG. 90 contains a bar graph showing results of an experiment where FL mouse GSDMD or WT, C192S or C39A GSDMD-NT were transiently expressed in HEK293T cells. Cell death was determined by CytoTox96 cytotoxicity assay 20 hrs after transfection. c shows the mean ± s.d. of 1 representative experiment of three independent experiments performed. *P < 0.05.

FIG. 91 contains a line plot showing results of GSDMD-mediated liposome leakage assay induced by 0.3 μM GSDMD plus 0.15 μM caspase-11 for compound necrosulfonamide (dose response curve).

FIG. 92 contains a line plot showing results of GSDMD-mediated liposome leakage assay induced by 0.3 μM GSDMD plus 0.15 μM caspase-11 for compound dimethyl fumarate (dose response curve).

FIG. 93 contains a line plot showing results of GSDMD-mediated liposome leakage assay induced by 0.3 μM GSDMD plus 0.15 μM caspase-11 for compound afatinib (dose response curve).

FIG. 94 contains a line plot showing results of GSDMD-mediated liposome leakage assay induced by 0.3 μM GSDMD plus 0.15 μM caspase-11 for compound ibrutinib (dose response curve).

FIG. 95 contains a line plot showing results of GSDMD-mediated liposome leakage assay induced by 0.3 μM GSDMD plus 0.15 μM caspase-11 for compound LDC7559 (dose response curve).

FIG. 96 contains a bar graph showing results of an experiment where LPS-primed THP-1 cells, pretreated or not with 30 μM disulfiram or z-VAD-fmk for 1 hr and stimulated with nigericin or medium, were analyzed for caspase-1 activity by a cell-permeable fluorescent caspase activity probe FAM-YVAD-FMK after 0.5 hr.

FIG. 97 contains a bar graph showing results of an experiment where LPS-primed THP-1 cells, after medium removal, were incubated with probe FAM-YVAD-FMK in FLICA assay buffer for another 0.5 hr before fluorescence reading. iBMDMs were pretreated with disulfiram, Bay 11-7082, necrosulfonamide (NSA) or z-VAD-fmk for 1 hr before treated or not with Nigericin for 0.5 hr. Whole cell lysates and culture supernatants were immunoblotted with the indicated antibodies.

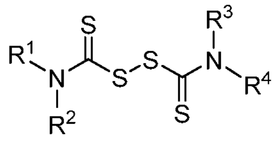
FIG. 98 contains a bar graph showing results of an experiment where LPS-primed THP-1 cells, after medium removal, were incubated with probe FAM-YVAD-FMK in FLICA assay buffer for another 0.5 hr before fluorescence reading. iBMDMs were pretreated with disulfiram, Bay 11-7082, necrosulfonamide (NSA) or z-VAD-fmk for 1 hr before treated or not with Nigericin for 1 hr. Whole cell lysates and culture supernatants were immunoblotted with the indicated antibodies.

DETAILED DESCRIPTION

As discussed more fully below, the pore-forming protein gasdermin (such as gasdermin D) is the final pyroptosis executioner downstream of inflammasome activation. The compounds of the present application potently inhibit gasdermin pore formation and subsequent secretion of inflammatory mediators such as IL-1β. As such, the compounds of the present application are useful, for example, in treating diseases and conditions mediated by inflammation such as sepsis. Pharmaceutical compositions containing compounds of the present disclosure, as well as various methods using and making these compounds are described below.

Therapeutic compounds

In one general aspect, the present disclosure provides a compound of Formula (I):



(I),

or a pharmaceutically acceptable salt thereof, wherein:

R^1, R^2, R^3, and R^4 are each independently selected from H, C_{1-6} alkyl, C_{1-6} haloalkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, Cy^1, $C(O)R^{b1}$, $C(O)NR^{c1}R^{d1}$, $C(O)OR^{a1}$, $S(O)_2R^{b1}$, and $S(O)_2NR^{c1}R^{d1}$; wherein said C_{1-6} alkyl, C_{2-6} alkenyl, and C_{2-6} alkynyl are each optionally substituted with 1, 2 or 3 substituents independently selected from Cy^1, halo, CN, NO_2, OR^{a1}, SR^{a1}, $C(O)R^{b1}$, $C(O)NR^{c1}R^{d1}$, $C(O)OR^{a1}$, $NR^{c1}R^{d1}$, $NR^{c1}C(O)R^{b1}$, $NR^{c1}C(O)OR^{a1}$, $NR^{c1}C(O)NR^{c1}R^{d1}$, $NR^{c1}S(O)_2R^{b1}$, $NR^{c1}S(O)_2NR^{c1}R^{d1}$, $S(O)_2R^{b1}$ and $S(O)_2NR^{c1}R^{d1}$;

or R^1 and R^2 together with the N atom to which they are attached form a 4-12 membered heterocycloalkyl, which is optionally substituted with 1, 2, 3, 4, or 5 substituents independently selected from R^{Cy2};

or R^3 and R^4 together with the N atom to which they are attached form a 4-12 membered heterocycloalkyl, which is optionally substituted with 1, 2, 3, 4, or 5 substituents independently selected from R^{Cy3};

each Cy^1 is independently selected from C_{6-10} aryl, C_{3-10} cycloalkyl, 5-10 membered heteroaryl, and 4-12 membered heterocycloalkyl, each of which is optionally substituted with 1, 2, 3, 4, or 5 substituents independently selected from R^{Cy1};

each R^{Cy1}, R^{Cy2}, and R^{Cy3} is independently selected from C_{1-6} alkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, C_{1-6} haloalkyl, halo, CN, NO_2, OR^{a2}, $C(O)R^{b2}$, $C(O)NR^{c2}R^{d2}$, $C(O)OR^{a2}$, $NR^{c2}R^{d2}$, $NR^{c2}C(O)R^{b2}$, $NR^{c2}C(O)OR^{a2}$, $NR^{c2}C(O)NR^{c2}R^{d2}$, $S(O)_2R^{b2}$ and $S(O)_2NR^{c2}R^{d2}$;

R^{a1}, R^{a2}, R^{c1}, R^{c2}, R^{d1}, and R^{d2} are each independently selected from H, C_{1-6} alkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, C_{1-4} haloalkyl, Cy^1, $C(O)R^{b3}$, $C(O)NR^{c3}R^{d3}$, $C(O)OR^{a3}$, $S(O)_2R^{b3}$, and $S(O)_2NR^{c3}R^{d3}$; wherein said C_{1-6} alkyl, C_{2-6} alkenyl, and C_{2-6} alkynyl are each optionally substituted with 1, 2, 3, 4, or 5 substituents independently selected from Cy^1, halo, CN, NO_2, OR^{a3}, SR^{a3}, $C(O)R^{b3}$, $C(O)NR^{c3}R^{d3}$, $C(O)OR^{a3}$, $NR^{c3}R^{d3}$, $NR^{c3}C(O)R^{b3}$, $NR^{c3}C(O)OR^{a3}$, $NR^{c3}C(O)NR^{c3}R^{d3}$, $NR^{c3}S(O)_2R^{b3}$, $NR^{c3}S(O)_2NR^{c3}R^{d3}$, $S(O)_2R^{b3}$ and $S(O)_2NR^{c3}R^{d3}$;

R^{b1} and R^{b2} are each independently selected from C_{1-6} alkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, C_{1-4} haloalkyl and Cy^1; wherein said C_{1-6} alkyl, C_{2-6} alkenyl, and C_{2-6} alkynyl are each optionally substituted with 1, 2, 3, 4, or 5 substituents independently selected from Cy^1, halo, CN, NO_2, OR^{a3}, SR^{a3}, $C(O)R^{b3}$, $C(O)NR^{c3}R^{d3}$, $C(O)OR^{a3}$, $NR^{c3}R^{d3}$, $NR^{c3}C(O)R^{b3}$, $NR^{c3}C(O)OR^{a3}$, $NR^{c3}C(O)NR^{c3}R^{d3}$, $NR^{c3}S(O)_2R^{b3}$, $NR^{c3}S(O)_2NR^{c3}R^{d3}$, $S(O)_2R^{b3}$ and $S(O)_2NR^{c3}R^{d3}$;

R^{a3}, R^{c3}, and R^{d3} are each independently selected from H, C_{1-6} alkyl, C_{1-4} haloalkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, C_{6-10} aryl, C_{3-10} cycloalkyl, 5-10 membered heteroaryl, 4-12 membered heterocycloalkyl, C_{6-10} aryl-C_{1-4} alkylene, C_{3-10} cycloalkyl-C_{1-4} alkylene, (5-10 membered heteroaryl)-C_{1-4} alkylene, (4-12 membered heterocycloalkyl)-C_{1-4} alkylene, $C(O)R^{b4}$, $C(O)NR^{c4}R^{d4}$, $C(O)OR^{a4}$, $NR^{c4}R^{d4}$, $S(O)_2R^{b4}$, and $S(O)_2NR^{c4}R^{d4}$; wherein said C_{1-6} alkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, C_{6-10}

aryl, C_{3-10} cycloalkyl, 5-10 membered heteroaryl, 4-12 membered heterocycloalkyl, C_{6-10} aryl-C_{1-4} alkylene, C_{3-10} cycloalkyl-C_{1-4} alkylene, (5-10 membered heteroaryl)-C_{1-4} alkylene, and (4-12 membered heterocycloalkyl)-C_{1-4} alkylene are each optionally substituted with 1, 2, 3, 4, or 5 substituents independently selected from oxo, C_{1-6} alkyl, C_{1-4} haloalkyl, C_{1-4} hydroxyalkyl, C_{1-6} cyanoalkyl, halo, CN, NO_2, OR^{a4}, SR^{a4}, $C(O)R^{b4}$, $C(O)NR^{c4}R^{d4}$, $C(O)OR^{a4}$, $NR^{c4}R^{d4}$, $NR^{c4}C(O)R^{b4}$, $NR^{c4}C(O)OR^{a4}$, $NR^{c4}C(O)NR^{c4}R^{d4}$, $NR^{c4}S(O)_2R^{b4}$, $NR^{c4}S(O)_2NR^{c4}R^{d4}$, $S(O)_2R^{b4}$, and $S(O)_2NR^{c4}R^{d4}$;

each R^{b3} is independently selected from C_{1-6} alkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, C_{1-4} haloalkyl, C_{6-10} aryl, C_{3-10} cycloalkyl, 5-10 membered heteroaryl, 4-12 membered heterocycloalkyl, C_{6-10} aryl-C_{1-4} alkylene, C_{3-10} cycloalkyl-C_{1-4} alkylene, (5-10 membered heteroaryl)-C_{1-4} alkylene, and (4-12 membered heterocycloalkyl)-C_{1-4} alkylene, wherein said C_{1-6} alkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, C_{6-10} aryl, C_{3-10} cycloalkyl, 5-10 membered heteroaryl, 4-12 membered heterocycloalkyl, C_{6-10} aryl-C_{1-4} alkylene, C_{3-10} cycloalkyl-C_{1-4} alkylene, (5-10 membered heteroaryl)-C_{1-4} alkylene, and (4-12 membered heterocycloalkyl)-C_{1-4} alkylene are each optionally substituted with 1, 2, 3, 4, or 5 substituents independently selected from C_{1-6} alkyl, C_{1-4} haloalkyl, C_{1-4} hydroxyalkyl, C_{1-6} cyanoalkyl, halo, CN, NO_2, OR^{a4}, SR^{a4}, $C(O)R^{b4}$, $C(O)NR^{c4}R^{d4}$, $C(O)OR^{a4}$, $NR^{c4}R^{d4}$, $NR^{c4}C(O)R^{b4}$, $NR^{c4}C(O)OR^{a4}$, $NR^{c4}C(O)NR^{c4}R^{d4}$, $NR^{c4}S(O)_2R^{b4}$, $NR^{c4}S(O)_2NR^{c4}R^{d4}$, $S(O)_2R^{b4}$, and $S(O)_2NR^{c4}R^{d4}$;

R^{a4}, R^{c4}, and R^{d4} are each independently selected from H, C_{1-6} alkyl, C_{1-4} haloalkyl, C_{1-4} hydroxyalkyl, C_{1-4} cyanoalkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, C_{6-10} aryl, C_{3-10} cycloalkyl, 5-10 membered heteroaryl, 4-12 membered heterocycloalkyl, C_{6-10} aryl-C_{1-4} alkylene, C_{3-10} cycloalkyl-C_{1-4} alkylene, (5-10 membered heteroaryl)-C_{1-4} alkylene, (4-12 membered heterocycloalkyl)-C_{1-4} alkylene and R^g, wherein said C_{1-6} alkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, C_{6-10} aryl, C_{3-10} cycloalkyl, 5-10 membered heteroaryl, 4-12 membered heterocycloalkyl, C_{6-10} aryl-C_{1-4} alkylene, C_{3-10} cycloalkyl-C_{1-4} alkylene, (5-10 membered heteroaryl)-C_{1-4} alkylene, and (4-12 membered heterocycloalkyl)-C_{1-4} alkylene are each optionally substituted with 1, 2, 3, 4, or 5 substituents independently selected from R^g;

each R^{b4} is independently selected from C_{1-6} alkyl, C_{1-4} haloalkyl, C_{1-4} hydroxyalkyl, C_{1-4} cyanoalkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, C_{6-10} aryl, C_{3-10} cycloalkyl, 5-10 membered heteroaryl, 4-12 membered heterocycloalkyl, C_{6-10} aryl-C_{1-4} alkylene, C_{3-10} cycloalkyl-C_{1-4} alkylene, (5-10 membered heteroaryl)-C_{1-4} alkylene, (4-12 membered heterocycloalkyl)-C_{1-4} alkylene and R^g, wherein said C_{1-6} alkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, C_{6-10} aryl, C_{3-10} cycloalkyl, 5-10 membered heteroaryl, 4-12 membered heterocycloalkyl, C_{6-10} aryl-C_{1-4} alkylene, C_{3-10} cycloalkyl-C_{1-4} alkylene, (5-10 membered heteroaryl)-C_{1-4} alkylene, and (4-12 membered heterocycloalkyl)-C_{1-4} alkylene is optionally substituted with 1, 2, 3, 4, or 5 substituents independently selected from R^g; and

each R^g is independently selected from OH, NO_2, CN, halo, C_{1-6} alkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, C_{1-4} haloalkyl, C_{1-6} alkoxy, C_{1-6} haloalkoxy, cyano-C_{1-3} alkylene, HO-C_{1-3} alkylene, C_{6-10} aryl, C_{6-10} aryloxy, C_{3-10} cycloalkyl, 5-10 membered heteroaryl, 4-12 membered heterocycloalkyl, C_{6-10} aryl-C_{1-4} alkylene, C_{3-10} cycloalkyl-C_{1-4} alkylene, (5-10 membered heteroaryl)-C_{1-4} alkylene, (4-12 membered heterocycloalkyl)-C_{1-4} alkylene, amino, C_{1-6} alkylamino, di(C_{1-6} alkyl)amino, thio, C_{1-6} alkylthio, C_{1-6} alkylsulfinyl, C_{1-6} alkylsulfonyl, carbamyl, C_{1-6} alkylcarbamyl, di(C_{1-6} alkyl)carbamyl, carboxy, C_{1-6} alkylcarbonyl, C_{1-6} alkoxycarbonyl, C_{1-6} alkylcarbonylamino, C_{1-6} alkylsulfonylamino, aminosulfonyl, C_{1-6} alkylaminosulfonyl, di(C_{1-6} alkyl)aminosulfonyl, aminosulfonylamino, C_{1-6} alkylaminosulfonylamino, di(C_{1-6} alkyl)aminosulfonylamino, aminocarbonylamino, C_{1-6} alkylaminocarbonylamino, and di(C_{1-6} alkyl)aminocarbonylamino.

In some embodiments, R^1 is selected from H, C_{1-6} alkyl, C_{1-6} haloalkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, and Cy^1; wherein said C_{1-6} alkyl, C_{2-6} alkenyl, and C_{2-6} alkynyl are each optionally substituted with 1, 2 or 3 substituents independently selected from Cy^1, halo, CN, NO_2, OR^{a1}, SR^{a1}, $C(O)R^{b1}$, $C(O)NR^{c1}R^{d1}$, $C(O)OR^{a1}$, $NR^{c1}R^{d1}$, $NR^{c1}C(O)R^{b1}$, $NR^{c1}C(O)OR^{a1}$, $NR^{c1}C(O)NR^{c1}R^{d1}$, $NR^{c1}S(O)_2R^{b1}$, $NR^{c1}S(O)_2NR^{c1}R^{d1}$, $S(O)_2R^{b1}$ and $S(O)_2NR^{c1}R^{d1}$.

In some embodiments, R^1 is selected from H, C_{1-6} alkyl, C_{1-6} haloalkyl, and Cy^1; wherein said C_{1-6} alkyl is optionally substituted with 1, 2 or 3 substituents independently selected from Cy^1, halo, CN, NO_2, OR^{a1}, $C(O)NR^{c1}R^{d1}$, $C(O)OR^{a1}$, $NR^{c1}R^{d1}$, $NR^{c1}C(O)R^{b1}$, $NR^{c1}C(O)OR^{a1}$, and $NR^{c1}S(O)_2R^{b1}$.

In some embodiments, R^1 is C_{1-6} alkyl optionally substituted with Cy^1. In some aspects of these embodiments, R^1 is selected from methyl, ethyl, propyl, isopropyl, *n*-butyl, and *t*-butyl, each of which is optionally substituted with Cy^1. In other aspects of these embodiments, R^1 is methyl substituted with Cy^1. In some embodiments, R^1 is Cy^1. In some embodiments, R^1 is selected from Cy^1 and C_{1-6} alkyl optionally substituted with Cy^1.

In some embodiments, R^2 is selected from H, C_{1-6} alkyl, C_{1-6} haloalkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, and Cy^1; wherein said C_{1-6} alkyl, C_{2-6} alkenyl, and C_{2-6} alkynyl are each optionally substituted with 1, 2 or 3 substituents independently selected from Cy^1, halo, CN, NO_2, OR^{a1}, SR^{a1}, $C(O)R^{b1}$, $C(O)NR^{c1}R^{d1}$, $C(O)OR^{a1}$, $NR^{c1}R^{d1}$, $NR^{c1}C(O)R^{b1}$, $NR^{c1}C(O)OR^{a1}$, $NR^{c1}C(O)NR^{c1}R^{d1}$, $NR^{c1}S(O)_2R^{b1}$, $NR^{c1}S(O)_2NR^{c1}R^{d1}$, $S(O)_2R^{b1}$ and $S(O)_2NR^{c1}R^{d1}$.

In some embodiments, R^2 is selected from H, C_{1-6} alkyl, C_{1-6} haloalkyl, and Cy^1; wherein said C_{1-6} alkyl is optionally substituted with 1, 2 or 3 substituents independently selected from Cy^1, halo, CN, NO_2, OR^{a1}, $C(O)NR^{c1}R^{d1}$, $C(O)OR^{a1}$, $NR^{c1}R^{d1}$, $NR^{c1}C(O)R^{b1}$, $NR^{c1}C(O)OR^{a1}$, and $NR^{c1}S(O)_2R^{b1}$.

In some embodiments, R^2 is C_{1-6} alkyl optionally substituted with Cy^1. In some aspects of these embodiments, R^2 is selected from methyl, ethyl, propyl, isopropyl, *n*-butyl, and *t*-butyl, each of which is optionally substituted with Cy^1. In other aspects of these embodiments, R^2 is methyl substituted with Cy^1. In some embodiments, R^2 is Cy^1. In some embodiments, R^2 is selected from Cy^1 and C_{1-6} alkyl optionally substituted with Cy^1.

In some embodiments, R^3 is selected from H, C_{1-6} alkyl, C_{1-6} haloalkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, and Cy^1; wherein said C_{1-6} alkyl, C_{2-6} alkenyl, and C_{2-6} alkynyl are each optionally substituted with 1, 2 or 3 substituents independently selected from Cy^1, halo, CN, NO_2, OR^{a1}, SR^{a1}, $C(O)R^{b1}$, $C(O)NR^{c1}R^{d1}$, $C(O)OR^{a1}$, $NR^{c1}R^{d1}$, $NR^{c1}C(O)R^{b1}$, $NR^{c1}C(O)OR^{a1}$, $NR^{c1}C(O)NR^{c1}R^{d1}$, $NR^{c1}S(O)_2R^{b1}$, $NR^{c1}S(O)_2NR^{c1}R^{d1}$, $S(O)_2R^{b1}$ and $S(O)_2NR^{c1}R^{d1}$.

In some embodiments, R^3 is selected from H, C_{1-6} alkyl, C_{1-6} haloalkyl, and Cy^1; wherein said C_{1-6} alkyl is optionally substituted with 1, 2 or 3 substituents independently selected from Cy^1, halo, CN, NO_2, OR^{a1}, $C(O)NR^{c1}R^{d1}$, $C(O)OR^{a1}$, $NR^{c1}R^{d1}$, $NR^{c1}C(O)R^{b1}$, $NR^{c1}C(O)OR^{a1}$, and $NR^{c1}S(O)_2R^{b1}$.

In some embodiments, R^3 is C_{1-6} alkyl optionally substituted with Cy^1. In some aspects of these embodiments, R^3 is selected from methyl, ethyl, propyl, isopropyl, *n*-butyl, and *t*-butyl, each of which is optionally substituted with Cy^1. In other aspects of these embodiments, R^3 is methyl substituted with Cy^1. In some embodiments, R^3 is Cy^1. In some embodiments, R^3 is selected from Cy^1 and C_{1-6} alkyl optionally substituted with Cy^1.

In some embodiments, R^4 is selected from H, C_{1-6} alkyl, C_{1-6} haloalkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, and Cy^1; wherein said C_{1-6} alkyl, C_{2-6} alkenyl, and C_{2-6} alkynyl are each optionally substituted with 1, 2 or 3 substituents independently selected from Cy^1, halo, CN, NO_2, OR^{a1}, SR^{a1}, $C(O)R^{b1}$, $C(O)NR^{c1}R^{d1}$, $C(O)OR^{a1}$, $NR^{c1}R^{d1}$, $NR^{c1}C(O)R^{b1}$, $NR^{c1}C(O)OR^{a1}$, $NR^{c1}C(O)NR^{c1}R^{d1}$, $NR^{c1}S(O)_2R^{b1}$, $NR^{c1}S(O)_2NR^{c1}R^{d1}$, $S(O)_2R^{b1}$ and $S(O)_2NR^{c1}R^{d1}$.

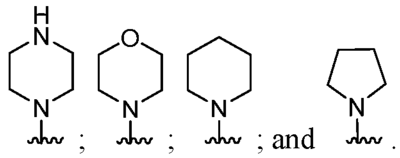
In some embodiments, R^4 is selected from H, C_{1-6} alkyl, C_{1-6} haloalkyl, and Cy^1; wherein said C_{1-6} alkyl is optionally substituted with 1, 2 or 3 substituents independently selected from Cy^1, halo, CN, NO_2, OR^{a1}, $C(O)NR^{c1}R^{d1}$, $C(O)OR^{a1}$, $NR^{c1}R^{d1}$, $NR^{c1}C(O)R^{b1}$, $NR^{c1}C(O)OR^{a1}$, and $NR^{c1}S(O)_2R^{b1}$.

In some embodiments, R^4 is C_{1-6} alkyl optionally substituted with Cy^1. In some aspects of these embodiments, R^4 is selected from methyl, ethyl, propyl, isopropyl, *n*-butyl, and *t*-butyl, each of which is optionally substituted with Cy^1. In other aspects of these embodiments, R^4 is methyl substituted with Cy^1. In some embodiments, R^4 is Cy^1. In some embodiments, R^4 is selected from Cy^1 and C_{1-6} alkyl optionally substituted with Cy^1.

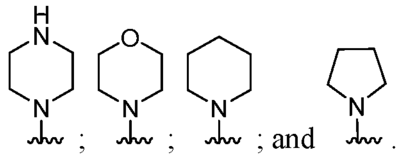
In some embodiments, R^1 and R^2 are each C_{1-6} alkyl optionally substituted with Cy^1. In some embodiments, R^1 and R^2 are each Cy^1. In some embodiments, R^1 is C_{1-6} alkyl optionally substituted with Cy^1, and R^2 is Cy^1. In some embodiments, R^1 is Cy^1; and R^2 is C_{1-6} alkyl optionally substituted with Cy^1.

In some embodiments, R^3 and R^4 are each C_{1-6} alkyl optionally substituted with Cy^1. In some embodiments, R^3 and R^4 are each Cy^1. In some embodiments, R^3 is C_{1-6} alkyl optionally substituted with Cy^1, and R^4 is Cy^1. In some embodiments, R^3 is Cy^1; and R^4 is C_{1-6} alkyl optionally substituted with Cy^1.

In some embodiments, R^1 and R^2 together with the N atom to which they are attached form a 4-12 membered heterocycloalkyl, which is optionally substituted with 1, 2, or 3 substituents independently selected from R^{Cy2}. In some aspects of the foregoing embodiments, 4-12 membered heterocycloalkyl is selected from any one of the following groups:



In some embodiments, R^3 and R^4 together with the N atom to which they are attached form a 4-12 membered heterocycloalkyl, which is optionally substituted with 1, 2, or 3 substituents independently selected from R^{Cy3}. In some aspects of the foregoing embodiments, 4-12 membered heterocycloalkyl is selected from any one of the following groups:



In some embodiments, Cy^1 is C_{6-10} aryl, optionally substituted with 1, 2, or 3 substituents independently selected from R^{Cy1}. In some aspects of these embodiments, C_{6-10} aryl is phenyl or naphthyl.

In some embodiments, each Cy^1 is independently selected from C_{6-10} aryl and 5-10 membered heteroaryl, each of which is optionally substituted with 1, 2, or 3 substituents independently selected from R^{Cy1}.

In some embodiments, Cy^1 is C_{3-10} cycloalkyl, optionally substituted with 1, 2, or 3 substituents independently selected from R^{Cy1}. In some aspects of these embodiments, C_{3-10} cycloalkyl is selected from cyclopropyl, cyclobutyl, cyclopentyl, and cyclohexyl.

In some embodiments, Cy^1 is 5-10 membered heteroaryl, optionally substituted with 1, 2, or 3 substituents independently selected from R^{Cy1}. In some aspects of these embodiments, 5-10 membered heteroaryl is selected from thienyl, furyl, pyrrolyl, imidazolyl, thiazolyl, oxazolyl, pyrazolyl, isothiazolyl, isoxazolyl, 1,2,3-triazolyl, tetrazolyl, 1,2,3-thiadiazolyl, 1,2,3-oxadiazolyl, 1,2,4-triazolyl, 1,2,4-thiadiazolyl, 1,2,4-oxadiazolyl, 1,3,4-triazolyl, 1,3,4-thiadiazolyl, 1,3,4-oxadiazolyl, pyridyl, pyrazinyl, pyrimidinyl, triazinyl and pyridazinyl. In other aspects of these embodiments, 5-10 membered heteroaryl is selected from pyridin-2-yl, pyridin-3-yl, and pyridin-4-yl.

In some embodiments, Cy^1 is 4-12 membered heterocycloalkyl, optionally substituted with 1, 2, or 3 substituents independently selected from R^{Cy1}. In some aspects of these embodiments, the 4-12 membered heterocycloalkyl is selected from tetrahydropuranyl, oxetanyl, azetidinyl, morpholinyl, thiomorpholinyl, piperazinyl, tetrahydrofuranyl, tetrahydrothienyl, piperidinyl, pyrrolidinyl, isoxazolidinyl, isothiazolidinyl, pyrazolidinyl, oxazolidinyl, thiazolidinyl, imidazolidinyl, azepanyl, and benzazapenyl.

In some embodiments, each R^{Cy1} is independently selected from C_{1-6} alkyl, C_{1-6} haloalkyl, halo, CN, NO_2, OR^{a2}, $C(O)R^{b2}$, $C(O)NR^{c2}R^{d2}$, $C(O)OR^{a2}$, $NR^{c2}R^{d2}$, $NR^{c2}C(O)R^{b2}$, and $NR^{c2}C(O)OR^{a2}$. In some embodiments, each R^{Cy1} is C_{1-6} alkyl.

In some embodiments, each R^{Cy2} is independently selected from C_{1-6} alkyl, C_{1-6} haloalkyl, halo, CN, NO_2, OR^{a2}, $C(O)R^{b2}$, $C(O)NR^{c2}R^{d2}$, $C(O)OR^{a2}$, $NR^{c2}R^{d2}$, $NR^{c2}C(O)R^{b2}$, and $NR^{c2}C(O)OR^{a2}$. In some embodiments, each R^{Cy2} is C_{1-6} alkyl.

In some embodiments, each R^{Cy3} is independently selected from C_{1-6} alkyl, C_{1-6} haloalkyl, halo, CN, NO_2, OR^{a2}, $C(O)R^{b2}$, $C(O)NR^{c2}R^{d2}$, $C(O)OR^{a2}$, $NR^{c2}R^{d2}$, $NR^{c2}C(O)R^{b2}$, and $NR^{c2}C(O)OR^{a2}$. In some embodiments, each R^{Cy3} is C_{1-6} alkyl.

In some embodiments, R^{a1}, R^{a2}, R^{c1}, R^{c2}, R^{d1}, and R^{d2} are each independently selected from H, C_{1-6} alkyl, Cy^1, $C(O)R^{b3}$, $C(O)NR^{c3}R^{d3}$, $C(O)OR^{a3}$, $S(O)_2R^{b3}$, $S(O)_2NR^{c3}R^{d3}$; wherein said C_{1-6} alkyl is optionally substituted with 1, 2, or 3 substituents independently selected from Cy^1, halo, CN, NO_2, OR^{a3}, $NR^{c3}R^{d3}$, $NR^{c3}C(O)R^{b3}$, $NR^{c3}C(O)OR^{a3}$, and $NR^{c3}S(O)_2R^{b3}$.

In some embodiments, R^{b1} and R^{b2} are each independently selected from C_{1-6} alkyl and Cy^1, wherein said C_{1-6} alkyl is optionally substituted with 1, 2, or 3 substituents independently selected from halo, Cy^1, CN, NO_2, OR^{a3}, $NR^{c3}R^{d3}$, $NR^{c3}C(O)R^{b3}$, $NR^{c3}C(O)OR^{a3}$, and $NR^{c3}S(O)_2R^{b3}$.

In some embodiments, R^{a3}, R^{c3}, and R^{d3} are each independently selected from H, C_{1-6} alkyl, C_{1-4} haloalkyl, C_{6-10} aryl, C_{3-10} cycloalkyl, 5-10 membered heteroaryl, 4-12 membered heterocycloalkyl, each of which is optionally substituted with 1, 2, or 3 substituents independently selected from C_{1-4} haloalkyl, C_{1-4} hydroxyalkyl, C_{1-6} cyanoalkyl, halo, CN, NO_2, OR^{a4}, $NR^{c4}R^{d4}$, $NR^{c4}C(O)R^{b4}$, $NR^{c4}C(O)OR^{a4}$, and $NR^{c4}S(O)_2R^{b4}$.

In some embodiments, each R^{b3} is independently selected from C_{1-6} alkyl, C_{1-4} haloalkyl, C_{6-10} aryl, C_{3-10} cycloalkyl, 5-10 membered heteroaryl, 4-12 membered heterocycloalkyl, each of which is optionally substituted with 1, 2, or 3 substituents independently selected from C_{1-6} alkyl, C_{1-4} haloalkyl, C_{1-4} hydroxyalkyl, C_{1-6}

cyanoalkyl, halo, CN, NO_2, OR^{a4}, $NR^{c4}R^{d4}$, $NR^{c4}C(O)R^{b4}$, $NR^{c4}C(O)OR^{a4}$, and $NR^{c4}S(O)_2R^{b4}$.

In some embodiments, R^{a4}, R^{c4}, and R^{d4} are each independently selected from H, C_{1-6} alkyl, C_{1-4} haloalkyl, C_{1-4} hydroxyalkyl, C_{1-4} cyanoalkyl, C_{6-10} aryl, C_{3-10} cycloalkyl, 5-10 membered heteroaryl, 4-12 membered heterocycloalkyl, each of which is optionally substituted with 1, 2, or 3 substituents independently selected from R^g.

In some embodiments, each R^{b4} is independently selected from C_{1-6} alkyl, C_{1-4} haloalkyl, C_{1-4} hydroxyalkyl, C_{1-4} cyanoalkyl, C_{6-10} aryl, C_{3-10} cycloalkyl, 5-10 membered heteroaryl, 4-12 membered heterocycloalkyl, each of which is optionally substituted with 1, 2, or 3 substituents independently selected from R^g.

In some embodiments, each R^g is independently selected from OH, NO_2, CN, halo, C_{1-6} alkyl, C_{1-4} haloalkyl, C_{1-6} alkoxy, C_{1-6} haloalkoxy, cyano-C_{1-3} alkylene, and HO-C_{1-3} alkylene.

In some embodiments:

each R^1, R^2, R^3, and R^4 is independently selected from H, C_{1-6} alkyl, C_{1-6} haloalkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, and Cy^1; wherein said C_{1-6} alkyl, C_{2-6} alkenyl, and C_{2-6} alkynyl are each optionally substituted with 1, 2 or 3 substituents independently selected from Cy^1, halo, CN, NO_2, OR^{a1}, SR^{a1}, $C(O)R^{b1}$, $C(O)NR^{c1}R^{d1}$, $C(O)OR^{a1}$, $NR^{c1}R^{d1}$, $NR^{c1}C(O)R^{b1}$, $NR^{c1}C(O)OR^{a1}$, $NR^{c1}C(O)NR^{c1}R^{d1}$, $NR^{c1}S(O)_2R^{b1}$, $NR^{c1}S(O)_2NR^{c1}R^{d1}$, $S(O)_2R^{b1}$ and $S(O)_2NR^{c1}R^{d1}$;

or R^1 and R^2 together with the N atom to which they are attached form a 4-12 membered heterocycloalkyl, which is optionally substituted with 1, 2, or 3 substituents independently selected from R^{Cy2};

or R^3 and R^4 together with the N atom to which they are attached form a 4-12 membered heterocycloalkyl, which is optionally substituted with 1, 2, or 3 substituents independently selected from R^{Cy3};

each Cy^1 is independently selected from C_{6-10} aryl and 5-10 membered heteroaryl, each of which is optionally substituted with 1, 2, or 3 substituents independently selected from R^{Cy1};

each R^{Cy1}, R^{Cy2}, and R^{Cy3} is independently selected from C_{1-6} alkyl, C_{1-6} haloalkyl, halo, CN, NO_2, OR^{a2}, $C(O)R^{b2}$, $C(O)NR^{c2}R^{d2}$, $C(O)OR^{a2}$, $NR^{c2}R^{d2}$, $NR^{c2}C(O)R^{b2}$, and $NR^{c2}C(O)OR^{a2}$;

R^{a1}, R^{a2}, R^{c1}, R^{c2}, R^{d1}, and R^{d2} are each independently selected from H, C_{1-6} alkyl, Cy^1, $C(O)R^{b3}$, $C(O)NR^{c3}R^{d3}$, $C(O)OR^{a3}$, $S(O)_2R^{b3}$, and $S(O)_2NR^{c3}R^{d3}$; wherein said C_{1-6} alkyl is optionally substituted with 1, 2, or 3 substituents independently selected from Cy^1, halo, CN, NO_2, OR^{a3}, $NR^{c3}R^{d3}$, $NR^{c3}C(O)R^{b3}$, $NR^{c3}C(O)OR^{a3}$, and $NR^{c3}S(O)_2R^{b3}$;

R^{b1} and R^{b2} are each independently selected from C_{1-6} alkyl and Cy^1, wherein said C_{1-6} alkyl is optionally substituted with 1, 2, or 3 substituents independently selected from halo, Cy^1, CN, NO_2, OR^{a3}, $NR^{c3}R^{d3}$, $NR^{c3}C(O)R^{b3}$, $NR^{c3}C(O)OR^{a3}$, and $NR^{c3}S(O)_2R^{b3}$;

R^{a3}, R^{c3}, and R^{d3} are each independently selected from H, C_{1-6} alkyl, C_{1-4} haloalkyl, C_{6-10} aryl, C_{3-10} cycloalkyl, 5-10 membered heteroaryl, and 4-12 membered heterocycloalkyl, each of which is optionally substituted with 1, 2, or 3 substituents independently selected from C_{1-4} haloalkyl, C_{1-4} hydroxyalkyl, C_{1-6} cyanoalkyl, halo, CN, NO_2, OR^{a4}, $NR^{c4}R^{d4}$, $NR^{c4}C(O)R^{b4}$, $NR^{c4}C(O)OR^{a4}$, and $NR^{c4}S(O)_2R^{b4}$;

each R^{b3} is independently selected from C_{1-6} alkyl, C_{1-4} haloalkyl, C_{6-10} aryl, C_{3-10} cycloalkyl, 5-10 membered heteroaryl, and 4-12 membered heterocycloalkyl, each of which is optionally substituted with 1, 2, or 3 substituents independently selected from C_{1-6} alkyl, C_{1-4} haloalkyl, C_{1-4} hydroxyalkyl, C_{1-6} cyanoalkyl, halo, CN, NO_2, OR^{a4}, $NR^{c4}R^{d4}$, $NR^{c4}C(O)R^{b4}$, $NR^{c4}C(O)OR^{a4}$, and $NR^{c4}S(O)_2R^{b4}$;

R^{a4}, R^{c4}, and R^{d4} are each independently selected from H, C_{1-6} alkyl, C_{1-4} haloalkyl, C_{1-4} hydroxyalkyl, C_{1-4} cyanoalkyl, C_{6-10} aryl, C_{3-10} cycloalkyl, 5-10 membered heteroaryl, and 4-12 membered heterocycloalkyl, each of which is optionally substituted with 1, 2, or 3 substituents independently selected from R^g;

each R^{b4} is independently selected from C_{1-6} alkyl, C_{1-4} haloalkyl, C_{1-4} hydroxyalkyl, C_{1-4} cyanoalkyl, C_{6-10} aryl, C_{3-10} cycloalkyl, 5-10 membered heteroaryl, and 4-12 membered heterocycloalkyl, each of which is optionally substituted with 1, 2, or 3 substituents independently selected from R^g; and

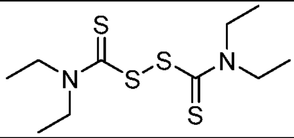
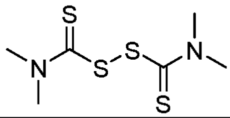
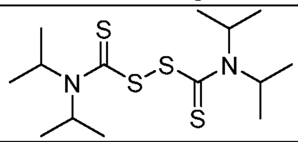
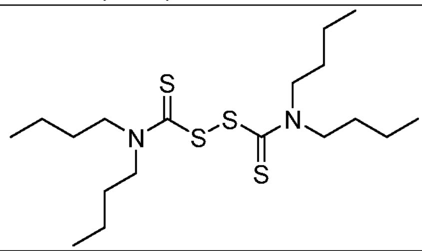
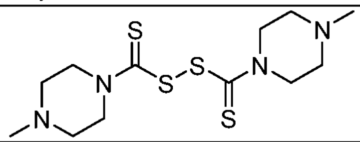
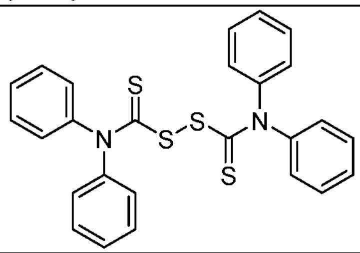
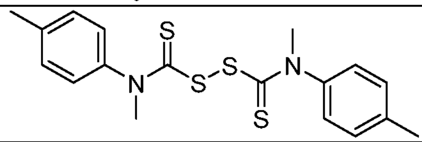
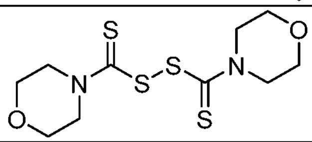
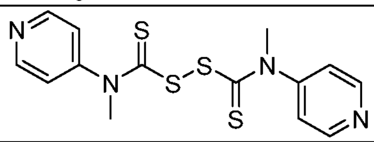
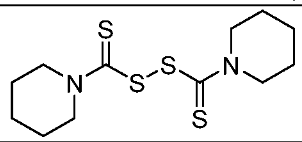
each R^g is independently selected from OH, NO_2, CN, halo, C_{1-6} alkyl, C_{1-4} haloalkyl, C_{1-6} alkoxy, C_{1-6} haloalkoxy, cyano-C_{1-3} alkylene, and HO-C_{1-3} alkylene.

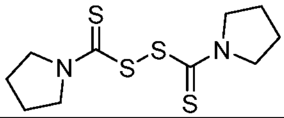
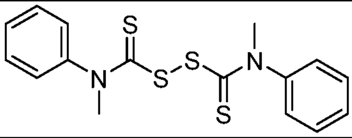
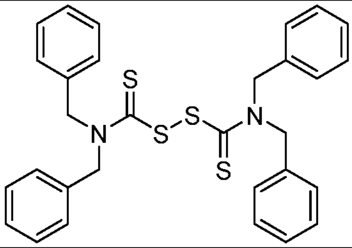
In some aspects of the foregoing embodiments:

R^1, R^2, R^3, and R^4 are each independently selected from Cy^1 and C_{1-6} alkyl optionally substituted with Cy^1.

In some embodiments, the compound of Formula (I) is selected from any one of compounds listed in Table A below:

Table A

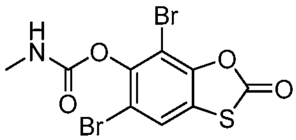
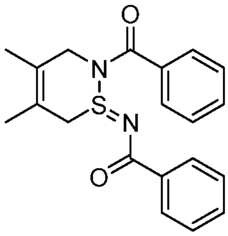
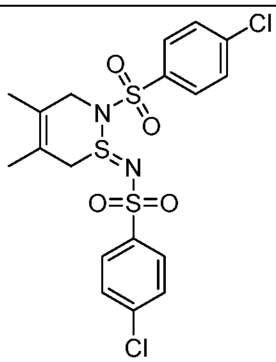
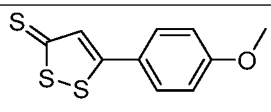
C-23	
C-23A1	
C-23A2	
C-23A3	
C-23A4	
C-23A5	
C-23A6	
C-23A7	
C-23A8	
C-23A9	

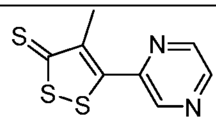
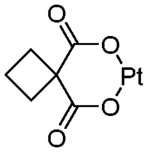
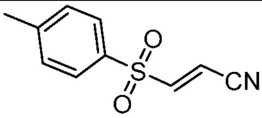
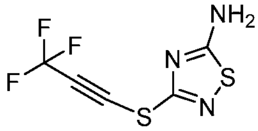
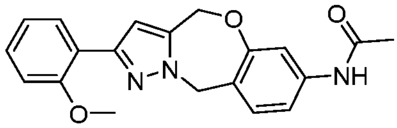
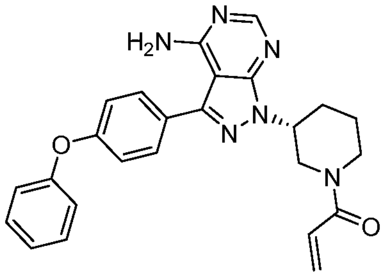
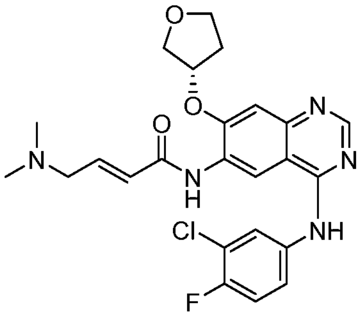
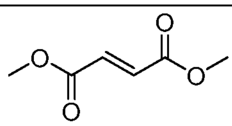
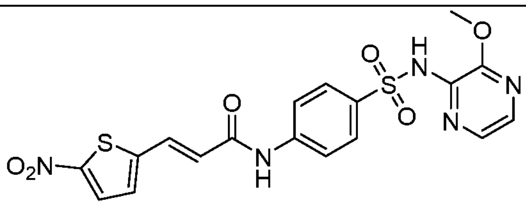
C-23A10	
C-23A11	
C-23A12	

, or a pharmaceutically acceptable salt thereof.

In some embodiments, the compound of Formula (I) is not any one of the compounds listed in Table (A).

In some embodiments, the present application provides any one of the following compounds:

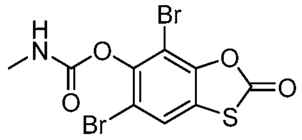
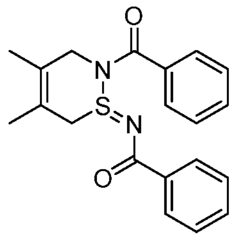
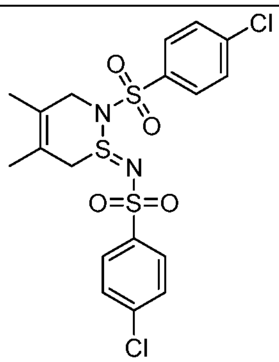
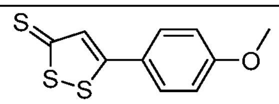
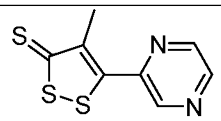
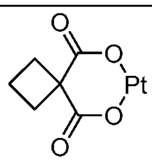
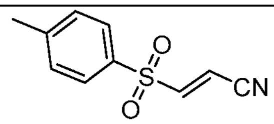
C-5	
C-7	
C-8	
C-22	

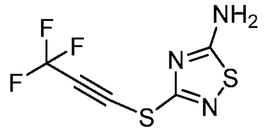
C-24	
C-25	
Bay 11-7082	
ASN-08966899	
LDC7559	
ibrutinib	
Afatinib	
Dimethyl fumarate	
Necrosulfonamide	

, or a pharmaceutically acceptable salt thereof.

In some embodiments, the compound of the present application is not C-5, C-7, C-8, C-22, C-24, C-25, Bay 11-7082, ASN-08966899, LDC7559, ibrutinib, afatinib, dimethyl fumarate, or necrosulfonamide.

In some embodiments, the present application provides any one of the following compounds:

C-5	
C-7	
C-8	
C-22	
C-24	
C-25	
Bay 11-7082	

ASN-08966899	

, or a pharmaceutically acceptable salt thereof.

In some embodiments, the compound of the present application is not C-5, C-7, C-8, C-22, C-24, C-25, Bay 11-7082, or ASN-08966899.

Pharmaceutically acceptable salts

In some embodiments, a salt of a compound disclosed herein is formed between an acid and a basic group of the compound, such as an amino functional group, or a base and an acidic group of the compound, such as a carboxyl functional group. According to another embodiment, the compound is a pharmaceutically acceptable acid addition salt.

In some embodiments, acids commonly employed to form pharmaceutically acceptable salts of the compounds of the present disclosure include inorganic acids such as hydrogen bisulfide, hydrochloric acid, hydrobromic acid, hydroiodic acid, sulfuric acid and phosphoric acid, as well as organic acids such as para-toluenesulfonic acid, salicylic acid, tartaric acid, bitartaric acid, ascorbic acid, maleic acid, besylic acid, fumaric acid, gluconic acid, glucuronic acid, formic acid, glutamic acid, methanesulfonic acid, ethanesulfonic acid, benzenesulfonic acid, lactic acid, oxalic acid, para-bromophenylsulfonic acid, carbonic acid, succinic acid, citric acid, benzoic acid and acetic acid, as well as related inorganic and organic acids. Such pharmaceutically acceptable salts thus include gluconate, sulfate, pyrosulfate, bisulfate, sulfite, bisulfite, phosphate, monohydrogenphosphate, dihydrogenphosphate, metaphosphate, pyrophosphate, chloride, bromide, iodide, acetate, propionate, decanoate, caprylate, acrylate, formate, isobutyrate, caprate, heptanoate, propiolate, oxalate, malonate, succinate, suberate, sebacate, fumarate, maleate, butyne-1,4-dioate, hexyne-l,6-dioate, benzoate, chlorobenzoate, methylbenzoate, dinitrobenzoate, hydroxybenzoate, methoxybenzoate, phthalate, terephthalate, sulfonate, xylene sulfonate, phenylacetate, phenylpropionate, phenylbutyrate, citrate, lactate, β-hydroxybutyrate, glycolate, maleate, tartrate, methanesu1fonate, propanesulfonate, naphthalene-1-sulfonate, naphthalene-2-sulfonate, mandelate and other salts. In one embodiment, pharmaceutically acceptable acid addition salts include those formed with mineral acids such as hydrochloric acid and hydrobromic acid, and especially those formed with organic acids such as maleic acid.

In some embodiments, bases commonly employed to form pharmaceutically acceptable salts of the compounds of the present disclosure include hydroxides of alkali metals, including sodium, potassium, and lithium; hydroxides of alkaline earth metals such as calcium and magnesium; hydroxides of other metals, such as aluminum and zinc; ammonia, organic amines such as unsubstituted or hydroxyl-substituted mono-, di-, or tri-alkylamines, dicyclohexylamine; tributyl amine; pyridine; N-methyl, N-ethylamine; diethylamine; triethylamine; mono-, bis-, or tris-(2-OH-(C1-C6)-alkylamine), such as N,N-dimethyl-N-(2-hydroxyethyl)amine or tri-(2-hydroxyethyl)amine; N-methyl-D-glucamine; morpholine; thiomorpholine; piperidine; pyrrolidine; and amino acids such as arginine, lysine, and the like.

In some embodiments, the compounds disclosed herein, or pharmaceutically acceptable salts thereof, are substantially isolated.

Methods of making

Compounds disclosed herein, including salts thereof, can be prepared using known organic synthesis techniques and can be synthesized according to any of numerous possible synthetic routes. A person skilled in the art knows how to select and implement appropriate synthetic protocols, and appreciates that a broad repertoire of synthetic organic reactions is available to be potentially employed in synthesizing compounds provided herein.

Suitable synthetic methods of starting materials, intermediates and products may be identified by reference to the literature, including reference sources such as: *Advances in Heterocyclic Chemistry*, Vols. 1-107 (Elsevier, 1963-2012); *Journal of Heterocyclic Chemistry* Vols. 1-49 (Journal of Heterocyclic Chemistry, 1964-2012); Carreira, *et al.* (Ed.) *Science of Synthesis*, Vols. 1-48 (2001-2010) and Knowledge Updates KU2010/1-4; 2011/1-4; 2012/1-2 (Thieme, 2001-2012); Katritzky, *et al.* (Ed.) *Comprehensive Organic Functional Group Transformations*, (Pergamon Press, 1996); Katritzky *et al.* (Ed.); *Comprehensive Organic Functional Group Transformations II* (Elsevier, 2nd Edition, 2004); Katritzky *et al.* (Ed.), *Comprehensive Heterocyclic Chemistry* (Pergamon Press, 1984); Katritzky *et al.*, *Comprehensive*

Heterocyclic Chemistry II, (Pergamon Press, 1996); Smith *et al.*, *March's Advanced Organic Chemistry: Reactions, Mechanisms, and Structure*, 6th Ed. (Wiley, 2007); Trost *et al.* (Ed.), *Comprehensive Organic Synthesis* (Pergamon Press, 1991).

The reactions for preparing the compounds provided herein can be carried out in suitable solvents which can be readily selected by one of skill in the art of organic synthesis. Suitable solvents can be substantially non-reactive with the starting materials (reactants), the intermediates, or products at the temperatures at which the reactions are carried out, e.g., temperatures which can range from the solvent's freezing temperature to the solvent's boiling temperature. A given reaction can be carried out in one solvent or a mixture of more than one solvent. Depending on the particular reaction step, suitable solvents for a particular reaction step can be selected by the skilled artisan.

Preparation of the compounds provided herein can involve the protection and deprotection of various chemical groups. The need for protection and deprotection, and the selection of appropriate protecting groups, can be readily determined by one skilled in the art. The chemistry of protecting groups can be found, for example, in P. G. M. Wuts and T. W. Greene, *Protective Groups in Organic Synthesis*, 4th Ed., Wiley & Sons, Inc., New York (2006).

Methods of use

Referring to Figure 67, the inflammatory cascade begin when pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs), also known as alarmins, are sensed by cell surface and endosomal pattern recognition receptors (PRR), such as Toll-like receptors (TLR) and C-type lectin receptors (CLR), and cytosolic sensors. Examples of PAMPs and DAMPs include LPS, bacterial toxins, bacterial proteins and nucleic acids, particulates (such as uric acid and cholesterol crystals and amyloid-β fibrils), hyaluronan, and extracellular ATP. In response, cellular mechanisms activate pro-caspase canonical or non-canonical inflammasomes, leading to the release of active inflammatory caspases. Examples of the inflammatory caspases include caspase-1, caspse-11, as well as caspase-4 and caspase-5. The activation of caspases in inflammasomes leads to caspase cleavage of cytoplasmic protein gasdermin, which produces a gasdermin N-terminal fragment (gasdermin-NT). In some instances, the caspase-cleavable gasdermin protein is selected from the following members of the gasdermin family: GSDMA, GSMDB, GSDMC, GSDMD, DFNA5, and DFNB59. The gasdermin-NT then binds to the cell membrane from the cytosolic side to form pores that permeabilize the cell membrane causing cytokine secretion and pyroptosis. DFNA5 is activated by caspase-3 during classical apoptosis. The proteases that activate the other gasdermins are currently not known, but are not caspases and may be activated independently of inflammasomes. Typically, gasdermin binds to acidic lipids that are restricted to the inner leaflet of mammalian membranes, such as phosphatidylinositol phosphates (PIPs), phosphatidylserine (PS) and phosphatidic acid (PA), and the bacterial and mitochondrial lipid cardiolipin. Typically, the gasdermin genes are expressed in epithelial and immune cells of a variety of tissues, and all are able to form pores when cleaved by an inflammatory caspase. In one example, canonical inflammasome activation activates caspase-1, which cleaves pro-IL-1β, pro-IL-18 and gasdermin D, which forms pores needed to release processed inflammatory cytokine IL-1β.

The compounds of the present disclosure efficiently block gasdermin pore formation and therefore block any of the individual downstream mediators. These compounds, therefore, are more efficient in inhibiting inflammation than anti-inflammatory agents that inhibits an individual upstream or downstream inflammatory pathway, such as those that have been clinically tested (IL-1 receptor antagonist, TNFα antibodies). The compounds are also more efficient in mediating multiple difficult-to-control dysregulatory events that kill the patient, such as disseminated intravascular coagulation (inhibited with activated protein C infusion). Inhibition of gasdermin (e.g., gasdermin D) by the compounds of the present application prevents cytokine storm. This is more effective than conventional anti-inflammatory treatments which try to reduce complications of cytokine storm once it is underway. Similarly, the compounds of the present application are also more efficient than agents that neutralize LPS or its extracellular receptors (TLR4, CD14). Since gram- bacteria elaborate many PAMPs (toxins, flagella, rod proteins), not all of which are known, neutralizing LPS may not prevent gram- sepsis, especially in humans who are LPS hypersensitive, if LPS inhibition is incomplete. TLR4 may be a less important sensor of LPS than the non-canonical inflammasome, which is constitutively expressed in humans not just in immune antigen-presenting cells, but also at mucosal epithelia.

LPS is a very important trigger and if inhibiting it or its first detection is unsuccessful, then inhibiting one of the other PAMP or DAMP sensors would also be effective in, e.g., pleiotropically triggered sepsis in humans, where the triggering PAMP is generally not known at the time treatment is needed. Additionally, the compounds of the present application are also more efficient than individual inhibitors of inflammatory caspases. This is because potential cross-reactivity of these inhibitors on apoptotic caspases and other cysteine proteases might result in unwanted toxicity (e.g., liver fibrosis). Unwanted inhibition of caspase-8 can also trigger necroptosis. In some embodiments, inhibition of gasdermin pore formation occurs as a result of the compound of the present application reacting with a cysteine in a gasdermin protein. In some embodiments, the cysteine is Cys191. In some embodiments, the compound also reacts with a cysteine of an inflammatory signaling molecule selected from: a sensor, an adaptor, and a transcription factor, or a regulator thereof. In some embodiments, the compound's promiscuous reactivity with the protein cysteine residues does not result in any undesired toxicity and does not negatively affect the compound's efficacy.

In some instances, the compounds of the present application are useful in treating or preventing inflammatory disorders or ameliorating symptoms associated with these disorders. Such disorders typically result in the immune system attacking the body's own cells or tissues and include sepsis (e.g., acute sepsis), alopecia, hearing loss syndrome, gout, arthritis, rheumatoid arthritis, sclerosis, inflammatory bowel disease, ankylosing spondylitis (AS), antiphospholipid antibody syndrome (APS), myositis, scleroderma, Sjogren's syndrome, systemic lupus erythematosus, vasculitis, familial mediterranean fever, neonatal onset multisystem inflammatory disease, Behçet's disease, dermatosis, type 1 diabetes, autoimmune disease, psoriasis, psoriatic arthritis, multiple sclerosis, Addison's disease, Graves' disease, Hashimoto's thyroiditis, myasthenia gravis, pernicious anemia, celiac disease, chronic inflammation, rheumatism, encephalomyelitis, postinfectious cerebellitis, neuromyelitis optica (e.g., Devic disease), encephalitis, metabolic encephalopathy, asthma, periodontitis, ulcerative colitis, Crohn's disease, sinusitis, atherosclerosis, hypercholesterolemia, and peptic ulcer. In some instances, the inflammatory diseases include eye diseases such as glaucoma, dry eye, and retinal ischemia-reperfusion. In some instances, the inflammatory diseases include chronic lung diseases and injuries, and NASH and other inflammatory liver diseases. In some instances, in inflammatory disease is a genetic auto-inflammatory condition.

Symptoms associated with inflammatory disorders typically include chronic pain, redness, swelling of joints and other tissues, stiffness, fever, buildup of blood protein in organs, hair loss, fatigue, and damage to normal tissues. The compounds of the present application are useful in ameliorating these symptoms.

In some instances, the compounds of the present application are useful in treating sepsis, or ameliorating symptoms associated with this condition. Examples of symptoms associated with sepsis include vascular leak, circulatory collapse, coagulation activation and multiorgan failure. Without proper treatment, sepsis is fatal in about a third of cases. It is the leading cause of death of newborns and small children in the world and contributes to 1 in every 2 or 3 deaths of hospitalized adults in the US. Current treatment of sepsis is limited to antibiotics and supportive care, and over 100 clinical trials designed to quiet the immune response to infection have failed to produce a single new effective therapy. Advantageously, the compound of the present application reduce innate immune response to disseminated and poorly controlled infection and successfully treat sepsis.

In some instances, the compounds of the present application may be used for preventing sepsis, for example, in patients that are at high risk for developing sepsis. Suitable examples of such patients include neutropenic patients undergoing bone marrow transplant.

In some instances, the compounds of the present disclosure are useful in treating or preventing a cardiovascular disease. Examples of such diseases include stroke, heart failure, hypertensive heart disease, rheumatic heart disease, cardiomyopathy, heart arrhythmia, congenital heart disease, valvular heart disease, carditis, aortic aneurysms, peripheral artery disease, thromboembolic disease, coronary artery disease, myocardial infarction and venous thrombosis.

In some instances, the compounds of the present disclosure are useful in treating or preventing a metabolic disorder. Examples of such disorders include metabolic syndrome, type II diabetes, cystinosis, cystinuria, Fabry disease, galactosemia, Gaucher disease (type I), Hartnup disease, homocystinuria, Hunter syndrome, Hurler syndrome, Lesch-Nyhan syndrome, maple syrup urine disease, Maroteaux-Lamy syndrome, Morquio syndrome, Niemann-Pick disease (type A), phenylketonuria, Pompe disease, porphyria, Scheie syndrome, Tay-Sachs disease, tyrosinemia (hepatorenal), and von Gierke disease.

In some instances, the compounds of the present application are useful in treating or preventing a neurodegenerative disease. Examples of such diseases include Alzheimer's disease, Parkinson's disease, multiple sclerosis, dementia, frontotemporal dementia, Huntington's disease, Amyotrophic lateral sclerosis (ALS), motor neuron disease, and schizophrenia.

There can be an inflammatory component to any disease, especially if infection or cell death is involved in the disease. Hence, the compounds of the present application are useful in treating or preventing such disease. Suitable examples of such disease include infections caused by a Gram-positive bacteria, polymicrobial infection, infections caused by parasites (e.g., malaria, toxoplasmosis, trypanosomiasis, leishmania), transplant rejections, inflammation in the eye (e.g., retinitis, uveitis), and cancer.

Combination treatments

In some instances, the method of using a compound described herein, or a pharmaceutically acceptable salt thereof, includes administering the compound to a subject in combination with at least one additional therapeutic agent. In this method, the compound and the additional therapeutic agent may be administered to the subject simultaneously (e.g., in the same dosage form or in separate dosage forms), or consecutively (e.g., additional therapeutic agent may be administered before or after the compound of the present disclosure, or a pharmaceutically acceptable salt thereof).

In some instances, an additional therapeutic agent includes an anti-inflammatory agent. Suitable examples include nonsteroidal anti-inflammatory drugs such as celecoxib, rofecoxib, ibuprofen, naproxen, aspirin, diclofenac, sulindac, oxaprozin, piroxicam, indomethacin, meloxicam, fenoprofen, diflunisal, BAY 11-7082, or a pharmaceutically acceptable salt thereof. Suitable examples of steroid (e.g., corticosteroid) anti-inflammatory agents include cortisol, corticosterone, hydrocortisone, aldosterone, deoxycorticosterone, triamcinolone, bardoxolone, bardoxolone methyl, triamcinolone, cortisone, prednisone, and methylprednisolone, or a pharmaceutically acceptable salt thereof. Other suitable examples of anti-inflammatory agents include proteins such as anti-inflammatory antibodies (e.g., anti-IL-1, anti-TNF), and integrins.

In some instances, an additional therapeutic agent is an antibiotic. Such an antibiotic may be selected from: a quinolone, a β-lactam, a cephalosporin, a penicillin, a carbapenem, a lipopetide, an aminoglycoside, a glycopeptide, a macrolide, an ansamycin, a sulfonamide, a monobactam, oxazolidinone, lipopeptide, macrolide, and a cationic antimicrobial peptide (CAMP).

Suitable examples of cationic antimicrobial peptides include a defensin peptide (e.g., defensin 1 such as beta-defensin 1 or alpha-defensin 1), or cecropin, andropin, moricin, ceratotoxin, melittin, magainin, dermaseptin, bombinin, brevinin (e.g., brevinin-1), esculentin, buforin II (e.g., from amphibians), CAP18 (e.g., from rabbits), LL37 (e.g., from humans), abaecin, apidaecins (e.g., from honeybees), prophenin (e.g., from pigs), indolicidin (e.g., from cattle), brevinins, protegrin (e.g., from pig), tachyplesins (e.g., from horseshoe crabs), and drosomycin (e.g., from fruit flies).

Suitable examples of quinoline antibiotics include levofloxacin, norfloxacin, ofloxacin, ciprofloxacin, perfloxacin, lomefloxacin, fleroxacin, sparfloxacin, grepafloxacin, trovafloxacin, clinafloxacin, gemifloxacin, enoxacin, sitafloxacin, nadifloxacin, tosulfloxacin, cinnoxacin, rosoxacin, miloxacin, moxifloxacin, gatifloxacin, cinnoxacin, enoxacin, fleroxacin, lomafloxacin, lomefloxacin, miloxacin, nalidixic acid, nadifloxacin, oxolinic acid, pefloxacin, pirimidic acid, pipemidic acid, rosoxacin, rufloxacin, temafloxacin, tosufloxacin, trovafloxacin, and besifloxacin.

Suitable examples of cephalosporin antibiotics include cefazolin, cefuroxime, ceftazidime, cephalexin, cephaloridine, cefamandole, cefsulodin, cefonicid, cefoperazine, cefoprozil, and ceftriaxone.

Suitable examples of penicillin antibiotics include penicillin G, penicillin V, procaine penicillin, and benzathine penicillin, ampicillin, and amoxicillin, benzylpenicillin, phenoxymethylpenicillin, oxacillin, methicillin, dicloxacillin, flucloxacillin, temocillin, azlocillin, carbenicillin, ricarcillin, mezlocillin, piperacillin, apalcillin, hetacillin, bacampicillin, sulbenicillin, mecicilam, pevmecillinam, ciclacillin, talapicillin, aspoxicillin, cloxacillin, nafcillin, and pivampicillin.

Suitable examples of carbapenem antibiotics include thienamycin, tomopenem, lenapenem, tebipenem, razupenem, imipenem, meropenem, ertapenem, doripenem, panipenem (betamipron), and biapenem.

Suitable examples of lipopeptide antibiotics include polymyxin B, colistin (polymyxin E), and daptomycin.

Suitable examples of aminoglycoside antibiotics include gentamicin, amikacin, tobramycin, debekacin, kanamycin, neomycin, netilmicin, paromomycin, sisomycin, spectinomycin, and streptomycin.

Suitable examples of glycopeptide antibiotics include vancomycin, teicoplanin, telavancin, ramoplanin, daptomycin, decaplanin, and bleomycin.

Suitable examples of macrolide antibiotics include azithromycin, clarithromycin, erythromycin, fidaxomicin, telithromycin, carbomycin A, josamycin, kitasamycin, midecamycin/midecamycinacetate, oleandomycin, solithromycin, spiramycin, troleandomycin, tylosin/tylocine, roxithromycin, dirithromycin, troleandomycin, spectinomycin, methymycin, neomethymycin, erythronolid, megalomycin, picromycin, narbomycin, oleandomycin, triacetyl-oleandomycin, laukamycin, kujimycin A, albocyclin and cineromycin B.

Suitable examples of ansamycin antibiotics is include streptovaricin, geldanamycin, herbimycin, rifamycin, rifampin, rifabutin, rifapentine and rifamixin.

Suitable examples of sulfonamide antibiotics include sulfanilamide, sulfacetarnide, sulfapyridine, sulfathiazole, sulfadiazine, sulfamerazine, sulfadimidine, sulfasomidine, sulfasalazine, mafenide, sulfamethoxazole, sulfamethoxypyridazine, sulfadimethoxine, sulfasymazine, sulfadoxine, sulfametopyrazine, sulfaguanidine, succinylsulfathiazole and phthalylsulfathiazole.

Pharmaceutical compositions

The present application also provides pharmaceutical compositions comprising an effective amount of a compound disclosed herein, or a pharmaceutically acceptable salt thereof; and a pharmaceutically acceptable carrier. The pharmaceutical composition may also comprise any one of the additional therapeutic agents described herein. In certain embodiments, the application also provides pharmaceutical compositions and dosage forms comprising any one the additional therapeutic agents described herein. The carrier(s) are “acceptable” in the sense of being compatible with the other ingredients of the formulation and, in the case of a pharmaceutically acceptable carrier, not deleterious to the recipient thereof in an amount used in the medicament.

Pharmaceutically acceptable carriers, adjuvants and vehicles that may be used in the pharmaceutical compositions of the present application include, but are not limited to, ion exchangers, alumina, aluminum stearate, lecithin, serum proteins, such as human serum albumin, buffer substances such as phosphates, glycine, sorbic acid, potassium sorbate, partial glyceride mixtures of saturated vegetable fatty acids, water, salts or electrolytes, such as protamine sulfate, disodium hydrogen phosphate, potassium hydrogen phosphate, sodium chloride, zinc salts, colloidal silica, magnesium trisilicate, polyvinyl pyrrolidone, cellulose-based substances, polyethylene glycol, sodium carboxymethylcellulose, polyacrylates, waxes, polyethylene-polyoxypropylene-block polymers, polyethylene glycol, and wool fat.

The compositions or dosage forms may contain any one of the compounds and therapeutic agents described herein in the range of 0.005% to 100% with the balance made up from the suitable pharmaceutically acceptable excipients. The contemplated compositions may contain 0.001%-100% of any one of the compounds and therapeutic agents provided herein, in one embodiment 0.1-95%, in another embodiment 75-85%, in a further embodiment 20-80%, wherein the balance may be made up of any pharmaceutically acceptable excipient described herein, or any combination of these excipients.

Routes of administration and dosage forms

The pharmaceutical compositions of the present application include those suitable for any acceptable route of administration. Acceptable routes of administration include, but are not limited to, buccal, cutaneous, endocervical, endosinusial, endotracheal, enteral, epidural, interstitial, intra-abdominal, intra-arterial, intrabronchial, intrabursal, intracerebral, intracisternal, intracoronary, intradermal, intraductal, intraduodenal, intradural, intraepidermal, intraesophageal, intragastric, intragingival, intraileal, intralymphatic, intramedullary, intrameningeal, intramuscular, intranasal, intraovarian, intraperitoneal, intraprostatic, intrapulmonary, intrasinal, intraspinal, intrasynovial, intratesticular, intrathecal, intratubular, intratumoral, intrauterine, intravascular, intravenous, nasal, nasogastric, oral, parenteral, percutaneous, peridural, rectal, respiratory (inhalation), subcutaneous, sublingual, submucosal, topical, transdermal, transmucosal, transtracheal, ureteral, urethral and vaginal.

Compositions and formulations described herein may conveniently be presented in a unit dosage form, e.g., tablets, sustained release capsules, and in liposomes, and may be prepared by any methods well known in the art of pharmacy. See, for example, Remington: The Science and Practice of Pharmacy, Lippincott Williams & Wilkins, Baltimore, MD (20th ed. 2000). Such preparative methods include the step of bringing into association with the molecule to be administered ingredients such as the carrier that constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers, liposomes or finely divided solid carriers, or both, and then, if necessary, shaping the product.

In some embodiments, any one of the compounds and therapeutic agents disclosed herein are administered orally. Compositions of the present application suitable for oral administration may be presented as discrete units such as capsules, sachets, granules or tablets each containing a predetermined amount (e.g., effective amount) of the active ingredient; a powder or granules; a solution or a suspension in an aqueous liquid or a non-aqueous liquid; an oil-in-water liquid emulsion; a water-in-oil liquid emulsion; packed in liposomes; or as a bolus, etc. Soft gelatin capsules can be useful for containing such suspensions, which may beneficially increase the rate of compound absorption. In the case of tablets for oral use, carriers that are commonly used include lactose, sucrose, glucose, mannitol, and silicic acid and starches. Other acceptable excipients may include: a) fillers or extenders such as starches, lactose, sucrose, glucose, mannitol, and silicic acid, b) binders such as, for example, carboxymethylcellulose, alginates, gelatin, polyvinylpyrrolidinone, sucrose, and acacia, c) humectants such as glycerol, d) disintegrating agents such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate, e) solution retarding agents such as paraffin, f) absorption accelerators such as quaternary ammonium compounds, g) wetting agents such as, for example, cetyl alcohol and glycerol monostearate, h) absorbents such as kaolin and bentonite clay, and i) lubricants such as talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, and mixtures thereof. For oral administration in a capsule form, useful diluents include lactose and dried corn starch. When aqueous suspensions are administered orally, the active ingredient is combined with emulsifying and suspending agents. If desired, certain sweetening and/or flavoring and/or coloring agents may be added. Compositions suitable for oral administration include lozenges comprising the ingredients in a flavored basis, usually sucrose and acacia or tragacanth; and pastilles comprising the active ingredient in an inert basis such as gelatin and glycerin, or sucrose and acacia.

Compositions suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions or infusion solutions which may contain antioxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example, sealed ampules and vials, and may be stored in a freeze dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example water for injections, saline (e.g., 0.9% saline solution) or 5% dextrose solution, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets. The injection solutions may be in the form, for example, of a sterile injectable aqueous or oleaginous suspension. This suspension may be formulated according to techniques known in the art using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example, as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are mannitol, water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose, any bland fixed oil may be employed including synthetic mono- or diglycerides. Fatty acids, such as oleic acid and its glyceride derivatives are useful in the preparation of injectables, as are natural pharmaceutically-acceptable oils, such as olive oil or castor oil, especially in their polyoxyethylated versions. These oil solutions or suspensions may also contain a long-chain alcohol diluent or dispersant.

The pharmaceutical compositions of the present application may be administered in the form of suppositories for rectal administration. These compositions can be prepared by mixing a compound of the present application with a suitable non-irritating excipient which is solid at room temperature but liquid at the rectal temperature and therefore will melt in the rectum to release the active components. Such materials include, but are not limited to, cocoa butter, beeswax, and polyethylene glycols.

The pharmaceutical compositions of the present application may be administered by nasal aerosol or inhalation. Such compositions are prepared according to techniques well-known in the art of pharmaceutical formulation and may be prepared as solutions in saline, employing benzyl alcohol or other suitable preservatives, absorption promoters to enhance bioavailability, fluorocarbons, and/or other solubilizing or dispersing agents known in the art. See, for example, U.S. Patent No. 6,803,031. Additional formulations and methods for intranasal administration are found in Ilium, L., *J Pharm Pharmacol*, 56:3-17, 2004 and Ilium, L., *Eur J Pharm Sci* 11:1-18, 2000.

The topical compositions of the present disclosure can be prepared and used in the form of an aerosol spray, cream, emulsion, solid, liquid, dispersion, foam, oil, gel, hydrogel, lotion, mousse, ointment, powder, patch, pomade, solution, pump spray, stick, towelette, soap, or other forms commonly employed in the art of topical administration and/or cosmetic and skin care formulation. The topical compositions can be in an emulsion form. Topical administration of the pharmaceutical compositions of the present application is especially useful when the desired treatment involves areas or organs readily accessible by topical application. In some embodiments, the topical composition comprises a combination of any one of the compounds and therapeutic agents disclosed herein, and one or more additional ingredients, carriers, excipients, or diluents including, but not limited to, absorbents, anti-irritants, anti-acne agents, preservatives, antioxidants, coloring agents/pigments, emollients (moisturizers), emulsifiers, film-forming/holding agents, fragrances, leave-on exfoliants, prescription drugs, preservatives, scrub agents, silicones, skin-identical/repairing agents, slip agents, sunscreen actives, surfactants/detergent cleansing agents, penetration enhancers, and thickeners.

The compounds and therapeutic agents of the present application may be incorporated into compositions for coating an implantable medical device, such as prostheses, artificial valves, vascular grafts, stents, or catheters. Suitable coatings and the general preparation of coated implantable devices are known in the art and are exemplified in U.S. Patent Nos. 6,099,562; 5,886,026; and 5,304,121. The coatings are typically biocompatible polymeric materials such as a hydrogel polymer, polymethyldisiloxane, polycaprolactone, polyethylene glycol, polylactic acid, ethylene vinyl acetate, and mixtures thereof. The coatings may optionally be further covered by a suitable topcoat of fluorosilicone, polysaccharides, polyethylene glycol, phospholipids or combinations thereof to impart controlled release characteristics in the composition. Coatings for invasive devices are to be included within the definition of pharmaceutically acceptable carrier, adjuvant or vehicle, as those terms are used herein.

According to another embodiment, the present application provides an implantable drug release device impregnated with or containing a compound or a therapeutic agent, or a composition comprising a compound of the present application or a therapeutic agent, such that said compound or therapeutic agent is released from said device and is therapeutically active.

Dosages and regimens

In the pharmaceutical compositions of the present application, a compound described herein is present in an effective amount (e.g., a therapeutically effective amount).

Effective doses may vary, depending on the diseases treated, the severity of the disease, the route of administration, the sex, age and general health condition of the subject, excipient usage, the possibility of co-usage with other therapeutic treatments such as use of other agents and the judgment of the treating physician.

In some embodiments, the compounds of the present application are used at concentrations that are readily and safely achieved in human blood and tissues.

In some embodiments, an effective amount of a compound of described herein can range, for example, from about 0.001 mg/kg to about 500 mg/kg (e.g., from about 0.001 mg/kg to about 200 mg/kg; from about 0.01 mg/kg to about 200 mg/kg; from about 0.01 mg/kg to about 150 mg/kg; from about 0.01 mg/kg to about 100 mg/kg; from about 0.01 mg/kg to about 50 mg/kg; from about 0.01 mg/kg to about 10 mg/kg; from about 0.01 mg/kg to about 5 mg/kg; from about 0.01 mg/kg to about 1 mg/kg; from about 0.01 mg/kg to about 0.5 mg/kg; from about 0.01 mg/kg to about 0.1

mg/kg; from about 0. 1 mg/kg to about 200 mg/kg; from about 0. 1 mg/kg to about 150 mg/kg; from about 0. 1 mg/kg to about 100 mg/kg; from about 0.1 mg/kg to about 50 mg/kg; from about 0. 1 mg/kg to about 10 mg/kg; from about 0.1 mg/kg to about 5 mg/kg; from about 0.1 mg/kg to about 2 mg/kg; from about 0.1 mg/kg to about 1 mg/kg; or from about 0.1 mg/kg to about 0.5 mg/kg).

In some embodiments, an effective amount of a compound described herein is about 0.1 mg/kg, about 0.5 mg/kg, about 1 mg/kg, about 2 mg/kg, about 5 mg/kg, about 10 mg/kg, about 15 mg/kg, about 20 mg/kg, about 25 mg/kg, about 30 mg/kg, about 35 mg/kg, about 40 mg/kg, about 45 mg/kg, about 50 mg/kg, about 60 mg/kg, about 70 mg/kg, about 80 mg/kg, about 90 mg/kg, about 100 mg/kg, or about 150 mg/kg.

The foregoing dosages can be administered on a daily basis (e.g., as a single dose or as two or more divided doses, e.g., once daily, twice daily, thrice daily) or non-daily basis (e.g., every other day, every two days, every three days, once weekly, twice weekly, once every two weeks, once a month).

Kits

The present disclosure also provides pharmaceutical kits useful, for example, in the treatment of disorders, diseases and conditions referred to herein, which include one or more containers containing a pharmaceutical composition comprising a therapeutically effective amount of a compound of the present disclosure. Such kits can further include, if desired, one or more of various conventional pharmaceutical kit components, such as, for example, containers with one or more pharmaceutically acceptable carriers, additional containers, etc. Instructions, either as inserts or as labels, indicating quantities of the components to be administered, guidelines for administration, and/or guidelines for mixing the components, can also be included in the kit. The kit may optionally include any one of the additional therapeutic agents described herein, or a pharmaceutically acceptable salt thereof, in any one of amounts and dosage forms described herein.

Screening assay

In some instances, the present application provides a screening assay to identify an inhibitor of, e.g., a gasdermin pore formation, inflammasome-mediated cell death (pyroptosis), cellular cytokine secretion, and/or an inflammatory caspase. Referring to Figure 1, in such an assay, a sample may include a liposome that is formed such that a metal cation is trapped inside the liposome. The sample may also include a full-length gasdermin protein containing a protease cleavage site, a test compound, and a ligand that is capable of forming a complex with the metal cation that is trapped inside the liposome. In order to determine that the compound inhibits pore formation, a protease enzyme is added to the sample. The protease enzyme cleaves an N-terminal gasdermin fragment from the full-length gasdermin protein. In the absence of the test compound or if the test compound is inactive in the assay, these NT fragments then bind to the lipids of the liposome and form a pore in the liposome, through which the metal cation leaks out of the liposome into the external buffer. In the external buffer, the metal cation binds to the chelating ligand to form a complex. This complex has higher fluorescence than the metal cation, or the chelating ligand, when the cation and the ligand are not bound to one another. The increased fluorescence of the sample can be detected using an appropriate instrument, thus indicating leakage of the metal cation from the liposome. In the presence of an active test compound, which, for example, chemically reacts with gasdermin, the NT gasdermin fragment that is chemically modified by the test compound does not form a pore in the liposome. Hence, the metal cation remains encapsulated in the liposome and does not bind with the chelating ligand in the external buffer. As such, there is no liposome leakage and no fluorescence increase is detected in the sample. An active compound may be identified in the assay by comparing fluorescence of the sample containing the test compound and fluorescence of a control sample that does not contain any test compound. When the compound is considered active in the assay, fluorescence of the sample is lower than fluorescence of the control. In some embodiments, when the compound is considered active, fluorescence of the sample is at least about 10%, about 20%, about 30%, about 40%, about 50%, or about 60% lower than the fluorescence of the control.

In some instances, the metal cation is selected from Ce^{3+}, Fe^{2+}, Fe^{3+}, Zn^{2+}, Cu^{2+}, Mg^{2+}, and Tb^{3+}. In some embodiments, the metal cation is Tb^{3+}. In some instances, the chelating ligand is selected from ethylenediaminetetraacetic acid (EDTA), dipicolinic acid (DPA), ethylenediamine, porphyrin, and dimercaptol. In some embodiments, the chelating ligand is dipicolinic acid (DPA).

In some instances, the gasdermin protein in the sample is selected from GSDMA, GSMDB, GSDMC, GSDMD, DFNA5, and DFNB59. In some instances, the gasdermin protein contains rhinovirus 3C protease cleavage site (GSDM-3C). For example, the gasdermin protein in the sample is gasdermin D protein with a 3C protease cleavage site (GSDMD-3C).

In some instances, the protease enzyme is selected from: an inflammatory caspase and rhinovirus 3C protease. The inflammatory caspase may be caspase 1 or caspase 11. In some embodiments, the gasdermin protein is GSDM-3C and the protease enzyme is 3C protease. In other embodiments, the gasdermin protein is GSDMD-3C and the protease enzyme is 3C protease.

In yet another general aspect, the present application provides a method of identifying a compound that:

- inhibits a gasdermin pore formation in a cell; and/or
- inhibits inflammasome-mediated death of a cell (pyroptosis); and/or
- inhibits cytokine secretion from a cell; and/or
- inhibits an inflammatory caspase in a cell; and/or
- covalently reacts with a cysteine of a gasdermin protein in a cell; and/or
- covalently reacts with a cysteine of an inflammatory signaling molecule selected from: a sensor, an adaptor, and a transcription factor, or a regulator thereof;

the method comprising:

d) providing a sample comprising a liposome comprising a metal cation capable of forming a complex with a chelating ligand, the chelating ligand, and a test compound;

e) contacting the test compound with an N-terminal gasdermin protein fragment; and

f) determining whether the test compound inhibits leakage of the metal cation from the liposome, wherein said inhibition of the leakage of the metal cation from the liposome is an indication that the test compound:

- inhibits a gasdermin pore formation in a cell; and/or
- inhibits inflammasome-mediated death of a cell (pyroptosis); and/or
- inhibits cytokine secretion from a cell; and/or
- inhibits an inflammatory caspase in a cell; and/or

- covalently reacts with a cysteine of a gasdermin protein in a cell; and/or
- covalently reacts with a cysteine of an inflammatory signaling molecule selected from: a sensor, an adaptor, and a transcription factor, or a regulator thereof.

Definitions

As used herein, the term "about" means "approximately" (e.g., plus or minus approximately 10% of the indicated value).

At various places in the present specification, substituents of compounds of the invention are disclosed in groups or in ranges. It is specifically intended that the invention include each and every individual subcombination of the members of such groups and ranges. For example, the term "C_{1-6} alkyl" is specifically intended to individually disclose methyl, ethyl, C_3 alkyl, C_4 alkyl, C_5 alkyl, and C_6 alkyl.

At various places in the present specification various aryl, heteroaryl, cycloalkyl, and heterocycloalkyl rings are described. Unless otherwise specified, these rings can be attached to the rest of the molecule at any ring member as permitted by valency. For example, the term "a pyridine ring" or "pyridinyl" may refer to a pyridin-2-yl, pyridin-3-yl, or pyridin-4-yl ring.

It is further appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, can also be provided in combination in a single embodiment. Conversely, various features of the invention which are, for brevity, described in the context of a single embodiment, can also be provided separately or in any suitable subcombination.

The term "aromatic" refers to a carbocycle or heterocycle having one or more polyunsaturated rings having aromatic character (i.e., having $(4n + 2)$ delocalized π (pi) electrons where n is an integer).

The term "n-membered" where n is an integer typically describes the number of ring-forming atoms in a moiety where the number of ring-forming atoms is n. For example, piperidinyl is an example of a 6-membered heterocycloalkyl ring, pyrazolyl is an example of a 5-membered heteroaryl ring, pyridyl is an example of a 6-membered heteroaryl ring, and 1,2,3,4-tetrahydro-naphthalene is an example of a 10-membered cycloalkyl group.

As used herein, the phrase "optionally substituted" means unsubstituted or substituted. The substituents are independently selected, and substitution may be at any chemically accessible position. As used herein, the term "substituted" means that a hydrogen atom is removed and replaced by a substituent. A single divalent substituent, *e.g.*, oxo, can replace two hydrogen atoms. It is to be understood that substitution at a given atom is limited by valency.

Throughout the definitions, the term "C_{n-m}" indicates a range which includes the endpoints, wherein n and m are integers and indicate the number of carbons. Examples include C_{1-4}, C_{1-6}, and the like.

As used herein, the term "C_{n-m} alkyl", employed alone or in combination with other terms, refers to a saturated hydrocarbon group that may be straight-chain or branched, having n to m carbons. Examples of alkyl moieties include, but are not limited to, chemical groups such as methyl, ethyl, *n*-propyl, isopropyl, *n*-butyl, *tert*-butyl, isobutyl, *sec*-butyl; higher homologs such as 2-methyl-1-butyl, *n*-pentyl, 3-pentyl, *n*-hexyl, 1,2,2-trimethylpropyl, and the like. In some embodiments, the alkyl group contains from 1 to 6 carbon atoms, from 1 to 4 carbon atoms, from 1 to 3 carbon atoms, or 1 to 2 carbon atoms.

As used herein, the term "C_{n-m} haloalkyl", employed alone or in combination with other terms, refers to an alkyl group having from one halogen atom to 2s+1 halogen atoms which may be the same or different, where "s" is the number of carbon atoms in the alkyl group, wherein the alkyl group has n to m carbon atoms. In some embodiments, the haloalkyl group is fluorinated only. In some embodiments, the alkyl group has 1 to 6, 1 to 4, or 1 to 3 carbon atoms.

As used herein, "C_{n-m} alkenyl" refers to an alkyl group having one or more double carbon-carbon bonds and having n to m carbons. Example alkenyl groups include, but are not limited to, ethenyl, *n*-propenyl, isopropenyl, *n*-butenyl, *sec*-butenyl, and the like. In some embodiments, the alkenyl moiety contains 2 to 6, 2 to 4, or 2 to 3 carbon atoms.

As used herein, "C_{n-m} alkynyl" refers to an alkyl group having one or more triple carbon-carbon bonds and having n to m carbons. Example alkynyl groups include, but are not limited to, ethynyl, propyn-1-yl, propyn-2-yl, and the like. In some embodiments, the alkynyl moiety contains 2 to 6, 2 to 4, or 2 to 3 carbon atoms.

As used herein, the term "C_{n-m} alkylene", employed alone or in combination with other terms, refers to a divalent alkyl linking group having n to m carbons. Examples of alkylene groups include, but are not limited to, ethan-1,1-diyl, ethan-1,2-diyl, propan-1,1,-diyl, propan-1,3-diyl, propan-1,2-diyl, butan-1,4-diyl, butan-1,3-diyl, butan-1,2-diyl, 2-methyl-propan-1,3-diyl, and the like. In some embodiments, the alkylene moiety contains 2 to 6, 2 to 4, 2 to 3, 1 to 6, 1 to 4, or 1 to 2 carbon atoms.

As used herein, the term "C_{n-m} alkoxy", employed alone or in combination with other terms, refers to a group of formula -O-alkyl, wherein the alkyl group has n to m carbons. Example alkoxy groups include, but are not limited to, methoxy, ethoxy, propoxy (e.g., *n*-propoxy and isopropoxy), butoxy (e.g., *n*-butoxy and *tert*-butoxy), and the like. In some embodiments, the alkyl group has 1 to 6, 1 to 4, or 1 to 3 carbon atoms.

As used herein, "C_{n-m} haloalkoxy" refers to a group of formula –O-haloalkyl having n to m carbon atoms. An example haloalkoxy group is OCF_3. In some embodiments, the haloalkoxy group is fluorinated only. In some embodiments, the alkyl group has 1 to 6, 1 to 4, or 1 to 3 carbon atoms.

As used herein, the term "amino" refers to a group of formula $–NH_2$.

As used herein, the term "C_{n-m} alkylamino" refers to a group of formula -NH(alkyl), wherein the alkyl group has n to m carbon atoms. In some embodiments, the alkyl group has 1 to 6, 1 to 4, or 1 to 3 carbon atoms. Examples of alkylamino groups include, but are not limited to, N-methylamino, N-ethylamino, N-propylamino (e.g., N-(*n*-propyl)amino and N-isopropylamino), N-butylamino (e.g., N-(*n*-butyl)amino and N-(*tert*-butyl)amino), and the like.

As used herein, the term "di(C_{n-m}-alkyl)amino" refers to a group of formula -$N(alkyl)_2$, wherein the two alkyl groups each has, independently, n to m carbon atoms. In some embodiments, each alkyl group independently has 1 to 6, 1 to 4, or 1 to 3 carbon atoms.

As used herein, the term "C_{n-m} alkoxycarbonyl" refers to a group of formula -C(O)O-alkyl, wherein the alkyl group has n to m carbon atoms. In some embodiments, the alkyl group has 1 to 6, 1 to 4, or 1 to 3 carbon atoms. Examples of alkoxycarbonyl groups include, but are not limited to, methoxycarbonyl,

ethoxycarbonyl, propoxycarbonyl (e.g., *n*-propoxycarbonyl and isopropoxycarbonyl), butoxycarbonyl (e.g., *n*-butoxycarbonyl and *tert*-butoxycarbonyl), and the like.

As used herein, the term "C_{n-m} alkylcarbonyl" refers to a group of formula -C(O)-alkyl, wherein the alkyl group has n to m carbon atoms. In some embodiments, the alkyl group has 1 to 6, 1 to 4, or 1 to 3 carbon atoms. Examples of alkylcarbonyl groups include, but are not limited to, methylcarbonyl, ethylcarbonyl, propylcarbonyl (e.g., *n*-propylcarbonyl and isopropylcarbonyl), butylcarbonyl (e.g., *n*-butylcarbonyl and *tert*-butylcarbonyl), and the like.

As used herein, the term "C_{n-m} alkylcarbonylamino" refers to a group of formula -NHC(O)-alkyl, wherein the alkyl group has n to m carbon atoms. In some embodiments, the alkyl group has 1 to 6, 1 to 4, or 1 to 3 carbon atoms.

As used herein, the term "C_{n-m} alkylsulfonylamino" refers to a group of formula -NHS$(O)_2$-alkyl, wherein the alkyl group has n to m carbon atoms. In some embodiments, the alkyl group has 1 to 6, 1 to 4, or 1 to 3 carbon atoms.

As used herein, the term "aminosulfonyl" refers to a group of formula -S$(O)_2NH_2$.

As used herein, the term "C_{n-m} alkylaminosulfonyl" refers to a group of formula -S$(O)_2$NH(alkyl), wherein the alkyl group has n to m carbon atoms. In some embodiments, the alkyl group has 1 to 6, 1 to 4, or 1 to 3 carbon atoms.

As used herein, the term "di(C_{n-m} alkyl)aminosulfonyl" refers to a group of formula -S$(O)_2$N$(alkyl)_2$, wherein each alkyl group independently has n to m carbon atoms. In some embodiments, each alkyl group has, independently, 1 to 6, 1 to 4, or 1 to 3 carbon atoms.

As used herein, the term "aminosulfonylamino" refers to a group of formula -NHS$(O)_2NH_2$.

As used herein, the term "C_{n-m} alkylaminosulfonylamino" refers to a group of formula -NHS$(O)_2$NH(alkyl), wherein the alkyl group has n to m carbon atoms. In some embodiments, the alkyl group has 1 to 6, 1 to 4, or 1 to 3 carbon atoms.

As used herein, the term "di(C_{n-m} alkyl)aminosulfonylamino" refers to a group of formula -NHS$(O)_2$N$(alkyl)_2$, wherein each alkyl group independently has n to m carbon atoms. In some embodiments, each alkyl group has, independently, 1 to 6, 1 to 4, or 1 to 3 carbon atoms.

As used herein, the term “aminocarbonylamino”, employed alone or in combination with other terms, refers to a group of formula -NHC(O)NH_2.

As used herein, the term “C_{n-m} alkylaminocarbonylamino” refers to a group of formula -NHC(O)NH(alkyl), wherein the alkyl group has n to m carbon atoms. In some embodiments, the alkyl group has 1 to 6, 1 to 4, or 1 to 3 carbon atoms.

As used herein, the term “di(C_{n-m} alkyl)aminocarbonylamino” refers to a group of formula -NHC(O)N$(alkyl)_2$, wherein each alkyl group independently has n to m carbon atoms. In some embodiments, each alkyl group has, independently, 1 to 6, 1 to 4, or 1 to 3 carbon atoms.

As used herein, the term “carbamyl” to a group of formula –C(O)NH_2.

As used herein, the term “C_{n-m} alkylcarbamyl” refers to a group of formula -C(O)-NH(alkyl), wherein the alkyl group has n to m carbon atoms. In some embodiments, the alkyl group has 1 to 6, 1 to 4, or 1 to 3 carbon atoms.

As used herein, the term “di(C_{n-m}-alkyl)carbamyl” refers to a group of formula –C(O)N$(alkyl)_2$, wherein the two alkyl groups each has, independently, n to m carbon atoms. In some embodiments, each alkyl group independently has 1 to 6, 1 to 4, or 1 to 3 carbon atoms.

As used herein, the term “thio” refers to a group of formula -SH.

As used herein, the term “C_{n-m} alkylthio” refers to a group of formula -S-alkyl, wherein the alkyl group has n to m carbon atoms. In some embodiments, the alkyl group has 1 to 6, 1 to 4, or 1 to 3 carbon atoms.

As used herein, the term “C_{n-m} alkylsulfinyl” refers to a group of formula -S(O)-alkyl, wherein the alkyl group has n to m carbon atoms. In some embodiments, the alkyl group has 1 to 6, 1 to 4, or 1 to 3 carbon atoms.

As used herein, the term “C_{n-m} alkylsulfonyl” refers to a group of formula -S$(O)_2$-alkyl, wherein the alkyl group has n to m carbon atoms. In some embodiments, the alkyl group has 1 to 6, 1 to 4, or 1 to 3 carbon atoms.

As used herein, the term “carbonyl”, employed alone or in combination with other terms, refers to a -C(=O)- group, which may also be written as C(O).

As used herein, the term “carboxy” refers to a -C(O)OH group.

As used herein, the term “cyano-C_{1-3} alkyl” refers to a group of formula -(C_{1-3} alkylene)-CN.

As used herein, the term "HO-C_{1-3} alkyl" refers to a group of formula -(C_{1-3} alkylene)-OH.

As used herein, "halo" refers to F, Cl, Br, or I. In some embodiments, a halo is F, Cl, or Br.

As used herein, the term "aryl," employed alone or in combination with other terms, refers to an aromatic hydrocarbon group, which may be monocyclic or polycyclic (e.g., having 2, 3 or 4 fused rings). The term "C_{n-m} aryl" refers to an aryl group having from n to m ring carbon atoms. Aryl groups include, e.g., phenyl, naphthyl, anthracenyl, phenanthrenyl, indanyl, indenyl, and the like. In some embodiments, aryl groups have from 6 to 10 carbon atoms. In some embodiments, the aryl group is phenyl or naphthyl.

As used herein, "cycloalkyl" refers to non-aromatic cyclic hydrocarbons including cyclized alkyl and/or alkenyl groups. Cycloalkyl groups can include mono- or polycyclic (e.g., having 2, 3 or 4 fused rings) groups and spirocycles. Ring-forming carbon atoms of a cycloalkyl group can be optionally substituted by 1 or 2 independently selected oxo or sulfide groups (e.g., C(O) or C(S)). Also included in the definition of cycloalkyl are moieties that have one or more aromatic rings fused (i.e., having a bond in common with) to the cycloalkyl ring, for example, benzo or thienyl derivatives of cyclopentane, cyclohexane, and the like. A cycloalkyl group containing a fused aromatic ring can be attached through any ring-forming atom including a ring-forming atom of the fused aromatic ring. Cycloalkyl groups can have 3, 4, 5, 6, 7, 8, 9, or 10 ring-forming carbons (C_{3-10}). In some embodiments, the cycloalkyl is a C_{3-10} monocyclic or bicyclic cycloalkyl. In some embodiments, the cycloalkyl is a C_{3-7} monocyclic cycloalkyl. Example cycloalkyl groups include cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, cyclopentenyl, cyclohexenyl, cyclohexadienyl, cycloheptatrienyl, norbornyl, norpinyl, norcarnyl, adamantyl, and the like. In some embodiments, cycloalkyl is cyclopropyl, cyclobutyl, cyclopentyl, or cyclohexyl.

As used herein, "heteroaryl" refers to a monocyclic or polycyclic aromatic heterocycle having at least one heteroatom ring member selected from sulfur, oxygen, and nitrogen. In some embodiments, the heteroaryl ring has 1, 2, 3, or 4 heteroatom ring members independently selected from nitrogen, sulfur and oxygen. In some embodiments, any ring-forming N in a heteroaryl moiety can be an N-oxide. In some embodiments, the heteroaryl is a 5-10 membered monocyclic or bicyclic heteroaryl having 1, 2, 3 or 4 heteroatom ring members independently selected from nitrogen, sulfur and oxygen. In some embodiments, the heteroaryl is a 5-6 monocyclic heteroaryl having 1 or 2 heteroatom ring members independently selected from nitrogen, sulfur and oxygen. In some embodiments, the heteroaryl is a five-membered or six-membered heteroaryl ring. A five-membered heteroaryl ring is a heteroaryl with a ring having five ring atoms wherein one or more (e.g., 1, 2, or 3) ring atoms are independently selected from N, O, and S. Exemplary five-membered ring heteroaryls are thienyl, furyl, pyrrolyl, imidazolyl, thiazolyl, oxazolyl, pyrazolyl, isothiazolyl, isoxazolyl, 1,2,3-triazolyl, tetrazolyl, 1,2,3-thiadiazolyl, 1,2,3-oxadiazolyl, 1,2,4-triazolyl, 1,2,4-thiadiazolyl, 1,2,4-oxadiazolyl, 1,3,4-triazolyl, 1,3,4-thiadiazolyl, and 1,3,4-oxadiazolyl. A six-membered heteroaryl ring is a heteroaryl with a ring having six ring atoms wherein one or more (e.g., 1, 2, or 3) ring atoms are independently selected from N, O, and S. Exemplary six-membered ring heteroaryls are pyridyl, pyrazinyl, pyrimidinyl, triazinyl and pyridazinyl.

As used herein, "heterocycloalkyl" refers to non-aromatic monocyclic or polycyclic heterocycles having one or more ring-forming heteroatoms selected from O, N, or S. Included in heterocycloalkyl are monocyclic 4-, 5-, 6-, 7-, 8-, 9- or 10-membered heterocycloalkyl groups. Heterocycloalkyl groups can also include spirocycles. Example heterocycloalkyl groups include pyrrolidin-2-one, 1,3-isoxazolidin-2-one, pyranyl, tetrahydropyran, oxetanyl, azetidinyl, morpholino, thiomorpholino, piperazinyl, tetrahydrofuranyl, tetrahydrothienyl, piperidinyl, pyrrolidinyl, isoxazolidinyl, isothiazolidinyl, pyrazolidinyl, oxazolidinyl, thiazolidinyl, imidazolidinyl, azepanyl, benzazapene, and the like. Ring-forming carbon atoms and heteroatoms of a heterocycloalkyl group can be optionally substituted by 1 or 2 independently selected oxo or sulfido groups (e.g., C(O), S(O), C(S), or $S(O)_2$, etc.). The heterocycloalkyl group can be attached through a ring-forming carbon atom or a ring-forming heteroatom. In some embodiments, the heterocycloalkyl group contains 0 to 3 double bonds. In some embodiments, the heterocycloalkyl group contains 0 to 2 double bonds. Also included in the definition of heterocycloalkyl are moieties that have one or more aromatic rings fused (*i.e.*, having a bond in common with) to the cycloalkyl ring, for example, benzo or thienyl derivatives of piperidine, morpholine, azepine, etc. A heterocycloalkyl group containing a fused aromatic ring can be attached through any ring-forming atom including a ring-forming atom of the fused aromatic ring. In some embodiments, the heterocycloalkyl is a monocyclic 4-6 membered heterocycloalkyl having 1 or 2 heteroatoms independently selected from nitrogen, oxygen, or sulfur and having one or more oxidized ring members. In some embodiments, the heterocycloalkyl is a monocyclic or bicyclic 4-10 membered heterocycloalkyl having 1, 2, 3, or 4 heteroatoms independently selected from nitrogen, oxygen, or sulfur and having one or more oxidized ring members.

At certain places, the definitions or embodiments refer to specific rings (e.g., an azetidine ring, a pyridine ring, etc.). Unless otherwise indicated, these rings can be attached to any ring member provided that the valency of the atom is not exceeded. For example, an azetidine ring may be attached at any position of the ring, whereas a pyridin-3-yl ring is attached at the 3-position.

As used herein, the term “oxo” refers to an oxygen atom as a divalent substituent, forming a carbonyl group when attached to a carbon (e.g., C=O), or attached to a heteroatom forming a sulfoxide or sulfone group.

The term “compound” as used herein is meant to include all stereoisomers, geometric isomers, tautomers, and isotopes of the structures depicted. Compounds herein identified by name or structure as one particular tautomeric form are intended to include other tautomeric forms unless otherwise specified.

The compounds described herein can be asymmetric (*e.g.*, having one or more stereocenters). All stereoisomers, such as enantiomers and diastereomers, are intended unless otherwise indicated. Compounds of the present invention that contain asymmetrically substituted carbon atoms can be isolated in optically active or racemic forms. Methods on how to prepare optically active forms from optically inactive starting materials are known in the art, such as by resolution of racemic mixtures or by stereoselective synthesis. Many geometric isomers of olefins, C=N double bonds, N=N double bonds, and the like can also be present in the compounds described herein, and all such stable isomers are contemplated in the present invention. *Cis* and *trans* geometric isomers of the compounds of the present invention are described and may be isolated as a mixture of isomers or as separated isomeric forms. In some embodiments, the compound has the *(R)*-configuration. In some embodiments, the compound has the *(S)*-configuration.

Compounds provided herein also include tautomeric forms. Tautomeric forms result from the swapping of a single bond with an adjacent double bond together with the concomitant migration of a proton. Tautomeric forms include prototropic tautomers which are isomeric protonation states having the same empirical formula and total charge. Example prototropic tautomers include ketone – enol pairs, amide - imidic acid pairs, lactam – lactim pairs, enamine – imine pairs, and annular forms where a proton can occupy two or more positions of a heterocyclic system, for example, 1H- and 3H-imidazole, 1H-, 2H- and 4H- 1,2,4-triazole, 1H- and 2H-isoindole, and 1H- and 2H-pyrazole. Tautomeric forms can be in equilibrium or sterically locked into one form by appropriate substitution.

As used herein, the term "cell" is meant to refer to a cell that is *in vitro*, *ex vivo* or *in vivo*. In some embodiments, an *ex vivo* cell can be part of a tissue sample excised from an organism such as a mammal. In some embodiments, an *in vitro* cell can be a cell in a cell culture. In some embodiments, an *in vivo* cell is a cell living in an organism such as a mammal.

As used herein, the term "contacting" refers to the bringing together of indicated moieties in an *in vitro* system or an *in vivo* system. For example, "contacting" the gasdermin with a compound of the invention includes the administration of a compound of the present invention to an individual or patient, such as a human, having gasdermin, as well as, for example, introducing a compound of the invention into a sample containing a cellular or purified preparation containing the gasdermin.

As used herein, the term "individual", "patient", or "subject" used interchangeably, refers to any animal, including mammals, preferably mice, rats, other rodents, rabbits, dogs, cats, swine, cattle, sheep, horses, or primates, and most preferably humans.

As used herein, the phrase "effective amount" or "therapeutically effective amount" refers to the amount of active compound or pharmaceutical agent that elicits the biological or medicinal response in a tissue, system, animal, individual or human that is being sought by a researcher, veterinarian, medical doctor or other clinician.

As used herein the term "treating" or "treatment" refers to 1) inhibiting the disease; for example, inhibiting a disease, condition or disorder in an individual who is experiencing or displaying the pathology or symptomatology of the disease, condition or disorder (*i.e.*, arresting further development of the pathology and/or symptomatology), or 2) ameliorating the disease; for example, ameliorating a disease, condition or disorder in an individual who is experiencing or displaying the pathology or symptomatology of the disease, condition or disorder (*i.e.*, reversing the pathology and/or symptomatology).

As used herein, the term “preventing” or “prevention” of a disease, condition or disorder refers to decreasing the risk of occurrence of the disease, condition or disorder in a subject or group of subjects (e.g., a subject or group of subjects predisposed to or susceptible to the disease, condition or disorder). In some embodiments, preventing a disease, condition or disorder refers to decreasing the possibility of acquiring the disease, condition or disorder and/or its associated symptoms. In some embodiments, preventing a disease, condition or disorder refers to completely or almost completely stopping the disease, condition or disorder from occurring.

EXAMPLES

Cytosolic sensing of pathogens and danger by myeloid and barrier epithelial cells assembles large complexes, called inflammasomes, which activate inflammatory caspases to trigger cytokine maturation and inflammatory cell death (pyroptosis). Inflammation recruits immune cells to orchestrate a protective immune response but can also cause pathology. Pore formation by gasdermin D (GSDMD), an inflammatory caspase substrate, was recently identified as the mechanism responsible for pyroptosis and release of inflammatory mediators. Inhibiting GSDMD is an attractive strategy to curb inflammation. The experimental results described below show that disulfiram, a drug used to treat chronic alcohol addiction, as an inhibitor of pore formation by GSDMD, but not other members of the GSDM family. Disulfiram blocks inflammasome-mediated pyroptosis and cytokine release in cells and inhibits LPS-induced septic death in mice. At nanomolar concentration, disulfiram covalently modifies human Cys191 (mouse Cys192) in GSDMD to block pore formation and pyroptosis.

General methods

Mice. 8-week-old female C57BL/6 wild-type mice were purchased from The Jackson Laboratory and maintained at the SPF facility at Harvard Medical School. All mouse experiments were conducted using protocols approved by the Animal Care and Use Committees of Boston Children's Hospital and Harvard Medical School.

Drug administration and LPS-induced sepsis in mice. Mice were treated with disulfiram (C-23, DSF, 50 mg/kg) formulated in sesame oil or vehicle (Ctrl) by intraperitoneal injection at indicated times. In the indicated group of mice in Fig. 5h, copper gluconate (0.15 mg/kg) was administered intraperitoneally 6 hr prior to the first injection of DSF. Sepsis was induced in C57BL/6 mice (8-10 weeks old) by intraperitoneal injection of LPS (E. coli O111:B4) at indicated concentrations. In some experiments, mice were treated with copper gluconate (0.15 mg/kg) or vehicle by intraperitoneal injection 5 hr before LPS challenge and then given DSF (50 mg/kg) intraperitoneally dissolved in sesame oil or vehicle 4 hr before and just before LPS challenge (15 mg/kg intraperitoneally). Peritoneal cells were collected by rinsing the peritoneal cavity with ice cold PBS containing 3% FBS 6 hr after LPS challenge. To measure cytokines, blood samples were collected by tail vein bleed 12 hr post LPS challenge and allowed to clot at room temperature. Sera obtained after centrifugation at 2,000 x g for 10 min were analysed for inflammatory cytokines by ELISA.

Reagents. β-mercaptoethanol (2ME), dithiothreitol (DTT), terbium(III) chloride (TbCl3), dipicolinic acid (DPA) and copper gluconate were from Sigma-Aldrich. Compound C-23 and its analogues: Tetraethylthiuram disulfide (C-23), tetramethylthiuram disulfide (C-23A1), tetrabutylthiuram disulfide (C-23A3), 4-Methylpiperazine-1-carbothioic dithioperoxyanhydride (C-23A4), Tetraphenylthiuram disulfide (C-23A5), N,N’-Dimethyl-N,N’-(4,4’-dimethyldiphenyl)thiuram disulfide (C-23A6), di(4-morpholinyl)dithioperoxyanhydride (C-23A7), N,N’-Dimethyl-N,N’-di(4-pyridinyl)thiuram disulfide (C-23A8), pyrrolidine-1-carbothioic dithioperoxyanhydride (C-23A10), and dimethyldiphenylthiuram disulfide (C-23A11) were from Sigma-Aldrich. Tetraisopropylthiuram disulfide (C-23A2) and dicyclopentamethylenethiuram disulfide (C-23A9) were from Oakwood Chemicals. Tetrabenzylthiuram disulfide (C-23A12) was from AK Scientific. Phorbol 12-myristate 13-acetate (PMA) and DMSO were from Sigma-Aldrich. Ultra LPS and nigericin were from InvivoGen. The pan-caspase inhibitor z-VAD-fmk was from BD Bioscience. The complete protease inhibitor cocktail and the PhosSTOP phosphatase inhibitor cocktail were from Roche. Necrosulfonamide, Necrostatin-1, dimethyl fumarate, ibrutinib and afatinib were from Sigma-Aldrich. LDC7559 was synthesized by Intonation Research Labs.

Biomolecules: The monoclonal antibody against GSDMD was generated in house by immunizing 6 week-old BALB/c mice with recombinant human GSDMD and boosting with recombinant human GSDMD-NT according to standard protocols. Serum samples were collected to assess titers of reactive antibodies and spleen cells were fused with SP2/0 myeloma cells. Hybridomas were selected and supernatants from the resulting clones were screened by enzyme linked immunosorbent assay (ELISA), immunoblot and immunofluorescence microscopy. Tubulin antibody was from Sigma-Aldrich. Phospho-IκBα antibody, IκBα antibody, Phospho-NF-κB p65 antibody, cleaved human caspase-1 (Asp297) antibody and NLRP3 antibody were from Cell Signaling Technology. ASC antibody (AL177) and mouse caspase-1 p20 antibody were from AdipoGen. Human and mouse IL-1β antibodies were from R&D Systems. HMGB1 and mouse GSDMD antibodies were from Abcam.

Liposome leakage assay: fluorogenic liposome leakage assay detects leakage of Tb^{3+} from Tb^{3+}-loaded liposomes incubated with GSDMD and caspase-11 (See References 7 and 9). See Figure 1. Liposome leakage was detected by an increase in fluorescence when Tb^{3+} bound to dipicolinic acid (DPA) in Buffer C. Human GSDMD (0.3 μM) was dispensed into a well (Corning 3820) containing PC/PE/CL liposomes (50 μM liposome lipids) and incubated with a test compound for 1 hr before addition of caspase-11 (0.15 μM) to each well. The fluorescence intensity of the well was measured at 545 nm with an excitation of 276 nm 1 hr after addition of caspase-11 using a Perkin Elmer EnVision plate reader. The final percent inhibition was calculated as $[(\text{fluorescence}_{\text{test compound}} - \text{fluorescence}_{\text{negative control}})/(\text{fluorescence}_{\text{positive control}} - \text{fluorescence}_{\text{negative control}})] \times 100$, where a well with GSDMD without the test compound was used as positive control, and a well without caspase-11 was used as negative control. IC_{50} of the test compound was determined in concentration-response experiments in a dose range of 0.008–50 μM.

Protein expression and purification: full-length human GSDMD sequence was cloned into the pDB.His.MBP vector with a tobacco etch virus (TEV)-cleavable N-terminal His_6-MBP tag using NdeI and XhoI restriction sites. Human GSDMD-3C and mouse GSDMA3-3C mutants were constructed by QuikChange Mutagenesis (Agilent Technologies). For expression of full-length GSDMD, GSDMD-3C, GSDMA3, and GSDMA3-3C, *E. coli* BL21 (DE3) cells harbouring the indicated plasmids were grown at 18 °C overnight in LB medium supplemented with 50 μg ml^{-1} kanamycin after induction with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) when OD_{600} reached 0.8. Cells were ultrasonicated in lysis buffer containing 25 mM Tris-HCl at pH 8.0, 150 mM NaCl, 20 mM imidazole and 5 mM 2ME. The lysate was clarified by centrifugation at 40,000xg at 4 °C for 1 hr. The supernatant containing the target protein was incubated with Ni-NTA resin (Qiagen) for 30 min at 4 °C. After incubation, the resin–supernatant mixture was poured into a column and the resin was washed with lysis buffer. The protein was eluted using the lysis buffer supplemented with 100 mM imidazole. The His_6-MBP tag was removed by overnight TEV protease digestion at 16 °C. The cleaved protein was purified using HiTrap Q ion-exchange and Superdex 200 gel-filtration columns (GE Healthcare Life Sciences).

Caspase-11 sequence was cloned into the pFastBac-HTa vector with a TEV cleavable N-terminal His_6-tag using EcoRI and XhoI restriction sites. The baculoviruses were prepared using the Bac-to-Bac system (Invitrogen), and the protein was expressed in Sf9 cells following the manufacturer’s instructions. His–caspase-11 baculovirus (10 ml) was used to infect 1 L of Sf9 cells. Cells were collected 48 hrs after infection and His_6–caspase-11 was purified following the same protocol as for His_6-MBP–GSDMD. Eluate from Ni-NTA resin was collected for subsequent assays.

Liposome preparation: PC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine, 25 mg/mL in chloroform; 80 μL), PE (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine, 25 mg/mL in chloroform; 128 μL) and CL (1',3'-bis[1,2-dioleoyl-*sn*-glycero-3-phospho]-*sn*-glycerol (sodium salt), 25 mg/mL in chloroform; 64 μL) were mixed and the solvent was evaporated under a stream of N_2 gas. The lipid mixture was suspended in 1 mL Buffer A (20 mM HEPES, 150 mM NaCl, 50 mM sodium citrate, and 15 mM $TbCl_3$) for 3 min. The suspension was pushed through 100 nm Whatman® Nuclepore™ Track-Etched Membrane 30 times to obtain homogeneous liposomes. The filtered suspension was purified by size exclusion column (Superose 6, 10/300 GL) in Buffer B (20 mM HEPES, 150 mM NaCl) to remove $TbCl_3$ outside liposomes. Void fractions were pooled to produce a stock of PC/PE/CL liposomes (1.6 mM). The liposomes are diluted to 50 μM with

Buffer C (20 mM HEPES, 150 mM NaCl and 50 µM DPA) for use in high-throughput screening.

Fluorescent protein labelling and microscale thermophoresis binding assay: His_6-MBP-GSDMD was labeled with AlexaFluor-488 using the Molecular Probes protein labelling kit. Binding of inhibitors to GSDMD was evaluated using microscale thermophoresis (MST). Ligands (49 nM - 150 µM) were incubated with purified AlexaFluor-488-labeled protein (80 nM) for 30 min in assay buffer (20 mM HEPES, 150 mM NaCl, 0.05% Tween 20). The sample was loaded into NanoTemper Monolith NT.115 glass capillaries and MST carried out using 20% LED power and 40% MST power. K_d values were calculated using the mass action equation and NanoTemper software.

Caspase-1 and caspase-11 inhibition assays: the fluorogenic assay for caspase-1 and caspase-11 activity is based on release of 7-amino-4-methylcoumarin (AMC) from the caspase substrate Ac-YVAD-AMC. Compounds (8 nM - 50 µM) were incubated with 0.5 U of caspase-1 or caspase-11 for 30 min in assay buffer (20 mM HEPES, 150 mM NaCl) in 384-well plates (Corning 3820) before addition of Ac-YVAD-AMC (40 µM) to initiate the reactions. Reactions were monitored in a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, California USA) with excitation/emission wavelengths at 350/460 nm. The fluorescence intensity of each reaction was recorded every 2 min for 2 hrs.

Cell viability assay. THP-1 cells seeded at a density of 4000 cells per well in 96-well plates (Corning 3610), were differentiated by exposure to 50 nM PMA for 36 hrs before being primed with 100 ng/mL LPS. Primed THP-1 cells were pretreated with each test compound for 1 hr before addition of 20 µM nigericin or medium as control. The number of surviving cells was determined by CellTiter-Glo assay 1.5 hrs later. The final percent cell viability was calculated using the formula $[(\text{luminescence}_{\text{test compound}} - \text{luminescence}_{\text{negative control}})/(\text{luminescence}_{\text{positive control}} - \text{luminescence}_{\text{negative control}})] \times 100$, where wells with only LPS were used as positive controls and wells treated with LPS and nigericin were used as negative controls. The IC_{50} of each test compound in the cell viability assay was determined by concentration-response experiments in a dose range of 0.39 - 50 µM.

Mass spectrometry and sample preparation. Gel bands were cut into 1 mm size pieces and placed into separate 1.5 mL polypropylene tubes. 100 µl of 50%

acetonitrile in 50 mM ammonium bicarbonate buffer were added to each tube and the samples were then incubated at room temperature for 20 min. This step was repeated if necessary to destain gel. Then, the gel slice was incubated with 55 mM iodoacetamide (in 50 mM ammonium bicarbonate) for 45 min in the dark at room temperature, before the gel was washed sequentially with 50 mM ammonium bicarbonate, water and acetonitrile. Samples were then dried in a Speedvac for 20 min. Trypsin (Promega Corp.) (10 ng/µL in 25 mM ammonium bicarbonate, pH 8.0) was added to each sample tube to just cover the gel, and samples were then incubated at 37 °C for 6 hrs or overnight.

After digestion, samples were acidified with 0.1% formic acid (FA) and 3 µl of tryptic peptide solution was injected. Nano-LC/MS/MS was performed on a Thermo Scientific Orbitrap Fusion system, coupled with a Dionex Ultimat 3000 nano HPLC and auto sampler with 40 well standard trays. Samples were injected onto a trap column (300 µm i.d. x 5mm, C18 PepMap 100) and then onto a C18 reversed-phase nano LC column (Acclaim PepMap 100 75 µm × 25 cm), heated to 50 °C. Flow rate was set to 400 nL/min with 60 min LC gradient, using mobile phases A (99.9% water, 0.1% FA) and B (99.9% acetonitrile, 0.1% FA). Eluted peptides were sprayed through a charged emitter tip (PicoTip Emitter, New Objective, 10 +/- 1 µm) into the mass spectrometer. Parameters were: tip voltage, +2.2 kV; Fourier Transform Mass Spectrometry (FTMS) mode for MS acquisition of precursor ions (resolution 120,000); Ion Trap Mass Spectrometry (ITMS) mode for subsequent MS/MS via higher-energy collisional dissociation (HCD) on top speed in 3 s.

Proteome Discoverer 1.4 was used for protein identification and modification analysis. UniPort human database was used to analyze raw data. Other parameters include the following: selecting the enzyme as trypsin; maximum missed cleavages = 2; dynamic modifications are carbamidomethyl (control), diethyldithiocarbamate (from C-23) and Bay 11-7082 on cysteine; oxidized methionine, deaminated asparagine and glutamine; precursor tolerance set at 10 ppm; MS/MS fragment tolerance set at 0.6 Da; and +2 to +4 charged peptides are considered. Peptide false discovery rate (FDR) was set to be smaller than 1% for significant match.

Cell lines and treatments: THP-1 cells and HEK293T cells (obtained from ATCC) were grown in RPMI with 10% heat-inactivated fetal bovine serum, supplemented with 100 U/ml penicillin G, 100 µg/ml streptomycin sulfate, 6 mM

HEPES, 1.6 mM L-glutamine, and 50 μM 2ME. C57BL/6 mouse iBMDM cells were kindly provided by J. Kagan (Boston Children's Hospital) and cultured in DMEM with the same supplements. Cells were verified to be free of mycoplasma contamination. Transient transfection of HEK293T cells was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. iBMDM cells were transfected by nucleofection using the Amaxa Nucleofector kit (VPA-1009). Generally, THP-1 cells were first differentiated by incubation with 50 nM PMA for 36 hrs and then primed with LPS (1 μg/ml) for 4 hrs before treatment with nigericin (20 μM). To examine IκBα phosphorylation and degradation as well as IL-1β induction, PMA-differentiated THP-1 cells were stimulated with LPS (1 μg/ml) for 0.5, 1 and 4 hrs, respectively. For noncanoical inflammasome activation, 1 million iBMDM cells were electroporated with 1 μg ultra LPS.

Cytotoxicity and cell viability assays: cell death and cell viability were determined by the lactate dehydrogenase release assay using the CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega) and by measuring ATP levels using the CellTiter-Glo Luminescent Cell Viability Assay (Promega), respectively, according to the manufacturer's instructions. Luminescence and absorbance were measured on a BioTek Synergy 2 plate reader.

Pore reconstitution on nanodiscs and negative staining electron microscopy: the coding sequence of the membrane scaffold protein NW50 was cloned into a pET-28a vector, and the protein was expressed in *E. coli* BL21(DE3), purified via a refolding procedure, and covalently circularized with sortase according to a previously described protocol. A lipid mixture containing phosphatidylserine (PS) and phosphatidylcholine (PC) (molar ratio 3:7) was solubilized in 60 mM sodium cholate and incubated with circularized NW50 on ice for 1 h to assemble nanodiscs. Sodium cholate was then removed by incubation overnight at 4 °C with Bio-beads SM-2 (Bio-Rad). The Bio-beads were then removed using a 0.22 μm filter, and the assembled nanodiscs were further purified using a Superose 6 10/300 gel-filtration column (GE Healthcare Life Sciences) equilibrated with Buffer D (50 mM Tris-HCl at pH 8.0, 150 mM NaCl) to remove excess lipids. To form GSDMD pores on the nanodiscs, purified human GSDMD-3C was incubated with 3C protease in the presence of nanodiscs for 6 hrs on ice. The pores were further purified over a Superose 6 column equilibrated with Buffer D. To assess the effect of C-23, human

GSDMD-3C plus 3C protease was either incubated with C-23 (molar ratio 1:1) for 30 min on ice before adding to nanodiscs (pretreatment), or C-23 was added for 30 min on ice to already assembled pores (post-treatment). For negative staining electron microscopy, a 5-μl sample was placed onto a glow-discharged carbon-coated copper grid (Electron Microscopy Sciences), washed twice with Buffer A, stained with 1% uranyl formate for 1 min, and air-dried. The grids were imaged on the Tecnai G^2 Spirit BioTWIN electron microscope and recorded with an AMT 2k CCD camera (Harvard Medical School Electron Microscopy Facility).

Immunoblot analysis: cell extracts were prepared using RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate) supplemented with a complete protease inhibitor cocktail (Roche) and a PhosSTOP phosphatase inhibitor cocktail (Roche). Samples were subjected to SDS-PAGE and the resolved proteins were then transferred to a PVDF membrane (Millipore). Immunoblots were probed with indicated antibodies and visualized using a SuperSignal West Pico chemiluminescence ECL kit (Pierce).

Caspase-1 activity assay in cells: to measure caspase-1 activation, THP-1 cells were seeded into 96-well plates and differentiated with PMA. After the indicated treatments, cells were incubated with a fluorescent active caspase-1 substrate FAM-YVAD-FMK (Immunochemistry Technologies). Samples were read on a BioTek Synergy 2 plate reader.

Measurement of cytokines: concentrations of IL-1β in culture supernatants or mouse serum were measured by ELISA kit (R&D Systems) according to the manufacturer’s instructions.

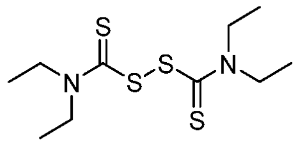
Immunostaining and confocal microscopy: cells grown on coverslips were fixed for 15 min with 4% paraformaldehyde in PBS, permeabilized for 5 min in 0.1% Triton X-100 in PBS and blocked using 5% BSA for 1 hr. Then, cells were stained with the indicated primary antibodies followed by incubation with fluorescent-conjugated secondary antibodies (Jackson ImmunoResearch). Nuclei were counterstained with DAPI (4,6-diamidino-2-phenylindole) (Sigma-Aldrich). Slides were mounted using Aqua-Poly/Mount (Dako). Images were captured using a laser scanning confocal microscope (Olympus Fluoview FV1000 Confocal System) with a

63× water immersion objective and Olympus Fluoview software (Olympus). All confocal images are representative of three independent experiments.

Statistics: student's t-test was used for the statistical analysis of two independent treatments. Mouse survival curves and statistics were analyzed using the Mantel-Cox Log-rank test.

Example 1 – inhibition of GSDMD pore formation by the test compounds

C-23 is a symmetrical molecule known as disulfiram, a drug used to treat alcohol addiction (See Reference 12):



(C-23).

IC_{50} values and GSDMD binding results for the test compounds (assessed by microscale thermophoresis (MST)) are presented in Table 1. Chemical structures of the tested compounds are shown in Figure 11.

Table 1

compound	In vitro IC_{50} (μM)	Binding K_D by MST (μM)
C-5	1.1 ± 0.4	
C-7	1.9 ± 0.1	
C-8	2.4 ± 0.3	
C-22	1.6 ± 0.3	27.9 ± 5.5
C-23	0.3 ± 0.0	12.8 ± 1.9
C-24	0.6 ± 0.1	8.6 ± 0.6
C-25	1.8 ± 0.6	

The test compounds were assessed for GSDMD binding by microscale thermophoresis (MST). Figure 3 shows MST measurement of the binding of Alexa 488-labeled His-MBP-GSDMD (80 nM) with C-22, C-23 or C-24.

To evaluate whether test compounds inhibit pyroptosis, test compounds were added to PMA-differentiated and LPS-primed human THP-1 cells or mouse immortalized bone marrow-derived macrophages (iBMDMs) before activating the canonical inflammasome with nigericin or the non-canonical inflammasome by LPS electroporation. As discussed in the following paragraph, C-23 blocked pyroptosis in cells, with IC_{50} values of 7.67 ± 0.29 μM and 10.33 ± 0.50 μM for canonical and non-canonical inflammasome-dependent pyroptosis, respectively, and impaired cell death triggered by the AIM2 inflammasome in mouse iBMDMs transfected with poly(dA:dT) (See Figure 10). Disulfiram also inhibited nigericin- or LPS transfection-induced IL-1β secretion with potency comparable to the pan-caspase inhibitor z-VAD-fmk.

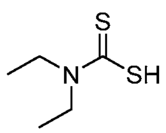
Experimental results: response curve of compound disulfiram (C-23) in liposome leakage assay is shown in Figure 2. In Figures 4, 6, and 8, PMA-differentiated LPS-primed human THP-1 were pre-treated with indicated concentrations of each compound for 1 h before adding nigericin or medium. The number of surviving cells was determined by CellTiter-Glo assay (figures 4 and 6) and IL-1β in culture supernatants was assessed by ELISA (figure 8) 2 hrs later. In Figures 5, 7, and 9, mouse iBMDMs were pre-treated with each test compound for 1 hr before electroporation with PBS or LPS. The number of surviving cells was determined by CellTiter-Glo assay (figures 5 and 7) and IL-1β in culture supernatants was assessed by ELISA (figure 9) 2.5 hrs later. In Figures 8 and 9, 40 μM concentration of test compounds were added. In Figure 10, mouse iBMDMs were pre-treated or not with 30 μM of C-23 for 1 h before transfection with PBS or poly(dA:dT) and analyzed for cell viability by CellTiter-Glo assay 4 h later. Graphs show the mean ± s.d. and data shown are representative of three independent experiments. **$P < 0.01$.

To confirm that C-23 inhibits pore formation, we reconstituted human GSDMD-NT pores on covalently circularized lipid nanodiscs constructed with phosphatidyl serine (PS), an acidic lipid, and phosphatidyl choline. Full-length GSDMD was engineered to replace the caspase cleavage site with a rhinovirus 3C protease cleavage site (GSDMD-3C) as previously described. 3C protease cleavage of the engineered GSDMD-3C liberates an active NT fragment. Adding GSDMD-3C plus 3C protease to assembled nanodiscs reconstituted pores that were visible by negative staining electron microscopy (EM). When pretreated with C-23 before being added to the nanodiscs, pore formation by GSDMD-3C plus 3C protease was completely blocked. However, C-23 addition after pore formation did not disrupt already assembled pores. Thus, disulfiram inhibits pore formation, but does not disassemble already formed pores.

To evaluate whether C-22, -23, and -24 inhibit pyroptosis, these compounds were added to PMA-differentiated and LPS-primed human THP-1 cells before activating the canonical NLRP3 inflammasome with nigericin or to mouse immortalized bone marrow-derived macrophages (iBMDMs) before activating the non-canonical inflammasome by LPS electroporation (See Figures). Only C-23 blocked pyroptosis, with similar IC_{50} values of 7.7 ± 0.3 μM and 10.3 ± 0.5 μM for canonical human and non-canonical mouse inflammasome-dependent pyroptosis, respectively. It also impaired cell death in a dose-dependent manner triggered by the AIM2 inflammasome in mouse iBMDMs transfected with poly(dA:dT), supporting its inhibition of the common downstream portion of inflammasome pathways. Inhibition was shown by cell survival, assessed by CellTiter-Glo ATP luminescence, and membrane permeabilization, assessed by uptake of the membrane-impermeable dye SYTOX Green. In addition, disulfiram inhibited nigericin-induced IL-1β secretion in THP-1 and LPS transfection-induced IL-1β secretion in iBMDM cells with potency comparable to the pan-caspase inhibitor z-VAD-fmk. In contrast, disulfiram had no effect on necroptosis induced in HT-29 cells by treatment with TNFα, SMAC mimetic, and z-VAD-fmk, which was blocked by either necrosulfonamide (NSA) or necrostatin-1 (Nec). These data show that disulfiram inhibits pyroptosis in both human and mouse cells triggered by canonical and non-canonical inflammasomes, but not necroptosis.

Example 2 - Disulfiram protects against LPS-induced sepsis

Disulfiram is being investigated as an anticancer agent because epidemiological studies showed that individuals taking disulfiram for alcohol addiction were less likely to die of cancer (See Reference 24). In cells disulfiram is rapidly metabolized to diethyldithiocarbamate (DTC) (See Reference 25 and 26):



The anti-cancer activity of DTC in vivo is greatly enhanced by complexation with copper (See, e.g., Reference 24), likely because of the enhanced electrophilicity of the DTC thiols. In liposome leakage assay, it was found that copper gluconate (Cu^{2+}) only weakly increased disulfiram or DTC inhibition. This is likely due to the high reactivity of the GSDMD Cys residue involved (see the following Example).

However, Cu^{2+} strongly promoted the ability of either disulfiram or DTC to protect LPS-primed THP-1 cells from pyroptosis (figure 13). With Cu^{2+}, the IC_{50} of C-23 for inhibiting pyroptosis decreased 24-fold to 0.41 ± 0.02 μM, which was similar to its potency for preventing liposome leakage. DTC became almost as active as C-23 in cells in the presence of Cu^{2+}.

Because C-23 inhibited pyroptosis and IL-1β release in cells, its ability to protect C57BL/6 mice from LPS-induced sepsis was also tested. Mice were treated with vehicle or disulfiram intraperitoneally before challenge with LPS. Whereas the lowest concentration of LPS (15 mg/kg) killed 3 of 8 control mice after 96 hrs, all the disulfiram-treated mice survived ($P < 0.05$) (Fig. 14). Serum IL-1β concentrations were strongly reduced 12 hrs after LPS challenge when all mice were alive (281 ± 149 ng/mL in disulfiram-pre-treated mice, 910 ± 140 ng/mL in control mice ($P < 0.0001$)) (Fig. 15). Following LPS challenge at the intermediate concentration (25 mg/kg), all the control mice died within 72 hrs, but 5 of 8 of the disulfiram-treated mice survived ($P < 0.01$) (Fig. 16). At the highest LPS challenge (50 mg/kg), while all the control mice died within a day, death was significantly delayed by disulfiram treatment and 1 of 8 mice survived ($P < 0.0001$) (Fig. 17). To determine if treatment could be delayed until after LPS challenge and whether adding copper could improve protection, mice were challenged with 25 mg/kg LPS intraperitoneally and administered C-23 with or without copper gluconate immediately and 24 hrs later. Post-LPS treatment still improved survival ($P = 0.041$ without copper and $P = 0.024$ with copper). All the control mice and mice treated without copper died, but 2 of 8 mice given copper-complexed disulfiram survived (Fig. 18). Thus, disulfiram given before or after LPS partially protected mice from septic death and reduced IL-1β secretion.

Experimental results: Figure 12 shows dose response curves of inhibition of liposome leakage by C-23 or its metabolite DTC in the presence or absence of Cu(II). In Figure 13, LPS-primed THP-1 were pre-treated with C-23 or DTC in the presence or absence of Cu(II) for 1 hr before adding nigericin or medium for 2 hrs. Cell death was determined by CytoTox96 assay. In Figures 14-17, mice were pre-treated with C-23 (50 mg/kg) or vehicle (Ctrl) by intraperitoneal injection 24 and 4 hrs before intraperitoneal LPS challenge (Figures 14 and 15: 15 mg/kg; Figure 16: 25 mg/kg; Figure 17: 50 mg/kg) and followed for survival. Statistical analysis was performed using the log-rank test (In figures 14, 16, 17, mice/group). In Figure 15, serum IL-1β measured by ELISA in mice (n = 5/group) pre-treated with C-23 as above and challenged with 15 mg/kg LPS. Serum was obtained 12 hrs post LPS challenge. Shown are mean ± s.d. In Figure 18, mice were treated with C-23 (50 mg/kg), C-23 (50 mg/kg) plus copper gluconate (0.15 mg/kg) or vehicle (Ctrl) by intraperitoneal injection 0 and 12 hrs post intraperitoneal LPS challenge (25 mg/kg). Statistical analysis was performed using the log-rank test (8 mice/group).

In cells, Cu(II) strongly promoted the ability of either disulfiram or DTC to protect LPS-primed THP-1 cells from pyroptosis, presumably because Cu(II) promoted the activity of the major cellular metabolite DTC. With Cu(II), the IC_{50} of disulfiram for inhibiting pyroptosis decreased 24-fold to 0.41 ± 0.02 μM, which was similar to its potency for preventing liposome leakage. DTC became almost as active as disulfiram in cells in the presence of Cu(II). The similar potency of disulfiram (when its principal cellular metabolite is stabilized) at inhibiting GSDMD pore formation in liposomes and pyroptosis in cells supports GSDMD as a major target of the mechanism of action of disulfiram.

Example 3 - Disulfiram covalently modifies GSDMD Cys191

Disulfiram has been shown to inactivate reactive Cys residues by covalent modification (See Reference 27). To probe the mechanism of GSDMD inhibition by disulfiram, nano-liquid chromatography-tandem mass spectrometry (nano-LC-MS/MS) was used to analyse disulfiram-treated human GSDMD. Tryptic fragments indicated a dithiodiethylcarbamoyl adduct of Cys191, in which half of the symmetrical disulfiram molecule is attached to the thiol (Figures 20, 21, 27, and 28). Indeed, Cys191 is required for GSDMD pore formation in cells, since oligomerization was blocked by Ala mutation of the corresponding Cys192 in mouse GSDMD (See Reference 8). This Cys residue, conserved in GSDMD, but not in other GSDM family members, is accessible in both the full-length autoinhibited structure model and the N-terminal pore form model, generated based on mouse GSDMA3 structures (References 7 and 14) (Figures 22 and 29). Corresponding to Leu183 of GSDMA3, Cys191 sits at the distal tip of the membrane spanning region at the beginning of the β8 strand within the β7-β8 hairpin, which is a key element in the β-barrel that forms the pore (Reference 14). Analysis of Cys reactivity using PROPKA (Reference 28) suggests that Cys191 is the most reactive among all Cys residues in GSDMD. Consistent with its high reactivity, a time course analysis showed that disulfiram inhibited liposome leakage within 2 min of incubation (Figure 30). To confirm that disulfiram acts on Cys191, Ala mutations of Cys191, and of Cys38 as a control, were generated. Whereas the disulfiram IC_{50} values for WT and C38A were both around 0.3 μM in the liposome leakage assay, the IC_{50} for C191A was about 8-fold higher (Figure 23). Disulfiram was also incubated with N-acetylcysteine (NAC), which contains a reactive Cys that can inactivate Cys-reactive drugs, before assessing whether disulfiram protects THP-1 cells from nigericin-mediated pyroptosis. As expected, NAC eliminated the activity of disulfiram (Fig. 24). These data together suggest that disulfiram inhibits GSDMD pore formation by selectively and covalently modifying Cys191.

Experimental results: Figures 20 and 21 show MS/MS spectra of the Cys191-containing human GSDMD peptide FSLPGATCLQGEGQGHLSQK (aa 184-103; 2057.00 Da) modified on Cys191 by carbamidomethyl (an increase of 57.0214 Da) [LC retention time, 22.85 min; a triplet charged precursor ion m/z 705.6827 (mass: 2114.0481 Da; delta M 2.27 ppm) was observed] (a) or of the corresponding GSDMD peptide after GSDMD incubation with C-23 (disulfiram), which was modified on Cys191 by the diethyldithiocarbamate moiety of C-23 (an increase of 147.0255 Da). [LC retention time. 28.93 min; a triplet charged precursor ion m/z 735.6802 (mass: 2204.0406 Da; delta M 0.53 ppm) was observed.] (b). Figure 22 shows models of full-length human GSDMD in its auto-inhibited form and of the pore form of GSDMD N-terminal fragment (GSDMD-NT) based on the corresponding structures of GSDMA3 (References 7 and 14) showing the location of Cys191, modified by compound C-23. GSDMD-NT in cyan; GSDMD-CT in grey. Figure 23 shows dose response curve of C-23 inhibition of liposome leakage induced by wild-type, C38A or C191A GSDMD (0.3 μM) plus caspase-11 (0.15 μM). Figure 24 shows C-23 inhibition of pyroptosis of LPS + nigericin treated THP-1 cells after C-23 preincubation for 1 hr with N-acetylcysteine (NAC, 500 μM) or medium. 2-fold dilutions of C-23 ranging from 5 to 40 μM were used. Graphs show the mean ± s.d. and data shown are representative of three independent experiments. $^{**}P < 0.01$. Figures 25 and 26 show dose response curve of compound C-23 in liposome leakage induced by human GSDMD-3C (0.3 µM) plus 3C protease (0.15 µM) (Figure 25) or mouse GSDMA3-3C (0.3 µM) plus 3C protease (0.15 µM) (Figure 26).

Figures 27 and 28 show MS/MS spectrum for the peptide containing Cys191 in human GSDMD. Figure 27 shows MS/MS spectrum for peptide FSLPGATCLQGEGQGHLSQK modified on cysteine by carbamidomethyl. Protein coverage is 73%. Figure 28 shows MS/MS spectrum for peptide FSLPGATCLQGEGQGHLSQK modified on cysteine by C-23. Protein coverage is 72%.

Figures 29 and 30 show that disulfiram covalently modifies GSDMD Cys191. In figure 29, sequence alignment of mouse GSDMA3, human GSDMA (hGSDMA), mouse GSDMD (mGSDMD) and human GSDMD (hGSDMD) shows Cys residues. In Figure 30, GSDMD (0.3 µM) was preincubated with the indicated concentrations of C-23 (0 - 50 µM) for different durations (2 -90 min) before caspase-11 (0.15 µM) in liposome (50 µM) was added.

To confirm that disulfiram acts on Cys191, the disulfiram IC_{50} values were compared for pore formation in liposomes treated with WT, C38A control or C191A human GSDMD plus caspase-11. The IC_{50} for disulfiram acting on C191A GSDMD was ~8-fold higher than on WT GSDMD, while the activity on C38A was similar to WT GSDMD, confirming the importance of Cys191 for disulfiram activity. The residual inhibition of the Cys191 mutant may be due to disulfiram modifications of other Cys residues in the mutant GSDMD. To confirm the importance of Cys191 in pore formation, cell death was measured by LDH release in HEK293T cells ectopically expressing full-length human WT or C191S mutant GSDMD with or without caspase-11. Although WT or C191S GSDMD alone did not compromise cell survival, WT GSDMD and caspase-11 together caused substantial cell death, which was reduced for C191S GSDMD and caspase-11. Similarly, cell death caused by ectopic expression of mouse GSDMD-NT (mGSDMD-NT) was significantly reduced in HEK293T cells expressing the analogous C192S mutant, but only modestly in cells expressing C39A mGSDMD-NT. These results confirm the role of Cys191 and Cys192 in GSDMD-NT pore formation in humans and mice, respectively, consistent with previous results.

To further confirm that disulfiram acts on Cys191, disulfiram inhibition of LDH release in HEK293T cells expressing caspase-11 and WT or C191S GSDMD was assessed. As expected, WT GSDMD-induced cell death was strongly inhibited by disulfiram in a dose-dependent manner beginning at the lowest concentration tested (10 μM), but the reduced cell death caused by expression of caspase-11 and C191S GSDMD was only inhibited when 4 times as much disulfiram was added. These data together indicate that disulfiram inhibits GSDMD pore formation by covalently modifying Cys191. In addition, the data suggests that disulfiram inhibits cell death mainly through its effect on GSDMD-NT pore formation because if disulfiram strongly inhibited caspase-11, it would have provided better protection from death of cells expressing caspase-11 and C191S GSDMD.

Example 4 - Disulfiram (C-23) inhibits caspase-1 and caspase-11

Disulfiram has been reported to inhibit caspases by binding to the catalytic Cys responsible for proteolysis (See Reference 29). It is therefore likely that disulfiram inhibits both caspases and GSDMD. Using a fluorogenic caspase activity assay that measures the release of 7-amino-4-methylcoumarin (AMC) from substrate Ac-YVAD-AMC, it was found that disulfiram indeed inhibited caspase-1 and caspase-11 with IC_{50} of 0.15 ± 0.04 μM and 0.73 ± 0.07 μM, respectively (Figures 31-38). Adding Cu(II) did not strongly change disulfiram caspase inhibition in vitro. To determine the relative contribution of caspase-11 inhibition versus GSDMD inhibition by disulfiram in pore formation, the caspase cleavage site in GSDMD was replaced with the rhinovirus 3C protease site (GSDMD-3C) and the 3C protease was used instead of caspase-11 in the liposome leakage assay. The resulting IC_{50} was 0.52 ± 0.03 μM, comparable to 0.30 ± 0.01 μM for caspase-11-triggered liposome leakage (Figures 2 and 25). By contrast, as mouse GSDMA3 lacks the conserved Cys191, disulfiram inhibited liposome leakage triggered by 3C-cleaved GSDMA3 containing a 3C protease site (GSDMA3-3C) with a much weaker IC_{50} of 12.14 ± 2.10 μM (Fig. 26). Thus, the inhibitory effect of disulfiram in the liposome leakage assay is mediated by direct inhibition of GSDMD.

Experimental results: Figures 31 and 32 show time course of caspase-1 and caspase-11 activity in the presence of indicated concentrations of compound C-23. Caspases (0.5 U) were incubated with compound C-23 (at indicated concentrations for 1 hr before adding Ac-YVAD-AMC (40 μM)). Figures 33 and 34 show dose response curve of compound C-23 in the caspase-1 and caspase-11 activity assay. Figures 35 and 36 show time course of caspase-1 and caspase-11 activity in the presence of indicated concentrations of compound C-23 + Cu(II). Caspases (0.5 U) were incubated with compound C-23 + Cu(II) (at indicated concentrations for 1 hr before adding Ac-YVAD-AMC (40 μM)). Figures 37 and 38 show dose response curve of compound C-23 + Cu(II) in the caspase-1 and caspase-11 activity assay. Fluorescence intensity at 460 nm was measured after excitation at 350 nm.

Example 5 – test compounds inhibit GSDMD pore formation

IC_{50} values of the test compounds shown in Figure 39 in liposome leakage assay are shown in Figures 40-42. Data shows that the tested compounds protected against nigericin-induced pyroptosis in THP-1. Results of the leakage assay are shown in Table 2. Chemical structures of compounds listed in Table 2 are shown in Figure 39.

Table 2

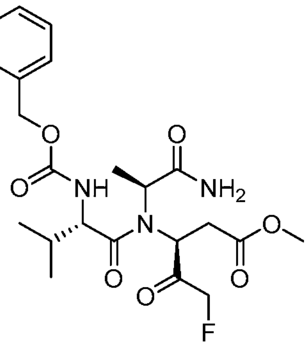
Compound	IC_{50} (μM)
C-23	0.30 ± 0.01
C-23A1	0.22 ± 0.01
C-23A2	0.37 ± 0.01
C-23A3	0.46 ± 0.08
C-23A4	0.26 ± 0.01
C-23A5	3.74 ± 1.06
C-23A6	0.35 ± 0.03
C-23A7	0.25 ± 0.01
C-23A8	1.25 ± 0.01
C-23A9	0.26 ± 0.003
C-23A10	0.26 ± 0.02
C-23A11	0.37 ± 0.01
C-23A12	2.93 ± 1.07

Experimental results: in Figure 40, PMA-differentiated LPS-primed THP-1 cells were treated with the indicated compounds (40 μM) for 3 hrs and tested for viability by CellTiter-Glo assay. In Figure 41, PMA-differentiated LPS-primed THP-1 cells were pretreated with 40 μM disulfiram or the indicated test compounds or z-

VAD-fmk for 1 hr before treatment or not with nigericin, and the cells were assessed for cell viability by CellTiter-Glo assay 2 hrs after adding nigericin. In Figure 42, PMA-differentiated LPS-primed THP-1 cells were pretreated with 40 μM disulfiram or z-VAD-fmk or with 2-fold serial dilutions (concentration range, 0.39-50 μM) of indicated test compounds for 1 hr before adding nigericin, and the cells were assessed for cell viability by CellTiter-Glo assay 2 hrs after adding nigericin. Graphs show the mean ± s.d. and data shown are representative of three independent experiments. **P < 0.01. None of the tested compounds was toxic to THP-1 cells (See Figures). The tested compounds also significantly protected against nigericin-induced pyroptosis in THP-1 cells.

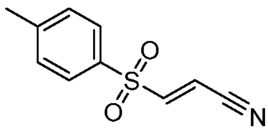
Example 6a - Disulfiram and Bay 11-7082 inhibit multiple steps in inflammasome activation cascade

It was found that pan-caspase inhibitor z-VAD-fmk (CAS Registry No. 187389-52-2):



inhibits the canonical inflammasome pathway in THP-1 cells.

It was also found that Bay 11-7082 (CAS Registry No. 19542-67-7):



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a previously known inhibitor of NF-κB activation (Reference 13) and the NLRP3 pathway (Reference 30) (Fig. 43) also inhibits the canonical inflammasome pathway in THP-1 cells. As discussed below, Bay 11-7082 inhibits, e.g., GSDMD, caspase-1 and caspase-11.

Bay 11-7082 bound to GSDMD according to MST (See Figures 55 and 56 and Figure 2). Bay 11-7082 inhibited caspase-1 and to lesser extend caspase-11 (See Figures 55-58). Surprisingly, like disulfiram, Bay 11-7082 functions by inactivating reactive Cys residues (See References 31 and 32), and Cys191 in GSDMD was covalently modified by Bay 11-7082 (See Figures 59 and 60). Bay 11-7082 inhibition of liposome leakage was reduced 2-fold by substituting C191A GSDMD for WT GSDMD in the liposome leakage assay (Figure 55). Much of Bay 11-7082 inhibition of liposome leakage could be attributed to caspase-11 inhibition, since Bay 11-7082 was less able to inhibit leakage caused by GSDMD-3C plus 3C protease than by GSDMD plus caspase-11 and its activity against mouse GSDMA3-3C, which lacks a comparable reactive cysteine, plus 3C protease was similar to its activity against GSDMD-3C (See Figures 61 and 62).

Bay 11-7082 inhibited pyroptosis triggered by both the canonical and non-canonical inflammasomes in THP-1 cells, but was more active in nigericin-treated than LPS-transfected cells (Figures 43 and 44). Bay 11-7082 was more effective at inhibiting canonical inflammasome-dependent pyroptosis than disulfiram in the absence of copper, and the two drugs together had an additive protective effect, although were cytotoxic at the highest concentration tested (Fig. 43). Bay 11-7082 was less active than disulfiram at inhibiting pyroptosis induced by non-canonical inflammasome activation (Fig. 44).

Because both disulfiram and Bay 11-7082 non-specifically modify reactive Cys, their effects on the steps leading to pyroptosis and inflammatory caspase activation were next analyzed. Some of the genes that participate in the canonical inflammasome pathway are not expressed in unstimulated cells and their expression needs to be induced, often by binding to cell surface sensors of pathogen and danger-associated molecular patterns, such as Toll-like receptors (TLR), in a process called priming. Bay 11-7082 is known to inhibit NF-κB activation, a key transcription factor in priming. The effect of disulfiram and Bay 11-7082 on priming were first examined (Fig. 45). NF-κB activation was assessed by examining IκBα phosphorylation and degradation and RelA (p65) phosphorylation. Induction of pro-IL-1β was assessed by immunoblot for pro-IL1β protein. In the absence of disulfiram or Bay 11-7082, phosphorylation of p65 was first detected 30 min after adding LPS and persisted for 4 hrs, phosphorylation and reduced IκBα were detected 1 hr after adding LPS, and increased pro-IL-1β was detected 4 hrs after adding LPS. Both tested compounds, added at 30 μM concentrations, inhibited NF-κB activation, but Bay 11-7082 had a stronger effect; both blocked pro-IL-1β induction. Thus, disulfiram and Bay 11-7082 both inhibit priming.

Nigericin activates the assembly of the NLRP3 canonical inflammasome using an adaptor called apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), which can be visualized in immunofluorescent microscopy as specks. When LPS-primed THP-1 cells were treated with nigericin in the absence of inhibitors, ASC specks were detected in 30% of cells (Fig. 36). As expected, speck formation was not inhibited by z-VAD-fmk, since caspase activation occurs downstream of inflammasome assembly. However, both test compounds, added after priming but one hour before nigericin, inhibited ASC speck formation, but not completely, and Bay 11-7082 was more potent than disulfiram when used at the same concentration. 1 μM disulfiram was completely inactive at blocking pyroptosis triggered by nigericin or transfected LPS (Figures 6 and 7), but the same concentration of disulfiram in combination with copper gluconate blocked pyroptosis completely and also reduced ASC puncta (Figures 48 and 49).

To assess which steps in NLRP3-mediated inflammation were inhibited post ASC speck formation, LPS-primed THP-1 cells were treated with vehicle or 30 μM z-VAD-fmk, disulfiram or Bay 11-7082 1 hr before adding nigericin, and cleavage and activation of caspase-1, GSDMD, and pro-IL-1β were analysed by immunoblot of whole cell lysates 30 min later (Figure 50). Secretion of processed IL-1β was also assessed by immunoblot of culture supernatants. Caspase-1, GSDMD and pro-IL-1β cleavage to their active forms was clearly detected in the absence of inhibitors, but was dramatically reduced in cells treated by any of the 3 inhibitors; moreover, processed IL-1β was only detected in the culture supernatants in the absence of any inhibitor. When the same experiment was repeated by treating cells with only 1 μM disulfiram in PBS or copper gluconate, disulfiram complexed with copper completely blocked caspase-1, GSDMD, and pro-IL-1β processing and IL-1β secretion, but disulfiram without copper had no effect (Figure 51). Because immunoblots are not quantitative, caspase-1 activity 30 min after adding nigericin was also assessed using a fluorescent substrate in intact cells. While caspase-1 activity was completely inhibited by z-VAD-fmk, it was only partially reduced by either disulfiram and Bay 11-7082, again more strongly by Bay 11-7082 (Figure 52). Next, the effect of z-VAD-fmk, disulfiram and Bay 11-7082 on LPS + nigericin-induced GSDMD pore formation was assessed by immunofluorescence microscopy using a monoclonal antibody that was generated that recognizes both uncleaved GSDMD and its pore form (Figures 53, 54, and 64-66). In the absence of any inhibitor, the GSDMD antibody stained both the cytosol and the plasma membrane of LPS plus nigericin treated cells, which formed characteristic pyroptotic bubbles (See Reference 10). All 3 inhibitors completely blocked GSDMD membrane staining and the appearance of pyroptotic bubbles. Thus, disulfiram and Bay 11-7082 inhibit multiple steps leading to canonical inflammasome-induced pyroptosis and inflammatory cytokine release, including priming, inflammasome assembly, inflammatory caspase activation, pro-inflammatory cytokine processing and GSDMD pore formation.

Experimental results: In Figure 43, PMA-differentiated LPS-primed THP-1 cells were pretreated with 2-fold serial dilutions (ranging from 0.3125 to 40 µM) of C-23 and/or Bay 11-7082 for 1 hr before treatment with nigericin. Cell death was determined by CytoTox96 assay. In Figure 44, mouse iBMDMs were pretreated with serial 2-fold dilutions of C-23 or Bay 11-7082 (ranging from 0.3125 to 40 µM) for 1 hr before electroporation with PBS or LPS. Cell death was determined by CytoTox96 assay. In Figure 45, THP-1 cells were pretreated with 30 µM C-23 or Bay 11-7082 for 1 hr before adding LPS. Shown are immunoblots of whole cell lysates harvested 0.5 hr later. In Figures 46, 47, 50, and 52, LPS-primed THP-1 were pretreated with 30 µM C-23, Bay 11-7082 or z-VAD-fmk for 1 hr before adding nigericin or medium. Representative images of ASC specks (arrowheads) and mean ± s.d. percent of cells with ASC specks analyzed 20 min later (Figure 47). Whole cell lysates (WCL) and culture supernatants (Sup) were harvested 30 min after adding nigericin and immunoblotted with the indicated antibodies (Figure 50). Caspase-1 activity was assayed 30 min after adding nigericin using a cell-permeable fluorescence dye FAM-YVAD-FMK (Figure 52). In Figures 48, 49 and 51, LPS-primed THP-1 were pretreated with 1 µM C-23 in the presence or absence of Cu(II) for 1 hr before adding nigericin or medium. Representative images of ASC specks (arrowheads) and mean ± s.d. percent of cells with ASC specks analyzed 20 min later (Figures 48 and 49). Whole cell lysates (WCL) and culture supernatants (Sup), harvested 30 min after adding nigericin, were analyzed by immunoblot (Figure 51). In Figures 53 and 54, LPS-primed THP-1 were pretreated with 30 µM C-23, Bay 11-7082 or z-VAD-fmk for 1 hr before adding nigericin or medium and stained with a mouse anti-GSDMD monoclonal antibody (see Figures 55-63) 30 min later. The Figurs show representative confocal microscopy images and quantification of proportion of cells with GSDMD membrane staining and pyroptotic bubbles. Arrows indicate GSDMD staining of pyroptotic bubbles. Figure 55 shows Bay 11-7082 dose response curve of inhibition of liposome leakage by wild-type, C38A or C191A GSDMD (0.3 μM) plus caspase-11 (0.15 μM). Figure 56 shows MST measurement of the direct binding of Alexa 488-labeled His-MBP-GSDMD (80 nM) with Bay 11-7082 by NanoTemper. Figures 57 and 58, dose response curve of the effect of Bay 11-7082 on caspase-1 (fig. 57) and caspase-11 (fig. 58) activity against a fluorescent peptide substrate. Figures 59 and 60 show MS/MS spectra of the Cys191-containing GSDMD peptide FSLPGATCLQGEGQGHLSQK (aa 184-103; 2057.00 Da) modified on Cys191 by carbamidomethyl (an increase of 57.0214 Da) [LC retention time, 22.85 min; a triplet charged precursor ion m/z 705.6827 (mass: 2114.0481 Da; delta M 2.27 ppm) was observed] (fig. 59) or of the corresponding GSDMD peptide after GSDMD incubation with Bay 11-7082, which was modified on Cys191 (an increase of 207.0354 Da). [LC retention time, 17.20 min; a triplet charged precursor ion m/z 756.0229 (mass: 2264.0688 Da; delta M 11.7 ppm) was observed.] (fig. 60). Figures 61 and 62 show dose response curve of the effect of Bay 11-7082 on liposome leakage induced by 0.3 μM human GSDMD-3C (fig. 61) or mouse GSDMA3-3C (fig. 62) plus 0.15 μM 3C protease. Figure 63 shows effect of 1 hr preincubation of Bay 11-7082 with N-acetylcysteine (NAC, 500 μM) on inhibition of pyroptosis of LPS + nigericin treated THP-1 cells. 2-fold dilutions of Bay 11-7082 from 5-40 μM were used. Graphs show the mean ± s.d; data are representative of three independent experiments. *P < 0.05, **P < 0.01.

In comparison with disulfiram, Bay 11-7082 bound to GSDMD with a lower affinity and was 23 times less active at inhibiting liposome leakage (IC_{50} 6.81 ± 0.10 μM vs 0.30 ± 0.01 μM). Bay 11-7082 also inhibited caspase-1, but was about 3 times less active against caspase-11 than disulfiram. Like disulfiram, Bay 11-7082 functions by inactivating reactive Cys residues29,30. By nano-LC-MS/MS, Bay 11-7082 was found to covalently modify Cys191 in GSDMD. However, Bay 11-7082 inhibition of liposome leakage was only reduced 2-fold by substituting C191A GSDMD for WT GSDMD in the assay. Hence, much of Bay 11-7082 inhibition of liposome leakage could be attributed to caspase-11 inhibition, since Bay 11-7082 was substantially less able to inhibit leakage caused by GSDMD-3C plus 3C protease than by GSDMD plus caspase-11 and its activity against mouse GSDMA3-3C, which lacks a comparable reactive cysteine, plus 3C protease was similar to its activity against GSDMD-3C. Therefore, unlike disulfiram, Bay 11-7082 is more of a caspase inhibitor than a GSDMD inhibitor in the liposome leakage assay.

Example 6b – inhibitors of inflammasome activation cascade

Recently the Cys-reactive necroptotic inhibitor NSA was shown to also inhibit GSDMD-mediated pyroptosis. The potency of disulfiram at inhibiting GSDMD and caspase-11-mediated liposome leakage with that of NSA and other Cys-reactive compounds was compared, including dimethyl fumarate (DMF, a drug for psoriasis and multiple sclerosis), afatinib (a drug that inhibits epidermal growth factor receptor tyrosine kinase), ibrutinib (a drug that inhibits Bruton's tyrosine kinase), and LDC7559. NSA moderately inhibited liposome leakage but was about 30-fold less potent than disulfiram (IC_{50} of 9.50 ± 0.43 μM).

Example 7 - Mouse monoclonal antibody recognizes full-length human GSDMD and the GSDMD-NT pore form on immunoblots and by immunofluorescence microscopy

The monoclonal antibody against GSDMD was generated by immunizing mice with recombinant human GSDMD and boosting with recombinant human GSDMD-NT as described in Methods. In Figure 64, HEK293T cells were transfected with the indicated plasmids and cell lysates were analyzed by immunoblot of reducing gels probed with the indicated antibodies. In Figure 65, cell lysates of HCT116, 293T and THP-1 cells, treated or not with nigericin, were immunoblotted with the indicated antibodies. 293T cells do not express endogenous GSDMD. In Figure 66, 293T and THP-1 cells were immunostained with the anti-GSDMD monoclonal antibody and co-stained with DAPI (blue). 293T cells that do not express GSDMD show no background staining.

Example 8 – mechanistic investigation

To elucidate the cellular mechanism of pyroptosis inhibition by disulfiram, its effects on the entire inflammasome activation pathway were analyzed. Some of the genes that participate in the canonical inflammasome pathway are not expressed in unstimulated cells and their expression needs to be induced, often by binding to cell surface sensors of pathogen and danger-associated molecular patterns, such as Toll-like receptors (TLR), in a process called priming. In previous experiments, disulfiram was added 4 hours after LPS priming and 1 hour before stimulating with nigericin and thus the effect of disulfiram in inflammasome priming was not investigated. To look at priming explicitly, THP-1 cells were pretreated with disulfiram for 1 hour before adding LPS for up to 4 hours. NF-κB activation, a key transcription factor in priming, was assessed by examining IκBα phosphorylation and degradation, and RelA (p65) phosphorylation. Induction of NLRP3 and pro-IL-1β expression was assessed by immunoblot. Bay 11-7082 was used as a positive control because of its known inhibitory effect on NF-κB activation. In the absence of disulfiram or Bay 11-7082, phosphorylation of p65 was first detected 30 min after adding LPS and persisted for 4 hours, phosphorylation and reduced IκBα were detected 1 hour after adding LPS, and increased NLRP3 and pro-IL-1β protein were detected 4 hours after adding LPS. Both drugs inhibited NF-κB activation, but Bay 11-7082 had a stronger effect; both blocked NLRP3 and pro-IL-1β induction.

Nigericin activates the assembly of the NLRP3 canonical inflammasome using an adaptor called apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), which can be visualized in immunofluorescence microscopy as specks. When LPS-primed THP-1 cells were treated with nigericin in the absence of inhibitors, ASC specks were detected in about 30% of cells. As expected, speck formation was not inhibited by z-VAD-fmk, since caspase activation occurs downstream of inflammasome assembly. Disulfiram, added after priming but one hour before nigericin, modestly inhibited ASC speck formation, to about 20% of cells. The modest reduction in speck formation is attributed to subtle inhibition of priming by disulfiram even though it was added after 4 hours of LPS priming. Indeed, immunoblot showed that the NLRP3 level was reduced by disulfiram added after priming compared to cells incubated in medium.

Canonical inflammasome assembly activates caspase-1, which cleaves pro-IL-1β and GSDMD, and the latter is needed to release processed IL-1β and to induce pyroptosis. To assess which steps in NLRP3-mediated inflammation were inhibited post ASC speck formation, LPS-primed THP-1 cells were treated with vehicle, 30 μM z-VAD-fmk or disulfiram 1 hour before adding nigericin, and cleavage and activation of caspase-1, GSDMD, and pro-IL-1β were analysed by immunoblot of whole cell lysates 30 min later and 1 hr later. Secretion of processed IL-1β was also assessed by immunoblot of culture supernatants. Caspase-1, GSDMD and pro-IL-1β cleavage to their active forms was clearly detected in the absence of inhibitors and their processing was reduced in cells treated by disulfiram or z-VAD-fmk at 30 min after nigericin. However, by 60 min, consistent with the weaker effects of disulfiram on caspases, processing of caspase-1, GSDMD and pro-IL-1β in disulfiram-treated samples caught up with what was detected in the absence of inhibitors, while the sample treated with z-VAD-fmk still showed little cleavage of these proteins. The 1 hr time point is relevant as the cell death and IL-1β release measurements used cells stimulated with nigericin for 1 and 2 hrs, respectively. These data suggest that disulfiram delayed, but did not inhibit, caspase-1 activation. However, processed IL-1β was only detected in culture supernatants in the absence of either inhibitor, suggesting that despite limited caspase-1 inhibition, disulfiram completely inhibited cytokine release by blocking GSDMD pore formation. Similar preferential effects of disulfiram on IL-1β release (but not processing) were found in mouse iBMDMs, while NSA, Bay 11-7082 and z-VAD-fmk still inhibited processing of caspase-1, GSDMD and IL-1β at the 1 hr time point.

The effect of z-VAD-fmk and disulfiram on LPS plus nigericin-induced GSDMD pore formation was assessed next by immunofluorescence microscopy using a monoclonal antibody that was generated in the previous example that recognizes both uncleaved GSDMD and its pore form. In the absence of any inhibitor, the GSDMD antibody stained both the cytosol and the plasma membrane of LPS plus nigericin treated cells, which formed characteristic pyroptotic bubbles. Both inhibitors completely blocked GSDMD membrane staining and the appearance of pyroptotic bubbles. Thus, while disulfiram inhibits priming and delays caspase-1 activation, its effects culminate at the bottleneck step of GSDMD pore formation to curtail both pyroptosis and inflammatory cytokine release in both THP-1 and iBMDM cells. In contrast, the control inhibitor z-VAD-fmk blocks exclusively caspase-1 activity.

To investigate the in vivo effect of disulfiram, the LPS-induced sepsis was examined in C57BL/6 mice. Mice were treated with vehicle or disulfiram intraperitoneally before challenge with LPS using a drug dose (50 mg/kg) that was equivalent, after allometric scaling to account for body surface area, to 284 mg/day in humans, which is within the 125-500 mg/day dose range clinically approved to treat alcohol dependence[32]. Whereas the lowest concentration of LPS (15 mg/kg) killed 3 of 8 control mice after 96 hours, all the disulfiram-treated mice survived (p = 0.045). Serum IL-1β, TNFα and IL-6 concentrations were strongly reduced 12 hours after LPS challenge when all mice were alive ($p \leqslant 0.0003$). Following LPS challenge at the intermediate concentration (25 mg/kg), all the control mice died within 72 hours, but 5 of 8 of the disulfiram-treated mice survived (p = 0.008). At the highest LPS challenge (50 mg/kg), while all the control mice died within a day, death was significantly delayed by disulfiram treatment and 1 of 8 mice survived (p = 0.007). LPS-induced sepsis in mice depends on GSDMD cleavage by caspase-11 in the non-canonical inflammasome. Consistent with previous studies, *Casp11*$^{-/-}$ and *Gsdmd*$^{-/-}$ mice, but not *Casp1*$^{-/-}$, mice were resistant to death from LPS-induced sepsis. As expected, disulfiram protected *Casp1*$^{-/-}$ mice from lethal LPS challenge but did not significantly affect the survival of *Casp11*$^{-/-}$ and *Gsdmd*$^{-/-}$ mice since all but 1 mouse in each undrugged control group survived.

To determine if complexation with Cu(II) could improve protection from sepsis in vivo, the effectiveness of disulfiram administered with or without Cu(II) was compared on survival of mice challenged with 25 mg/kg LPS intraperitoneally. To better mimic the clinical situation where sepsis is usually diagnosed only after the inflammatory cascade has begun, disulfiram administration was deferred until just after LPS injection and 12 hours later. Post-LPS disulfiram treatment significantly delayed death (p = 0.041 without Cu(II); p = 0.024 with copper). Although all the control mice and mice treated with disulfiram alone died, 2 of 8 mice given Cu(II)-complexed disulfiram survived. The difference in survival between disulfiram treatment with and without Cu(II), however, did not reach significance (p = 0.064). Thus, disulfiram given after LPS partially protected mice and administration with Cu(II) may have improved its activity.

LPS not only causes non-canonical inflammasome activation intracellularly, which does not need priming, but also primes NLRP3 inflammasome activation, which amplifies septic shock. Genetic deficiency of NLRP3, ASC, caspase-1, or the IL-1 receptor did not offer substantial survival advantages in mice challenged with LPS in previous studies, while caspase-11 or GSDMD deficiency protected mice from septic death. It is therefore reasoned that protection from LPS-induced sepsis likely depends on inhibiting GSDMD cleavage or pore formation, but not NLRP3 inflammasome priming. This reasoning is supported by our own finding that disulfiram protected *Casp1*$^{-/-}$ and WT mice similarly.

To determine whether disulfiram mainly inhibits GSDMD processing by caspase-11 or pore formation, four groups of mice were pretreated with disulfiram or vehicle 4 hrs before and immediately before challenge by LPS or vehicle intraperitoneally. Peritoneal macrophages were harvested 6 hrs later and analysed for NLRP3, GSDMD and HMGB1 by immunoblot. GSDMD was equally processed in LPS-challenged groups with or without disulfiram treatment, indicating that suppression of death was due to inhibition of GSDMD pore formation, rather than inhibition of GSDMD cleavage. Surprisingly, NLRP3 levels were also similar in LPS-challenged groups with or without disulfiram treatment, suggesting that even though disulfiram compromised NLRP3 priming in cells, it did not inhibit NLRP3 priming in mice. These results strongly suggest that inhibiting GSDMD pore formation to stop LPS-induced pyroptosis and release of inflammatory mediators is the main target of disulfiram in our model.

Disulfiram inhibition of GSDMD pore formation in mouse and human cells complements its activity in blocking inflammasome priming and caspase activity to suppress pyroptosis and inflammatory cytokine release triggered by both canonical and non-canonical pathways. The simultaneous targeting of three steps in the inflammasome pathway means that disulfiram, especially when given with Cu(II) to stabilize its intermediate, is an especially potent inhibitor of inflammation. The results presented herein indicate that inhibition of pore formation, a common mandatory final step in both pyroptosis and inflammatory mediator release, dominates disulfiram's anti-inflammatory activity. Its relatively weaker activity in inhibiting priming and caspases may have allowed disulfiram to be non-toxic to humans while more potent NF-κB inhibitors such as Bay 11-7082 and caspase inhibitors have both been associated with toxicity. Additionally, the non-canonical inflammasome does not require priming and in disease situations, priming of the relevant immune and epithelial cells may have already occurred by the time signs and symptoms of inflammation are clinically recognized, suggesting that inhibiting GSDMD to stop the most downstream step in pyroptosis and inflammatory mediator release will be especially useful. Finally, the relative selectivity of disulfiram is supported by the lack of activity against GSDMD of a number of other covalent Cys-reactive compounds, including the highly reactive DMF.

REFERENCES

1 Rathinam, V. A., Vanaja, S. K. & Fitzgerald, K. A. Regulation of inflammasome signaling. *Nat Immunol* **13**, 333-332 (2012).

2 Lamkanfi, M. & Dixit, V. M. Inflammasomes and their roles in health and disease. *Annu Rev Cell Dev Biol* **28**, 137-161 (2012).

3 Jo, E. K., Kim, J. K., Shin, D. M. & Sasakawa, C. Molecular mechanisms regulating NLRP3 inflammasome activation. *Cellular & molecular immunology* **13**, 148-159 (2016).

4 Frangogiannis, N. G., Smith, C. W. & Entman, M. L. The inflammatory response in myocardial infarction. *Cardiovasc Res* **53**, 31-47 (2002).

5 He, W. T., Wan, H., Hu, L., Chen, P., Wang, X., Huang, Z., Yang, Z. H., Zhong, C. Q. & Han, J. Gasdermin D is an executor of pyroptosis and required for interleukin-1beta secretion. *Cell Res* **25**, 1285-1298 (2015).

6 Kayagaki, N., Stowe, I. B., Lee, B. L., O'Rourke, K., Anderson, K., Warming, S., Cuellar, T., Haley, B., Roose-Girma, M., Phung, Q. T., Liu, P. S., Lill, J. R., Li, H., Wu, J., Kummerfeld, S., Zhang, J., Lee, W. P., Snipas, S. J., Salvesen, G. S., Morris, L. X., Fitzgerald, L., Zhang, Y., Bertram, E. M., Goodnow, C. C. & Dixit, V. M. Caspase-11 cleaves gasdermin D for non-canonical inflammasome signalling. *Nature* **526**, 666-671 (2015).

7 Ding, J., Wang, K., Liu, W., She, Y., Sun, Q., Shi, J., Sun, H., Wang, D. C. & Shao, F. Pore-forming activity and structural autoinhibition of the gasdermin family. *Nature* **535**, 111-116 (2016).

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

9 Sborgi, L., Ravotti, F., Dandey, V. P., Dick, M. S., Mazur, A., Reckel, S., Chami, M., Scherer, S., Huber, M., Bockmann, A., Egelman, E. H., Stahlberg, H., Broz, P., Meier, B. H. & Hiller, S. Structure and assembly of the mouse ASC inflammasome by combined NMR spectroscopy and cryo-electron microscopy. *Proc Natl Acad Sci U S A* **112**, 13237-13242 (2015).

10 Chen, X., He, W. T., Hu, L., Li, J., Fang, Y., Wang, X., Xu, X., Wang, Z., Huang, K. & Han, J. Pyroptosis is driven by non-selective gasdermin-D pore and its morphology is different from MLKL channel-mediated necroptosis. *Cell Res* **26**, 1007-1020 (2016).

11 Russo, H. M., Rathkey, J., Boyd-Tressler, A., Katsnelson, M. A., Abbott, D. W. & Dubyak, G. R. Active Caspase-1 Induces Plasma Membrane Pores That Precede Pyroptotic Lysis and Are Blocked by Lanthanides. *J Immunol* **197**, 1353-1367 (2016).

12 Wright, C. & Moore, R. D. Disulfiram treatment of alcoholism. *Am J Med* **88**, 647-655 (1990).

13 Pierce, J. W., Schoenleber, R., Jesmok, G., Best, J., Moore, S. A., Collins, T. & Gerritsen, M. E. Novel inhibitors of cytokine-induced IkappaBalpha phosphorylation and endothelial cell adhesion molecule expression show anti-inflammatory effects in vivo. *J Biol Chem* **272**, 21096-21103 (1997).

14 Ruan, J., Xia, S., Liu, X., Lieberman, J. & Wu, H. Cryo-EM structure of the gasdermin A3 membrane pore. *Nature* (2018).

15 Baldwin, A. G., Brough, D. & Freeman, S. Inhibiting the Inflammasome: A Chemical Perspective. *J Med Chem* **59**, 1691-1710 (2016).

16 Zhou, R., Yazdi, A. S., Menu, P. & Tschopp, J. A role for mitochondria in NLRP3 inflammasome activation. *Nature* **469**, 221-225 (2011).

17 Nakahira, K., Haspel, J. A., Rathinam, V. A., Lee, S. J., Dolinay, T., Lam, H. C., Englert, J. A., Rabinovitch, M., Cernadas, M., Kim, H. P., Fitzgerald, K. A., Ryter, S. W. & Choi, A. M. Autophagy proteins regulate innate immune responses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome. *Nat Immunol* **12**, 222-230 (2011).

18 Moon, J. S., Nakahira, K., Chung, K. P., DeNicola, G. M., Koo, M. J., Pabon, M. A., Rooney, K. T., Yoon, J. H., Ryter, S. W., Stout-Delgado, H. & Choi, A. M. NOX4-dependent fatty acid oxidation promotes NLRP3 inflammasome activation in macrophages. *Nat Med* **22**, 1002-1012 (2016).

19 Zanoni, I., Tan, Y., Di Gioia, M., Broggi, A., Ruan, J., Shi, J., Donado, C. A., Shao, F., Wu, H., Springstead, J. R. & Kagan, J. C. An endogenous caspase-11 ligand elicits interleukin-1 release from living dendritic cells. *Science* **352**, 1232-1236 (2016).

20 Lupfer, C. R., Anand, P. K., Liu, Z., Stokes, K. L., Vogel, P., Lamkanfi, M. & Kanneganti, T. D. Reactive oxygen species regulate caspase-11 expression and activation of the non-canonical NLRP3 inflammasome during enteric pathogen infection. *PLoS Pathog* **10**, e1004410 (2014).

21 Chu, L. H., Indramohan, M., Ratsimandresy, R. A., Gangopadhyay, A., Morris, E. P., Monack, D. M., Dorfleutner, A. & Stehlik, C. The oxidized phospholipid oxPAPC protects from septic shock by targeting the non-canonical inflammasome in macrophages. *Nature communications* **9**, 996 (2018).

22 Zhong, Z., Umemura, A., Sanchez-Lopez, E., Liang, S., Shalapour, S., Wong, J., He, F., Boassa, D., Perkins, G., Ali, S. R., McGeough, M. D., Ellisman, M. H., Seki, E., Gustafsson, A. B., Hoffman, H. M., Diaz-Meco, M. T., Moscat, J. & Karin, M. NF-kappaB Restricts Inflammasome Activation via Elimination of Damaged Mitochondria. *Cell* **164**, 896-910 (2016).

23 Zhang, J. H., Chung, T. D. & Oldenburg, K. R. A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. *J Biomol Screen* **4**, 67-73 (1999).

24 Skrott, Z., Mistrik, M., Andersen, K. K., Friis, S., Majera, D., Gursky, J., Ozdian, T., Bartkova, J., Turi, Z., Moudry, P., Kraus, M., Michalova, M., Vaclavkova, J., Dzubak, P., Vrobel, I., Pouckova, P., Sedlacek, J., Miklovicova, A., Kutt, A., Li, J., Mattova, J., Driessen, C., Dou, Q. P., Olsen, J., Hajduch, M., Cvek, B., Deshaies, R. J. & Bartek, J. Alcohol-abuse drug disulfiram targets cancer via p97 segregase adaptor NPL4. *Nature* **552**, 194-199 (2017).

25 Shen, M. L., Johnson, K. L., Mays, D. C., Lipsky, J. J. & Naylor, S. Determination of in vivo adducts of disulfiram with mitochondrial aldehyde dehydrogenase. *Biochem Pharmacol* **61**, 537-545 (2001).

26 Petersen, E. N. The pharmacology and toxicology of disulfiram and its metabolites. *Acta Psychiatr Scand Suppl* **369**, 7-13 (1992).

27 Castillo-Villanueva, A., Rufino-Gonzalez, Y., Mendez, S. T., Torres-Arroyo, A., Ponce-Macotela, M., Martinez-Gordillo, M. N., Reyes-Vivas, H. & Oria-Hernandez, J. Disulfiram as a novel inactivator of Giardia lamblia triosephosphate isomerase with antigiardial potential. *Int J Parasitol Drugs Drug Resist* **7**, 425-432 (2017).

28 Sanchez, R., Riddle, M., Woo, J. & Momand, J. Prediction of reversibly oxidized protein cysteine thiols using protein structure properties. *Protein Sci* **17**, 473-481 (2008).

29 Nobel, C. S., Kimland, M., Nicholson, D. W., Orrenius, S. & Slater, A. F. Disulfiram is a potent inhibitor of proteases of the caspase family. *Chem Res Toxicol* **10**, 1319-1324 (1997).

30 Irrera, N., Vaccaro, M., Bitto, A., Pallio, G., Pizzino, G., Lentini, M., Arcoraci, V., Minutoli, L., Scuruchi, M., Cutroneo, G., Anastasi, G. P., Ettari, R., Squadrito, F. & Altavilla, D. BAY 11-7082 inhibits the NF-kappaB and NLRP3 inflammasome pathways and protects against IMQ-induced psoriasis. *Clin Sci (Lond)* **131**, 487-498 (2017).

31 Juliana, C., Fernandes-Alnemri, T., Wu, J., Datta, P., Solorzano, L., Yu, J. W., Meng, R., Quong, A. A., Latz, E., Scott, C. P. & Alnemri, E. S. Anti-inflammatory compounds parthenolide and Bay 11-7082 are direct inhibitors of the inflammasome. *J Biol Chem* **285**, 9792-9802 (2010).

32 Krishnan, N., Bencze, G., Cohen, P. & Tonks, N. K. The anti-inflammatory compound BAY-11-7082 is a potent inhibitor of protein tyrosine phosphatases. *The FEBS journal* **280**, 2830-2841 (2013).

33 Kulkarni, R. A., Stanford, S. M., Vellore, N. A., Krishnamurthy, D., Bliss, M. R., Baron, R., Bottini, N. & Barrios, A. M. Thiuram disulfides as pseudo-irreversible inhibitors of lymphoid tyrosine phosphatase. *ChemMedChem* **8**, 1561-1568 (2013). Broz, P. & Dixit, V. M. Inflammasomes: mechanism of assembly, regulation and signalling. Nat Rev Immunol 16, 407-420 (2016).

34. Shi, J. et al. Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death. Nature 526, 660-665 (2015).

35. Hagn, F., Nasr, M. L. & Wagner, G. Assembly of phospholipid nanodiscs of controlled size for structural studies of membrane proteins by NMR. Nat Protoc 13, 79-98 (2018).

36. Sun, L. et al. Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase. Cell 148, 213-227 (2012).

37. Degterev, A. et al. Identification of RIP1 kinase as a specific cellular target of necrostatins. Nat Chem Biol 4, 313-321 (2008).

38. Rathkey, J. K. et al. Chemical disruption of the pyroptotic pore-forming protein gasdermin D inhibits inflammatory cell death and sepsis. Sci Immunol 3 (2018).

39. Sollberger, G. et al. Gasdermin D plays a vital role in the generation of neutrophil extracellular traps. Sci Immunol 3 (2018).

40. Nair, A. B. & Jacob, S. A simple practice guide for dose conversion between animals and human. J Basic Clin Pharm 7, 27-31 (2016).

41. Kayagaki, N. et al. Non-canonical inflammasome activation targets caspase-11. Nature 479, 117-121 (2011).

42. White, M. J. et al. Apoptotic caspases suppress mtDNA-induced STING-mediated type I IFN production. Cell 159, 1549-1562 (2014).

43. Rongvaux, A. et al. Apoptotic caspases prevent the induction of type I interferons by mitochondrial DNA. Cell 159, 1563-1577 (2014).

44. Rauert-Wunderlich, H. et al. The IKK inhibitor Bay 11-7082 induces cell death independent from inhibition of activation of NFkappaB transcription factors. PLoS One 8, e59292 (2013).

45. Nasr, M. L. et al. Covalently circularized nanodiscs for studying membrane proteins and viral entry. Nat Methods 14, 49-52 (2017).

OTHER EMBODIMENTS

It is to be understood that while the present application has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the present application, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

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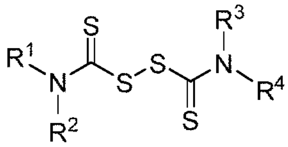
WHAT IS CLAIMED IS:

1. A method of treating sepsis, the method comprising administering to a subject a compound of Formula (I):



(I),

or a pharmaceutically acceptable salt thereof, wherein:

R^1, R^2, R^3, and R^4 are each independently selected from H, C_{1-6} alkyl, C_{1-6} haloalkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, and Cy^1; wherein said C_{1-6} alkyl is optionally substituted with 1, 2, or 3 substituents independently selected from Cy^1;

each Cy^1 is independently selected from C_6-C_{10} aryl, C_{3-10} cycloalkyl, 5-10 membered heteroaryl, and 4-12 membered heterocycloalkyl, each of which is optionally substituted with 1, 2, 3, 4, or 5 substituents independently selected from R^{Cy1};

or R^1 and R^2 together with an N atom to which they are attached form a 4-12 membered heterocycloalkyl, which is optionally substituted with 1, 2, 3, 4, or 5 substituents independently selected from R^{Cy2};

or R^3 and R^4 together with an N atom to which they are attached form a 4-12 membered heterocycloalkyl, which is optionally substituted with 1, 2, 3, 4, or 5 substituents independently selected from R^{Cy2}; and

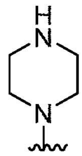
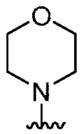
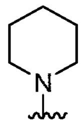
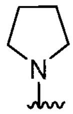
each R^{Cy1} and R^{Cy2} is independently selected from C_{1-6} alkyl, C_{1-6} haloalkyl, halo, CN, and NO_2.

2. The method of claim 1, wherein R^1, R^2, R^3, and R^4 are each independently selected from Cy^1 and C_{1-6} alkyl optionally substituted with Cy^1.

3. The method of claim 1 or 2, wherein R^1 and R^2 together with an N atom to which they are attached form a 4-12 membered heterocycloalkyl, which is optionally substituted with 1, 2, or 3 substituents independently selected from R^{Cy2}.

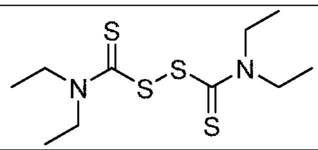
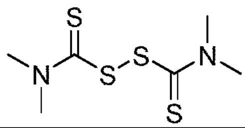
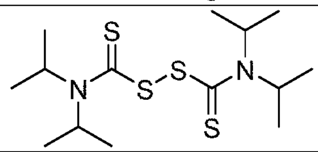
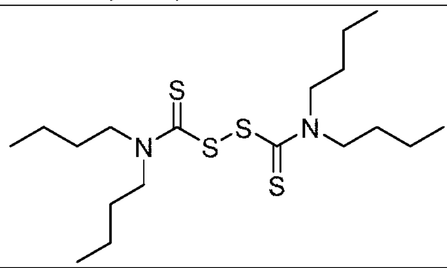
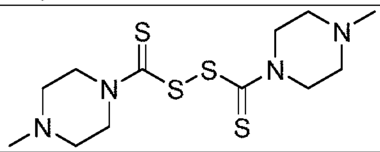
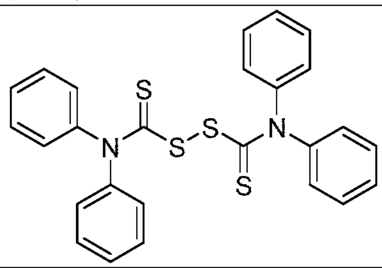
4. The method of any one of claims 1-3, wherein R^3 and R^4 together with an N atom to which they are attached form a 4-12 membered heterocycloalkyl, which is optionally substituted with 1, 2, or 3 substituents independently selected from R^{Cy2}.

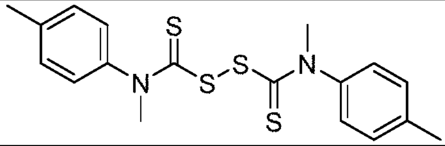
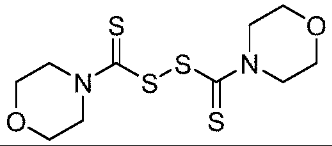
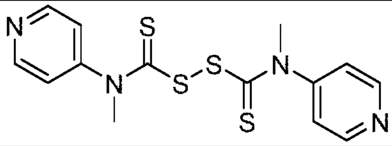
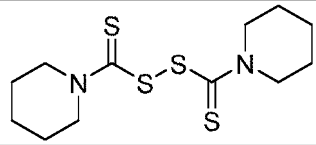
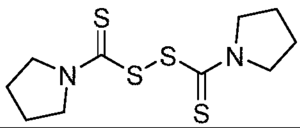
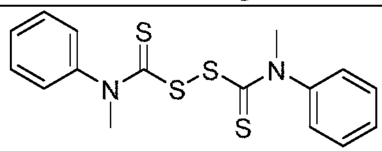
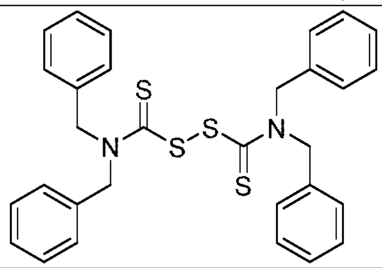
5. The method of claim 3 or claim 4, wherein the 4-12 membered heterocycloalkyl is selected from any one of the following groups:

 ;  ;  ; and  .

6. The method of any one of claims 1-5, wherein each Cy^1 is independently selected from C_{6-10} aryl and 5-10 membered heteroaryl, each of which is optionally substituted with 1, 2, or 3 substituents independently selected from R^{Cy1}.

7. The method of claim 1, wherein the compound of Formula (I) is selected from any one of the compounds listed in Table A:

C-23	
C-23A1	
C-23A2	
C-23A3	
C-23A4	
C-23A5	

C-23A6	
C-23A7	
C-23A8	
C-23A9	
C-23A10	
C-23A11	
C-23A12	

or a pharmaceutically acceptable salt thereof.

8. The method of any one of claims 1-7, wherein the compound, or a pharmaceutically acceptable salt thereof, is administered to the subject in combination with at least one additional therapeutic agent, or pharmaceutically acceptable salt thereof.

9. The method of claim 8, wherein the additional therapeutic agent is an anti-inflammatory agent.

10. The method of claim 9, wherein the anti-inflammatory agent is selected from: anti-IL1 antibody, an anti-TNF antibody, an NSAID, and a steroid anti-inflammatory agent.

11. The method of claim 8, wherein the additional therapeutic agent is an antibiotic.

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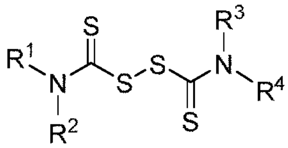
12. A pharmaceutical composition comprising:

a compound of Formula (I), or a pharmaceutically acceptable salt thereof, as recited in any one of claims 1-7; and

a pharmaceutically acceptable carrier.

13. Use of a compound of Formula (I):



(I),

or a pharmaceutically acceptable salt thereof, in the manufacture of a medicament for treating sepsis, wherein:

R^1, R^2, R^3, and R^4 are each independently selected from H, C_{1-6} alkyl, C_{1-6} haloalkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, and Cy^1; wherein said C_{1-6} alkyl is optionally substituted with 1, 2, or 3 substituents independently selected from Cy^1;

each Cy^1 is independently selected from C_6-C_{10} aryl, C_{3-10} cycloalkyl, 5-10 membered heteroaryl, and 4-12 membered heterocycloalkyl, each of which is optionally substituted with 1, 2, 3, 4, or 5 substituents independently selected from R^{Cy1};

or R^1 and R^2 together with an N atom to which they are attached form a 4-12 membered heterocycloalkyl, which is optionally substituted with 1, 2, 3, 4, or 5 substituents independently selected from R^{Cy2};

or R^3 and R^4 together with an N atom to which they are attached form a 4-12 membered heterocycloalkyl, which is optionally substituted with 1, 2, 3, 4, or 5 substituents independently selected from R^{Cy2}; and

each R^{Cy1} and R^{Cy2} is independently selected from C_{1-6} alkyl, C_{1-6} haloalkyl, halo, CN, and NO_2.

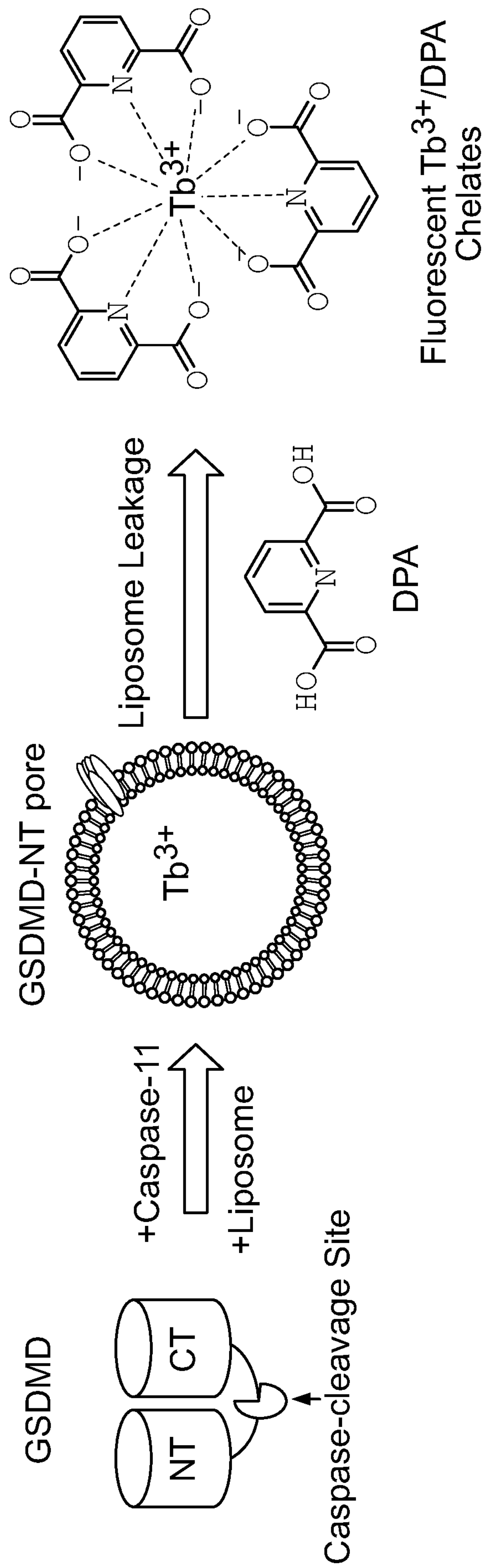

GSDMD
NT
CT
Caspase-cleavage Site
+Caspase-11
+Liposome
GSDMD-NT pore
Tb3+
Liposome Leakage
DPA
Fluorescent Tb3+/DPA Chelates

FIG. 1

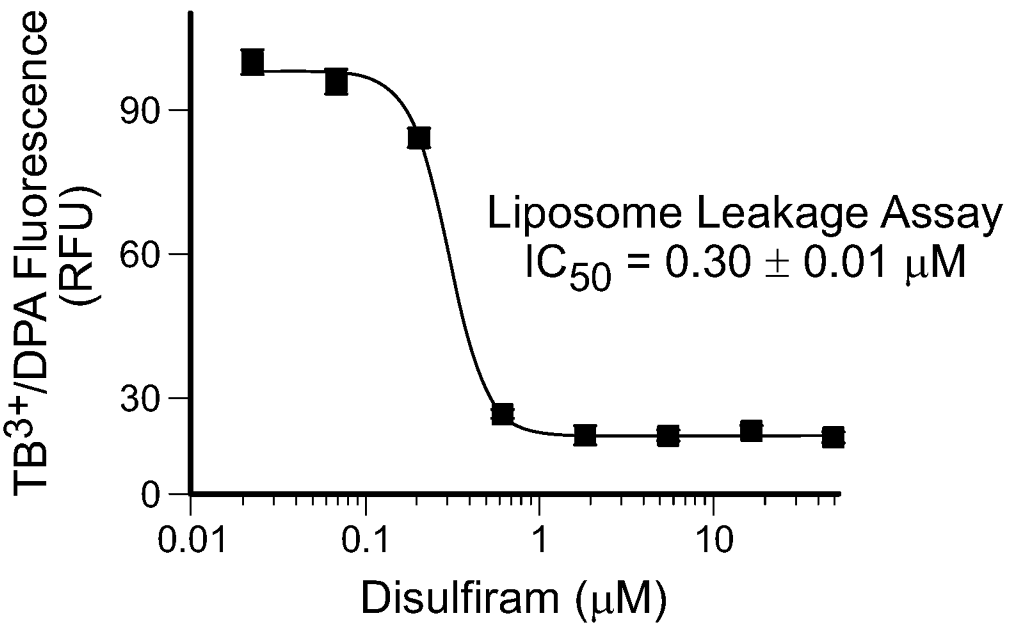

TB3+/DPA Fluorescence (RFU)
90
60
30
0
0.01
0.1
1
10
Disulfiram (μM)
Liposome Leakage Assay
IC50 = 0.30 ± 0.01 μM

FIG. 2

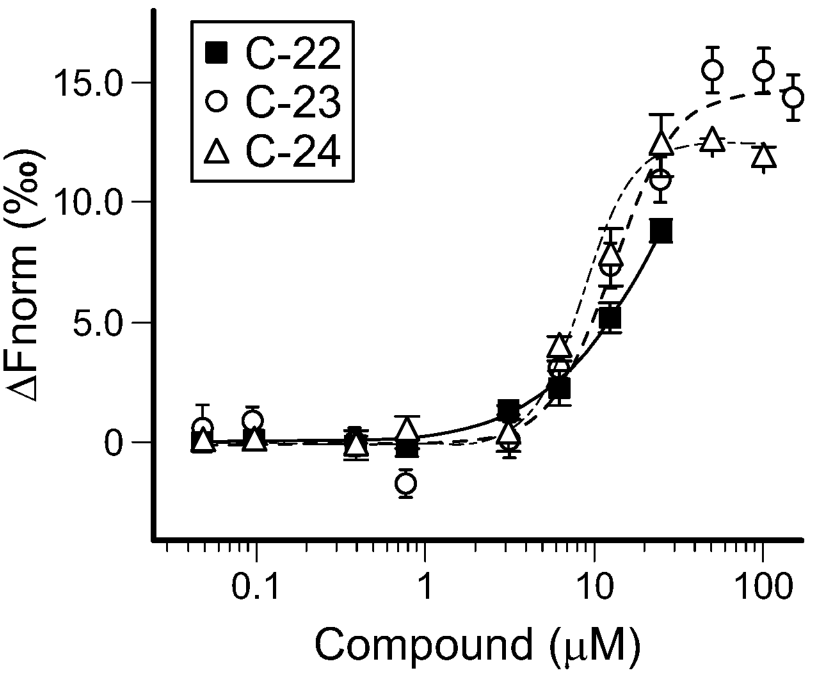
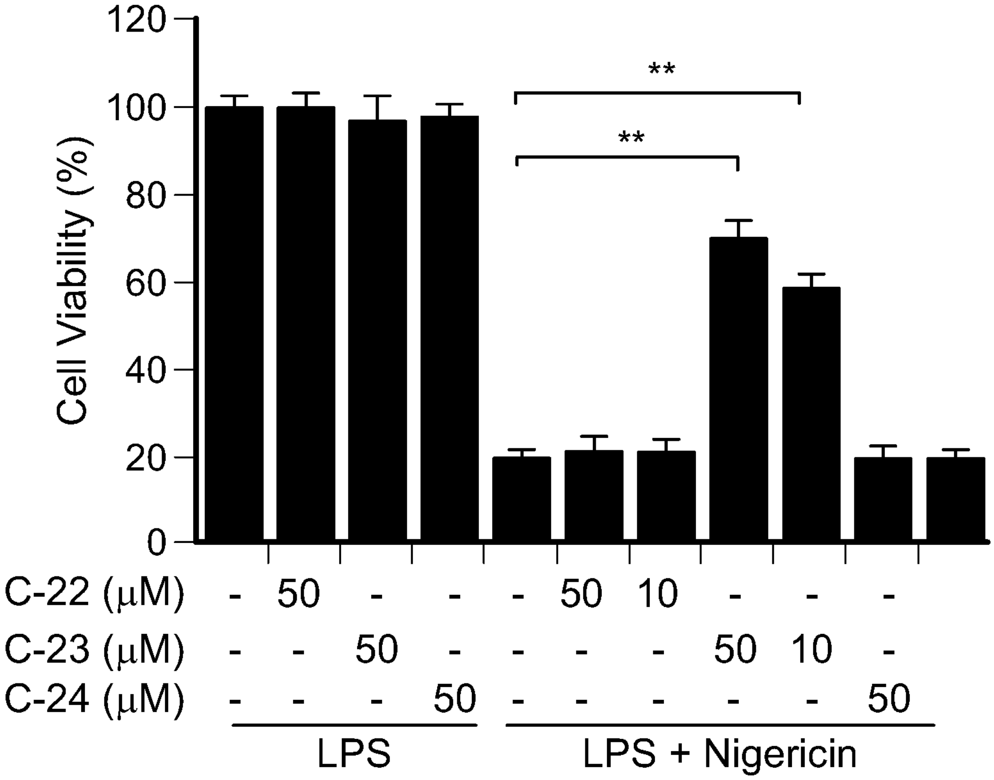
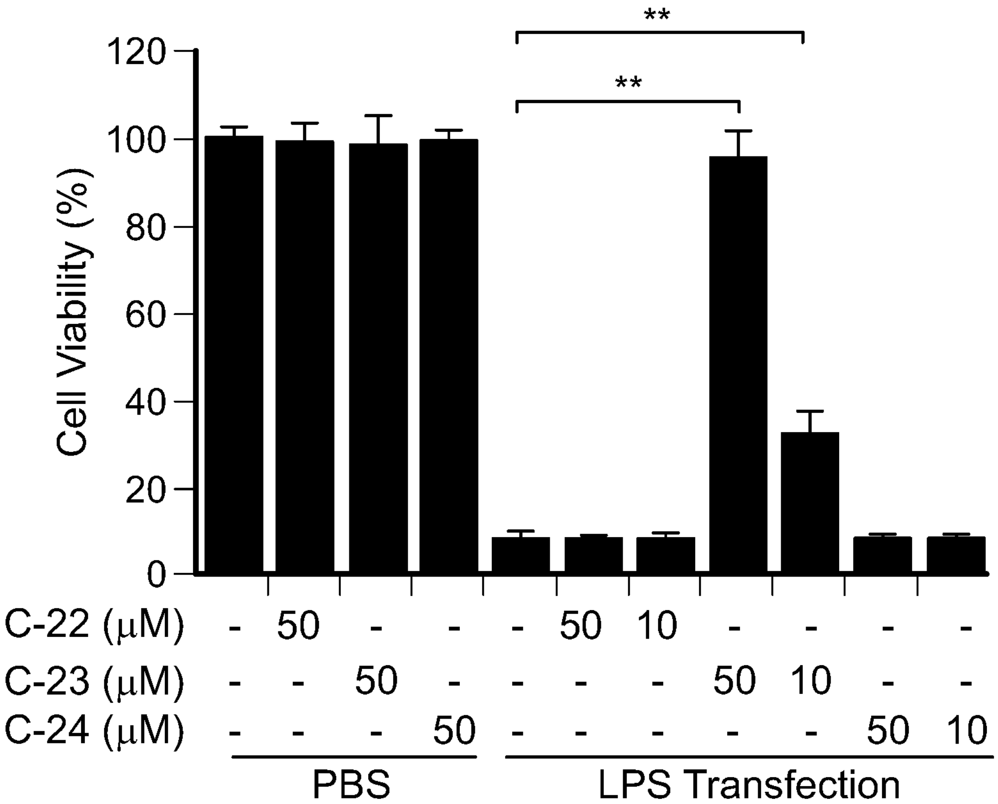

C-22
C-23
C-24
15.0
10.0
5.0
0
ΔFnorm (‰)
0.1
1
10
100
Compound (μM)

FIG. 3



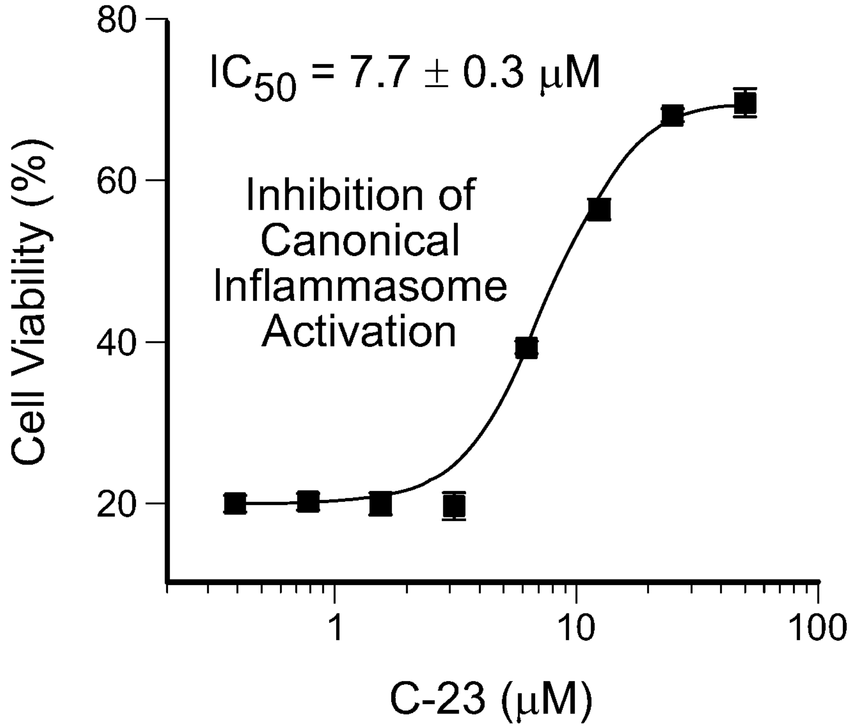
120
100
80
60
40
20
0
Cell Viability (%)
**
**
C-22 (μM) - 50 - - - 50 10 - - -
C-23 (μM) - - 50 - - - - 50 10 -
C-24 (μM) - - - 50 - - - - - 50
LPS
LPS + Nigericin

FIG. 4



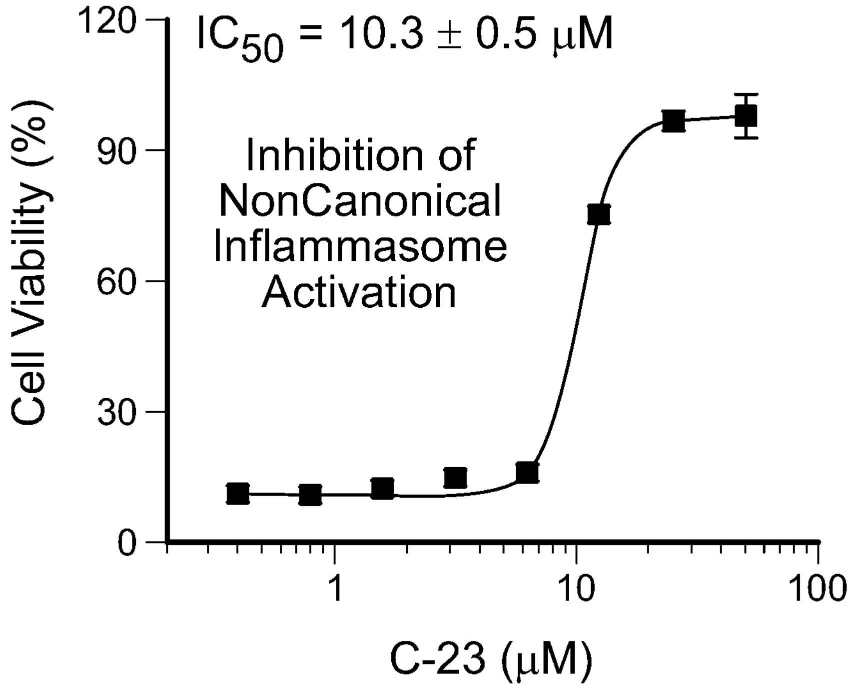
120
100
80
60
40
20
0
Cell Viability (%)
**
**
C-22 (μM) - 50 - - - 50 10 - - - -
C-23 (μM) - - 50 - - - - 50 10 - -
C-24 (μM) - - - 50 - - - - - 50 10
PBS
LPS Transfection

FIG. 5



80
60
40
20
Cell Viability (%)
IC50 = 7.7 ± 0.3 µM
Inhibition of Canonical Inflammasome Activation
1
10
100
C-23 (µM)

FIG. 6



120
90
60
30
0
Cell Viability (%)
IC50 = 10.3 ± 0.5 µM
Inhibition of NonCanonical Inflammasome Activation
1
10
100
C-23 (µM)

FIG. 7

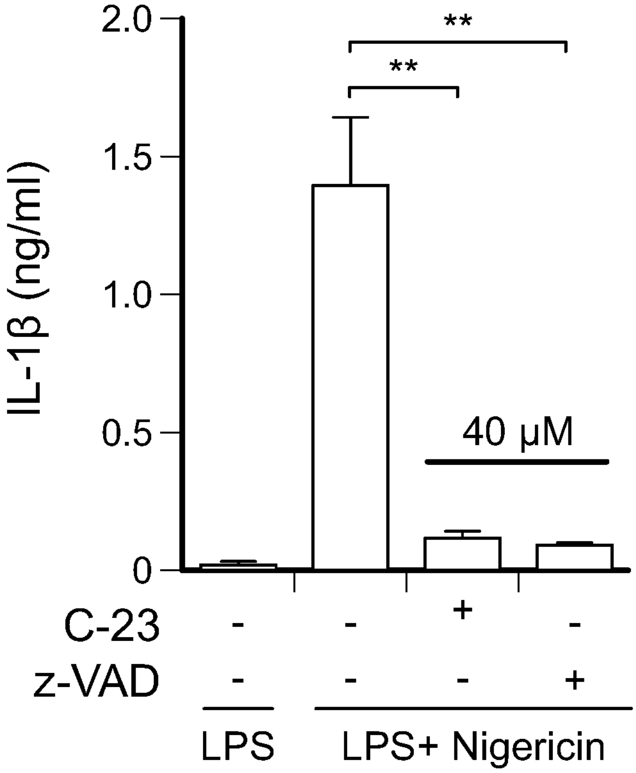

2.0
1.5
1.0
0.5
0
IL-1β (ng/ml)
**
**
40 μM
C-23
z-VAD
LPS
LPS+ Nigericin

FIG. 8

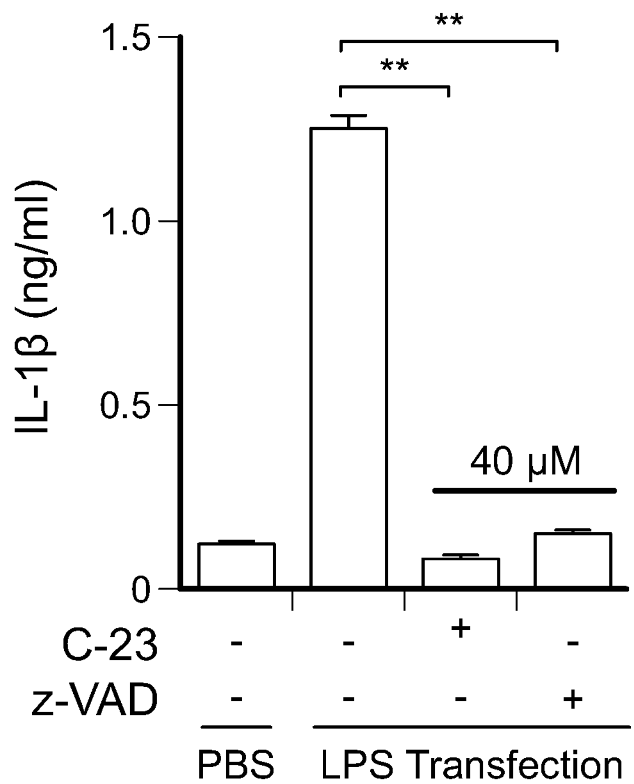
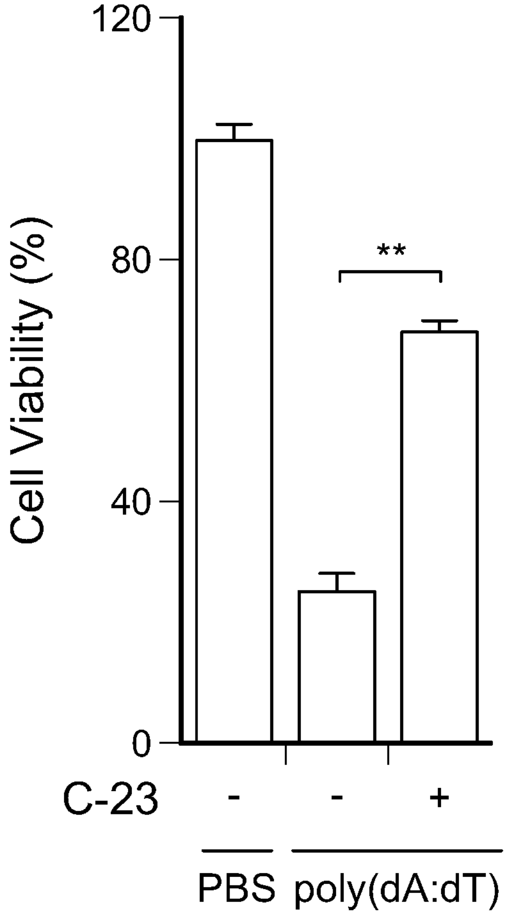
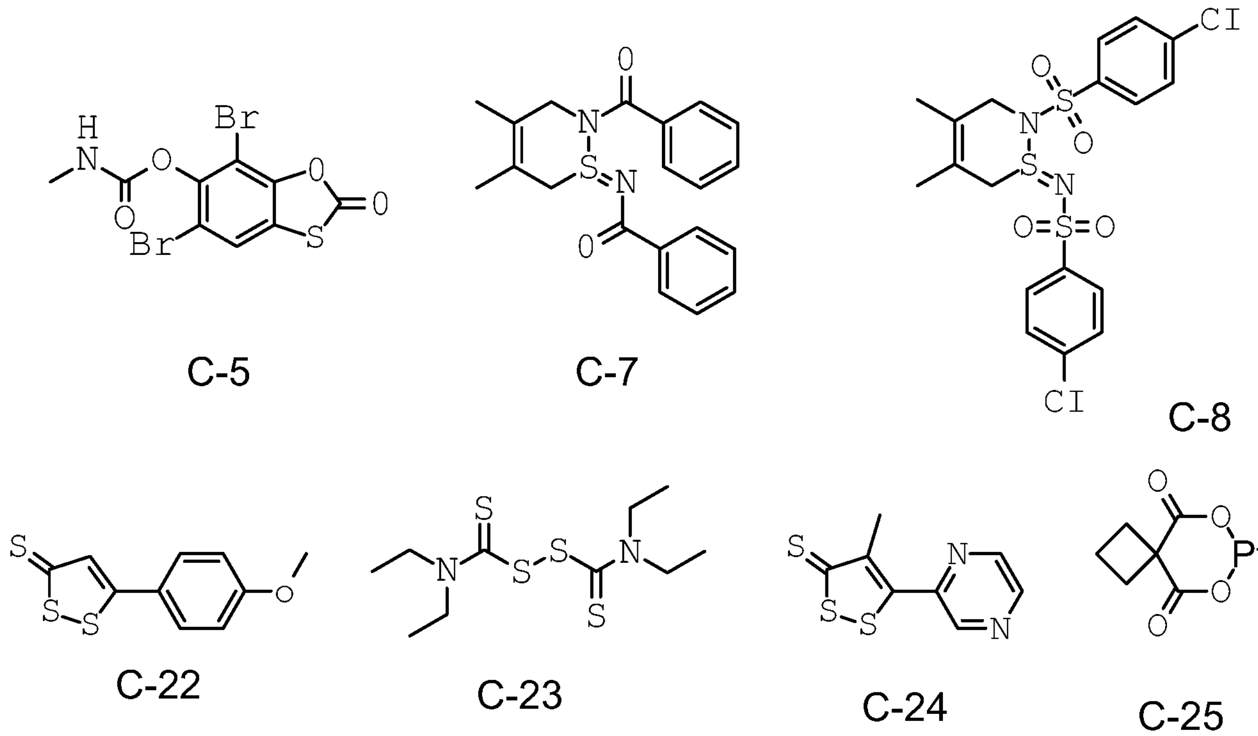

1.5
1.0
0.5
0
IL-1β (ng/ml)
**
**
40 μM
C-23
z-VAD
PBS
LPS Transfection

FIG. 9



120
80
40
0
Cell Viability (%)
**
C-23
-
-
+
PBS
poly(dA:dT)

FIG. 10



C-5
C-7
C-8
C-22
C-23
C-24
C-25

FIG. 11

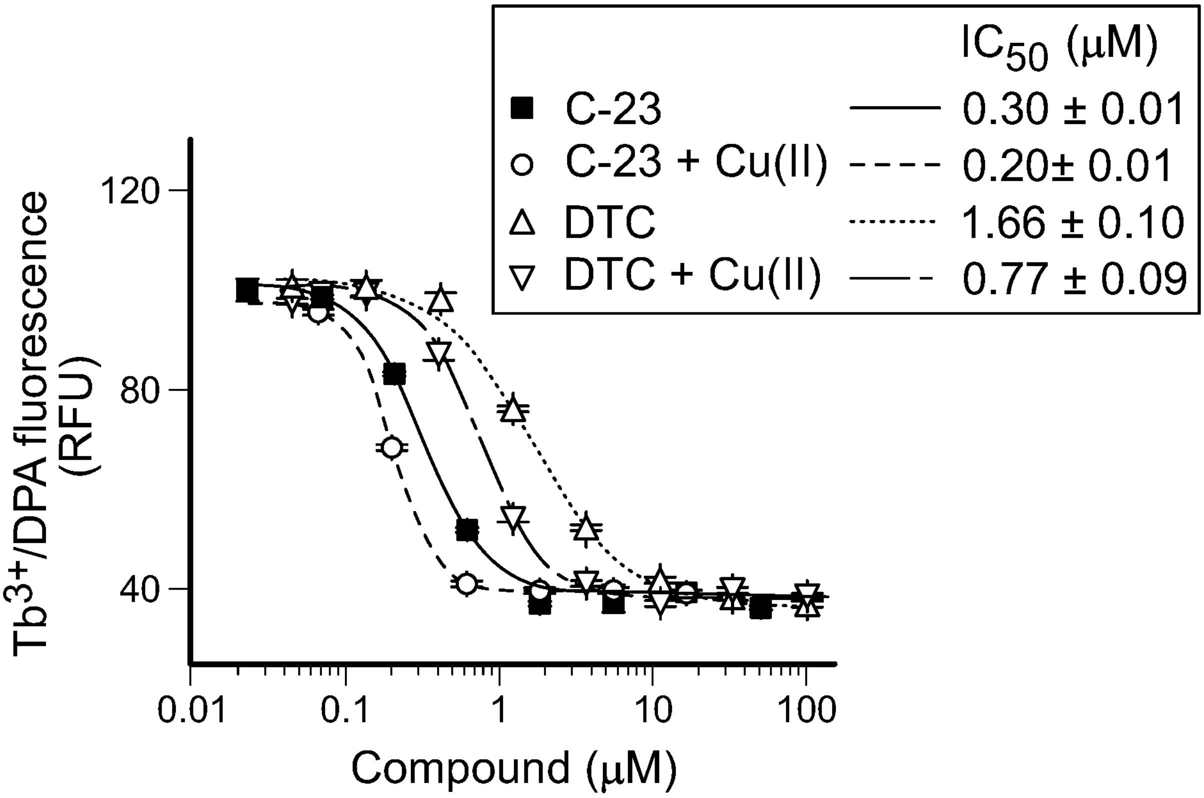

IC50 (μM)
C-23 0.30 ± 0.01
C-23 + Cu(II) 0.20± 0.01
DTC 1.66 ± 0.10
DTC + Cu(II) 0.77 ± 0.09
Tb3+/DPA fluorescence (RFU)
120
80
40
0.01
0.1
1
10
100
Compound (μM)

FIG. 12

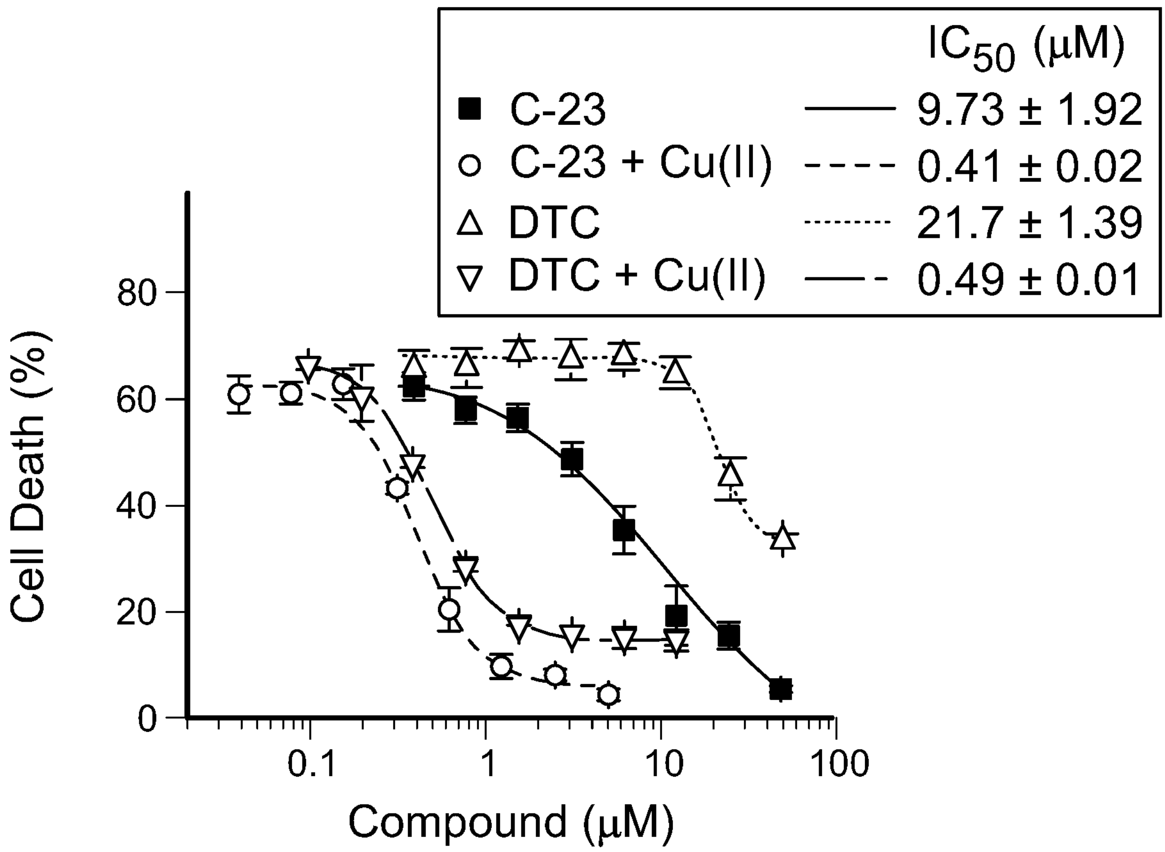
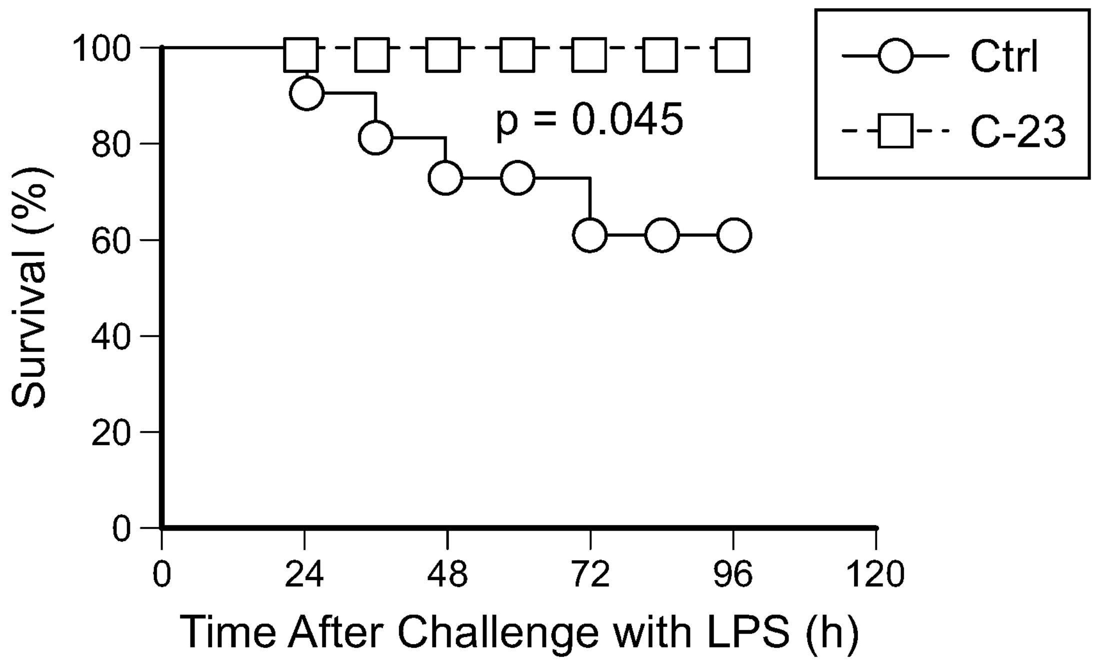
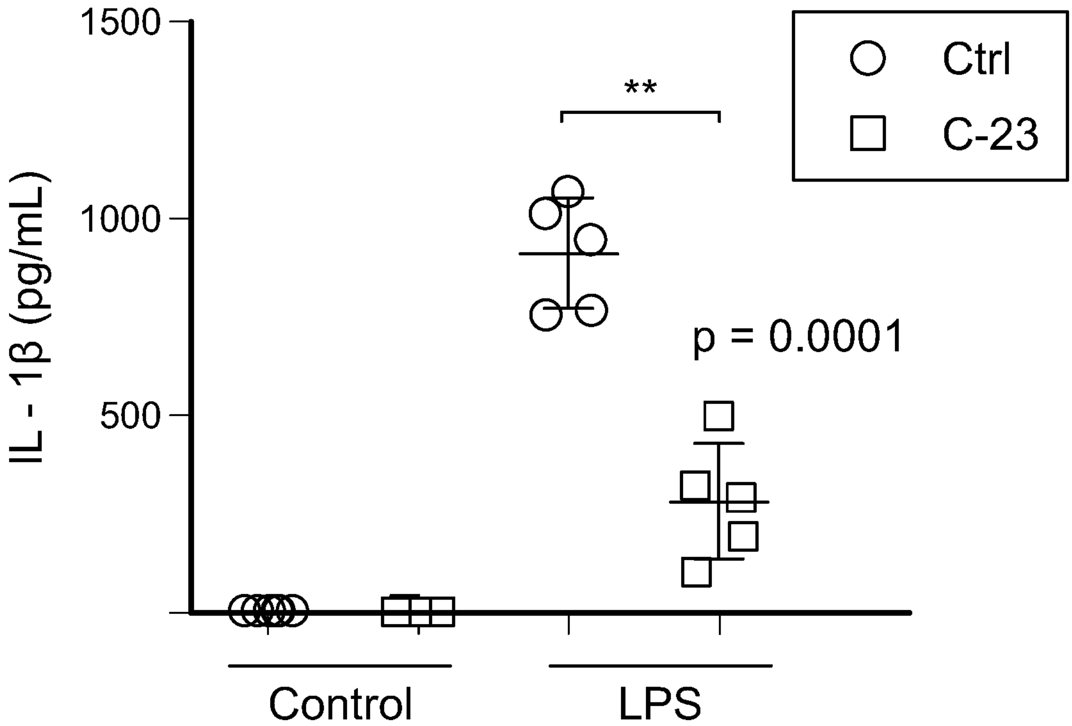

IC50 (μM)
C-23 9.73 ± 1.92
C-23 + Cu(II) 0.41 ± 0.02
DTC 21.7 ± 1.39
DTC + Cu(II) 0.49 ± 0.01
Cell Death (%)
80
60
40
20
0
0.1
1
10
100
Compound (μM)

FIG. 13



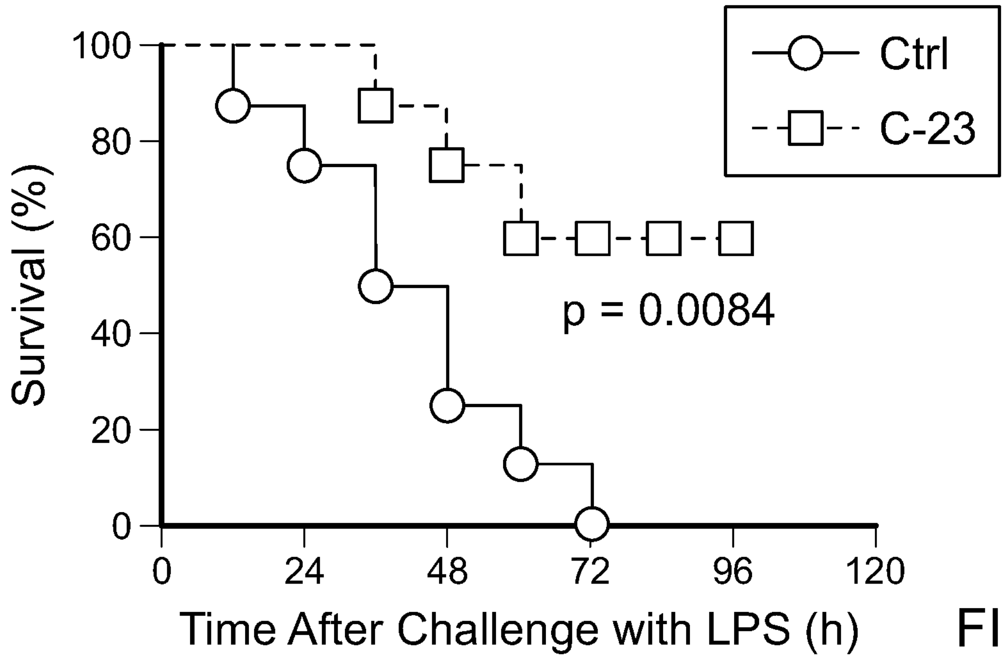
100
80
60
40
20
0
Survival (%)
0
24
48
72
96
120
Time After Challenge with LPS (h)
p = 0.045
Ctrl
C-23

FIG. 14



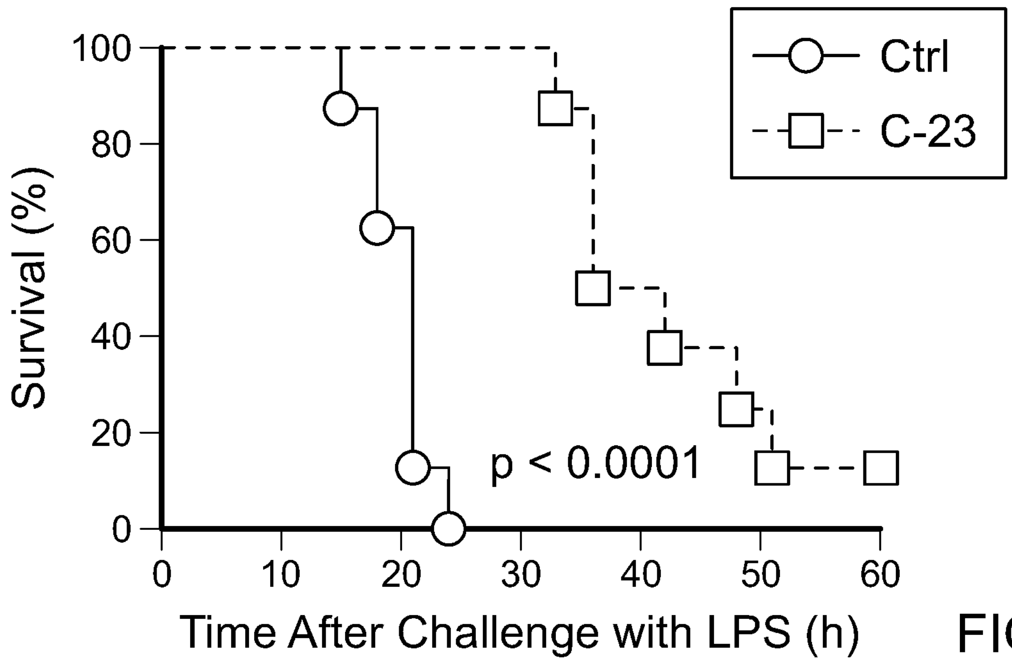
1500
1000
500
IL - 1β (pg/mL)
**
p = 0.0001
Control
LPS
Ctrl
C-23

FIG. 15



Ctrl
C-23
100
80
60
40
20
0
Survival (%)
p = 0.0084
0
24
48
72
96
120
Time After Challenge with LPS (h)

FIG. 16



Ctrl
C-23
100
80
60
40
20
0
Survival (%)
p < 0.0001
0
10
20
30
40
50
60
Time After Challenge with LPS (h)

FIG. 17

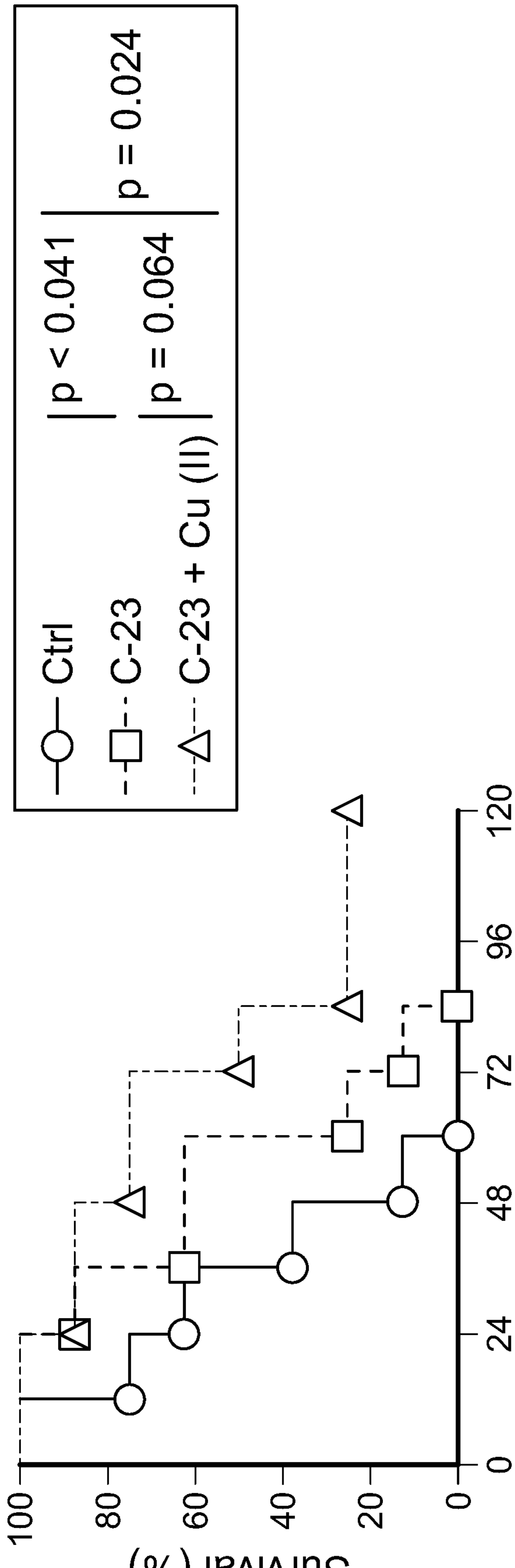

Survival (%)
100
80
60
40
20
0
0
24
48
72
96
120
Ctrl
C-23
C-23 + Cu (II)
p < 0.041
p = 0.064
p = 0.024

FIG. 18

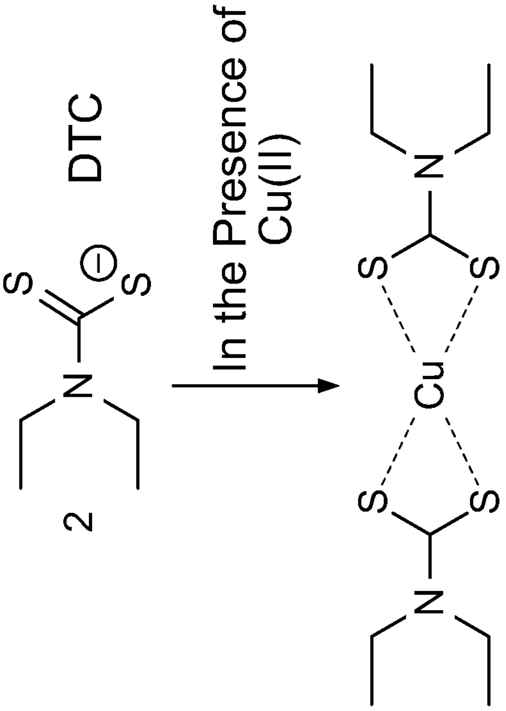

2
DTC
In the Presence of
Cu(II)
Cu

FIG. 19

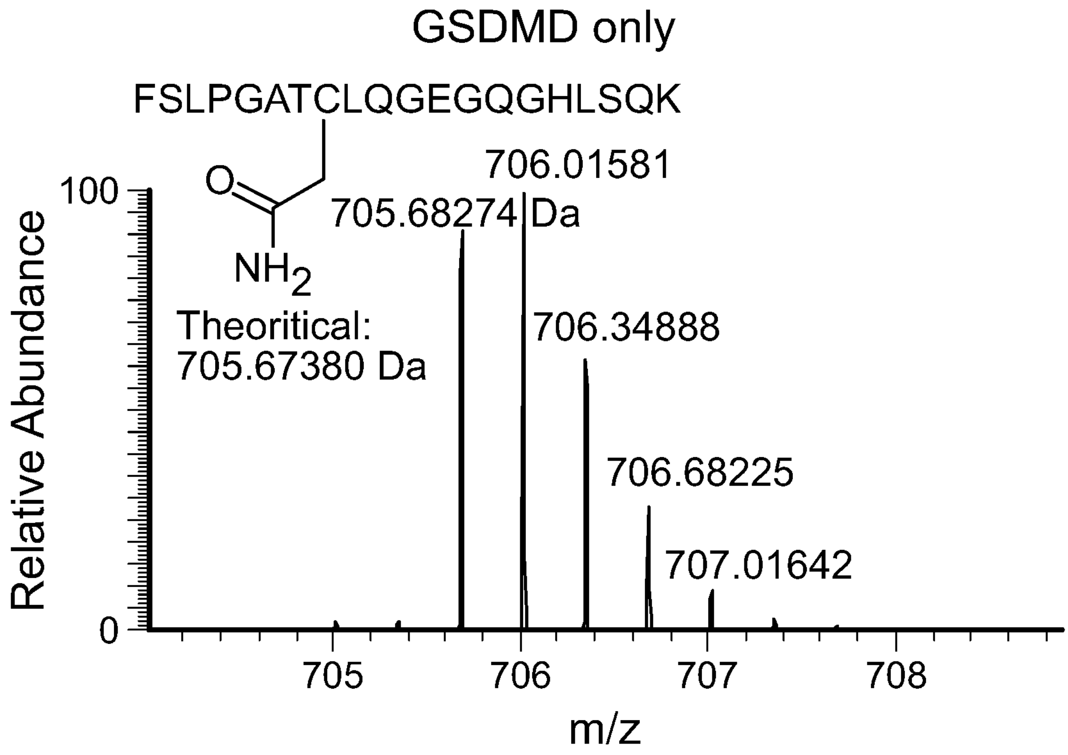

GSDMD only
FSLPGATCLQGEGQGHLSQK
O
NH_2
Theoritical:
705.67380 Da
706.01581
705.68274 Da
706.34888
706.68225
707.01642
Relative Abundance
100
0
705
706
707
708
m/z

FIG. 20

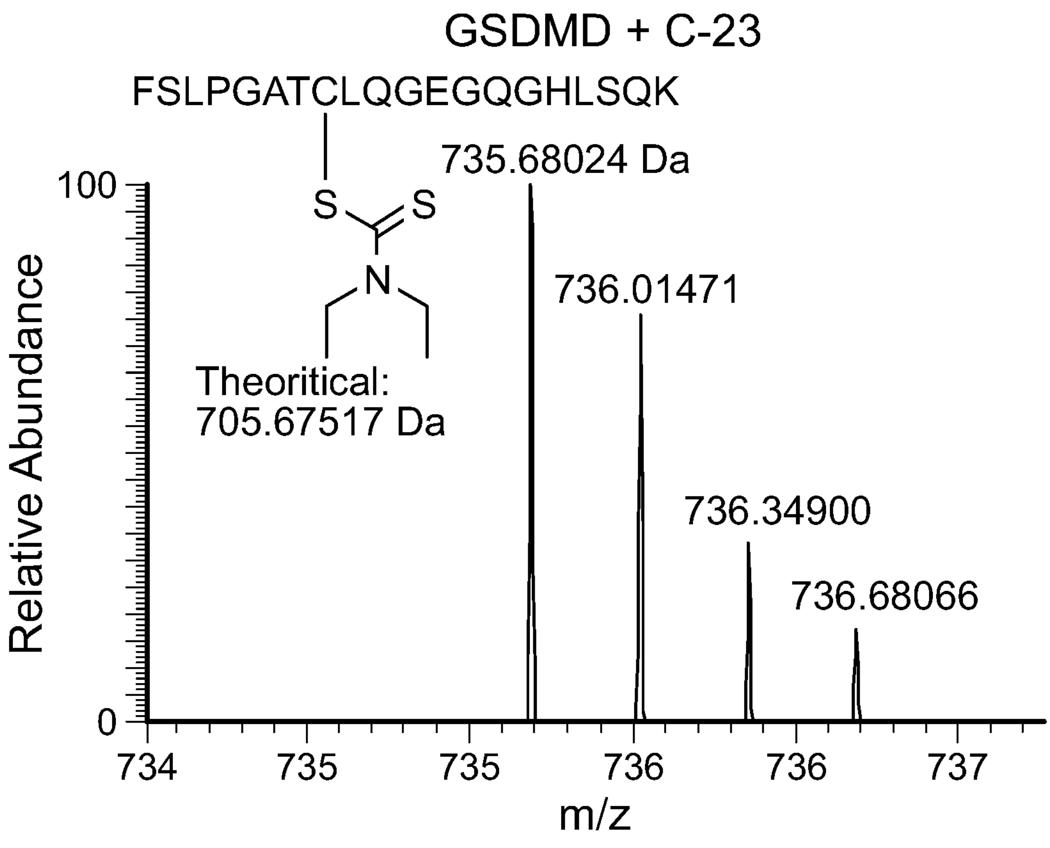

GSDMD + C-23
FSLPGATCLQGEGQGHLSQK
S
S
N
Theoritical:
705.67517 Da
735.68024 Da
736.01471
736.34900
736.68066
Relative Abundance
100
0
734
735
735
736
736
737
m/z

FIG. 21

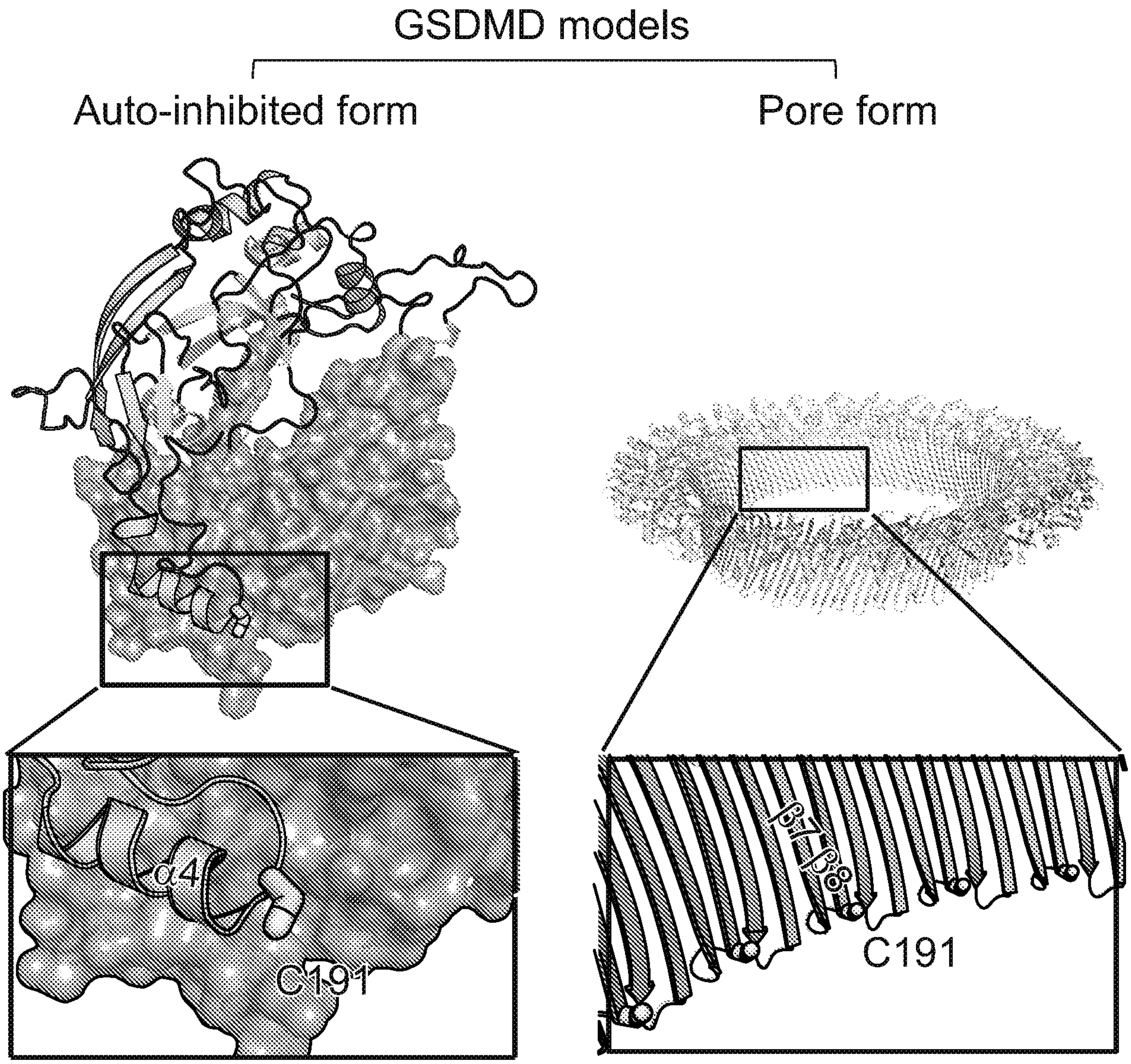

GSDMD models
Auto-inhibited form
Pore form
α4
C191
β7-β8
C191

FIG. 22

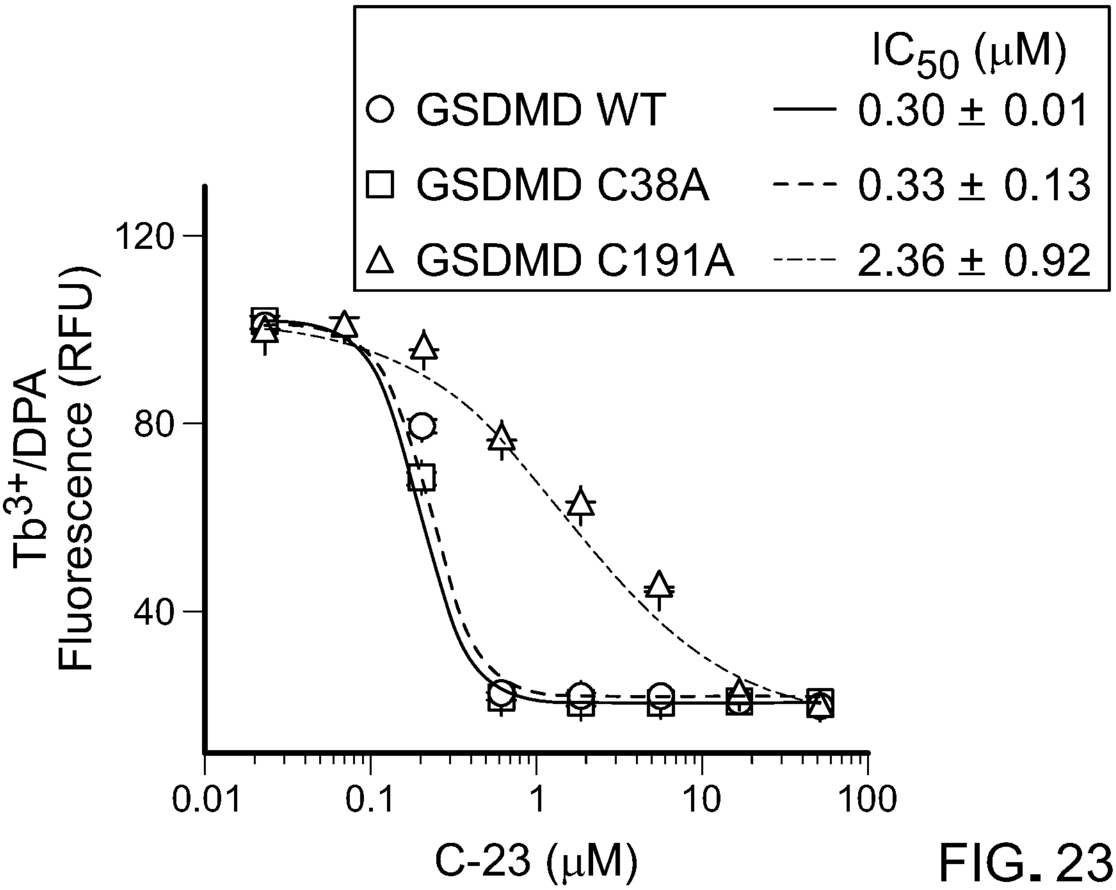

IC50 (μM)
GSDMD WT 0.30 ± 0.01
GSDMD C38A 0.33 ± 0.13
GSDMD C191A 2.36 ± 0.92
Tb3+/DPA Fluorescence (RFU)
120
80
40
0.01
0.1
1
10
100
C-23 (μM)

FIG. 23

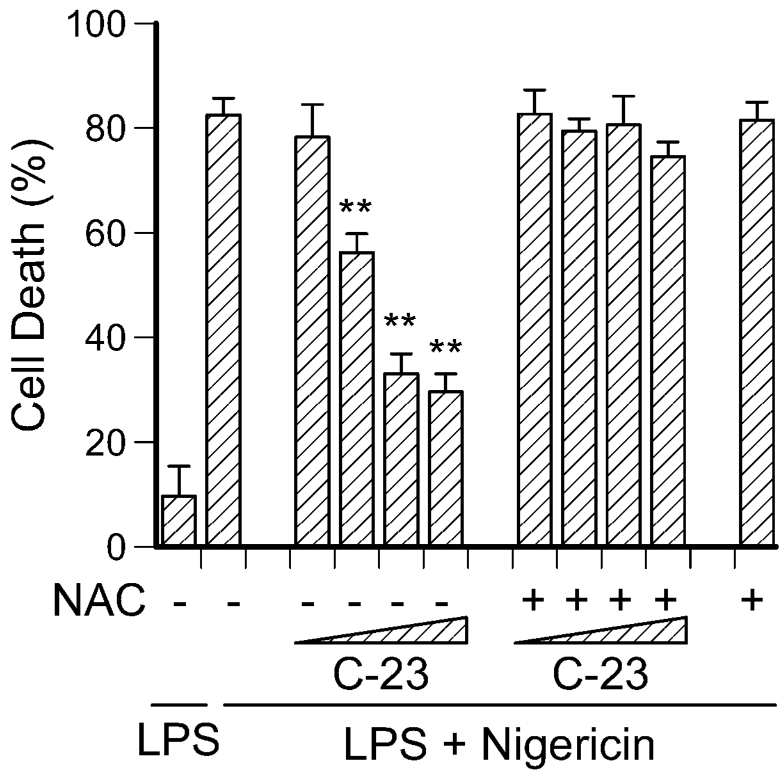

Cell Death (%)
100
80
60
40
20
0
**
**
**
NAC - - - - - - + + + + +
C-23
C-23
LPS
LPS + Nigericin

FIG. 24

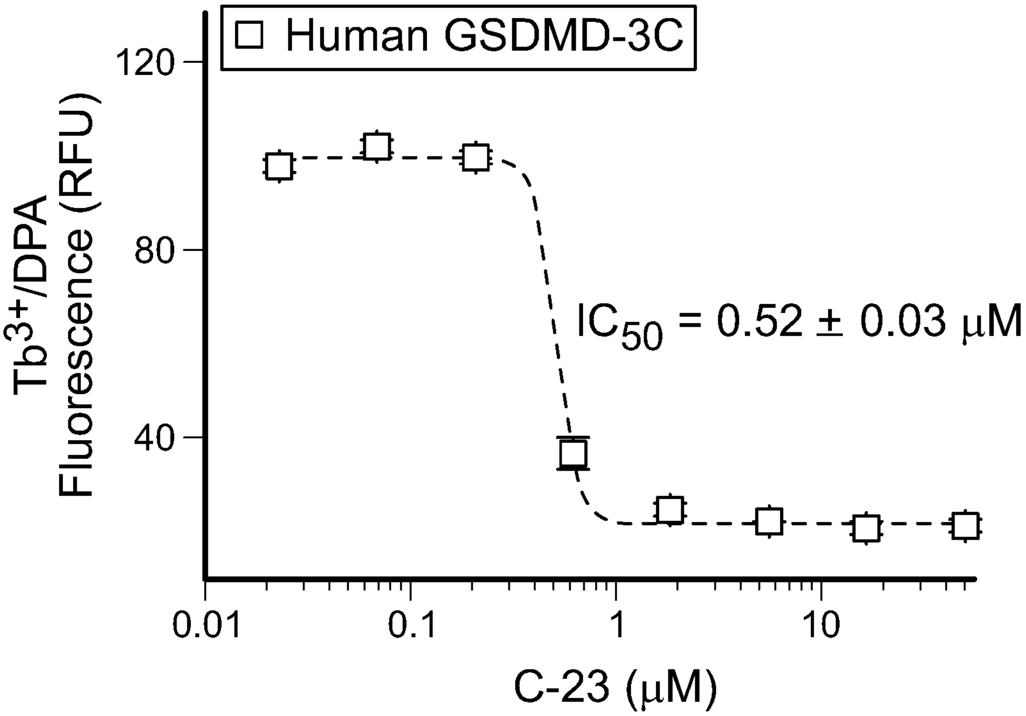

Human GSDMD-3C
120
80
40
Tb3+/DPA Fluorescence (RFU)
IC50 = 0.52 ± 0.03 μM
0.01
0.1
1
10
C-23 (μM)

FIG. 25

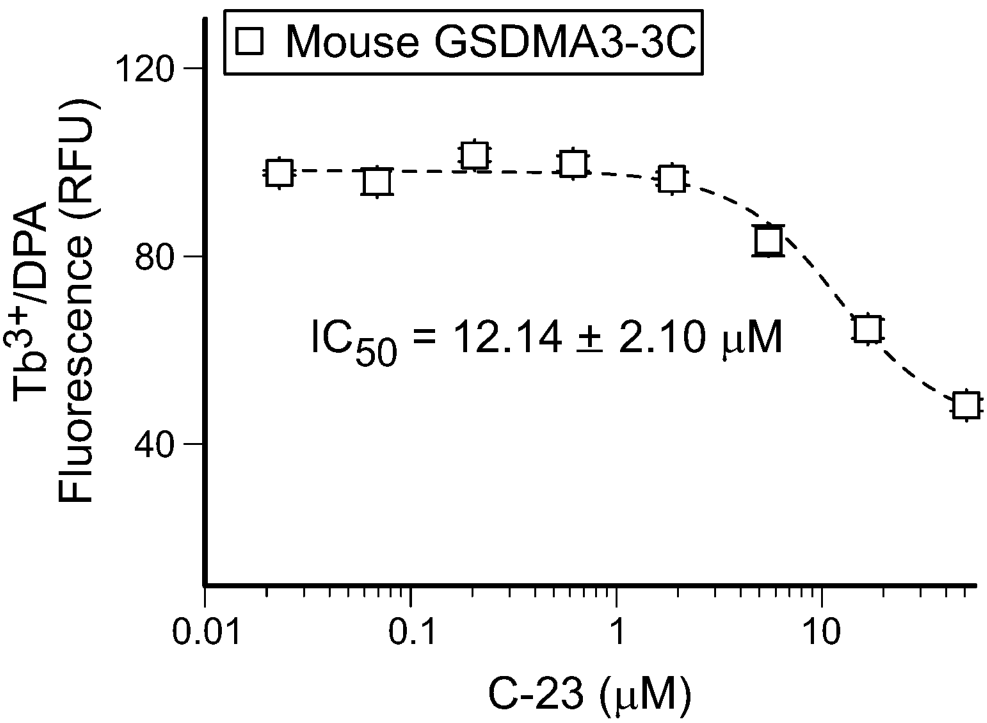

Mouse GSDMA3-3C
120
80
40
Tb3+/DPA Fluorescence (RFU)
IC50 = 12.14 ± 2.10 μM
0.01
0.1
1
10
C-23 (μM)

FIG. 26

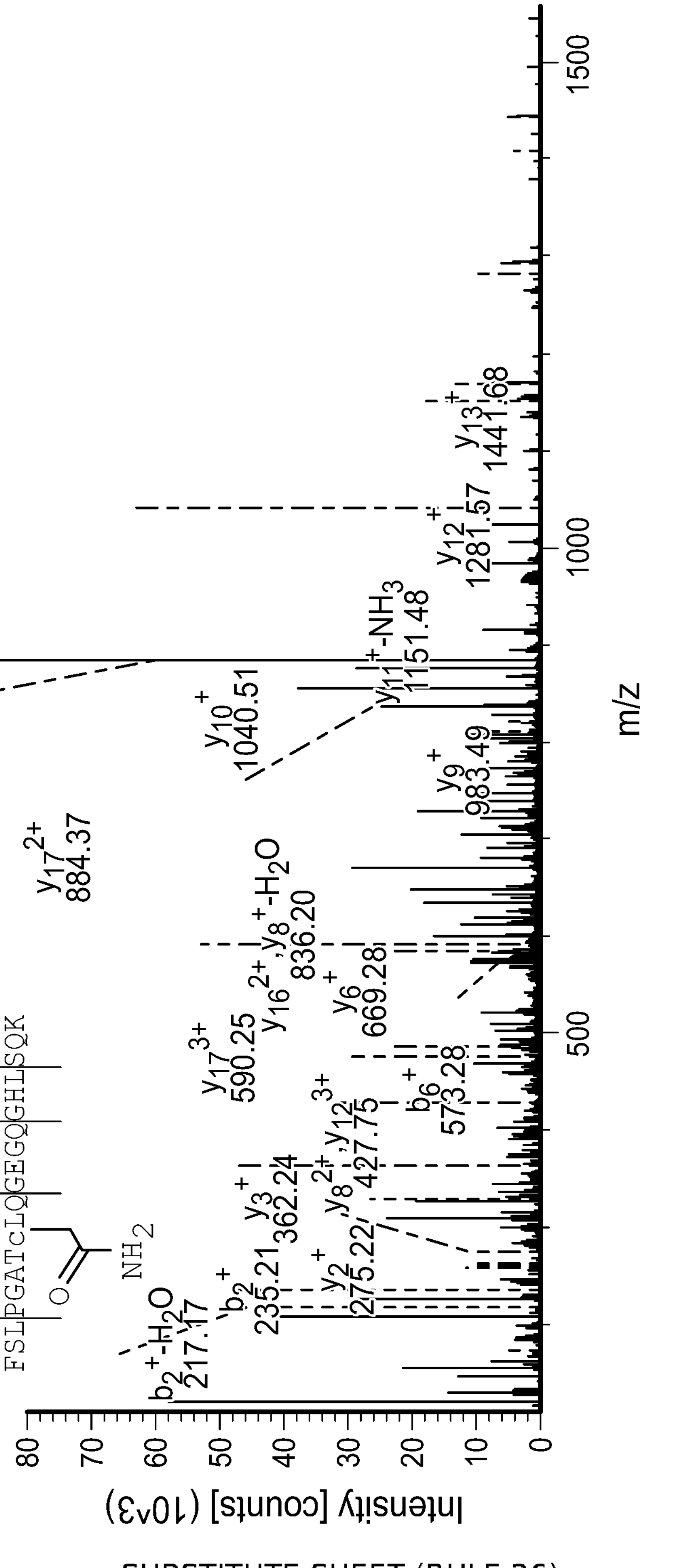

FSLPGATcLQGEGQGHLSQK
y17+
y10+
y6+
y3+
b2+-H2O 217.17
b2+ 235.21
y2+ 275.22
y3+ 362.24
y8 2+, y12 3+ 427.75
b6+ 573.28
y17 3+ 590.25
y6+ 669.28
y16 2+, y8+-H2O 836.20
y17 2+ 884.37
y9+ 983.49
y10+ 1040.51
y11+-NH3 1151.48
y12+ 1281.57
y13+ 1441.68
Intensity [counts] (10^3)
m/z

FIG. 27

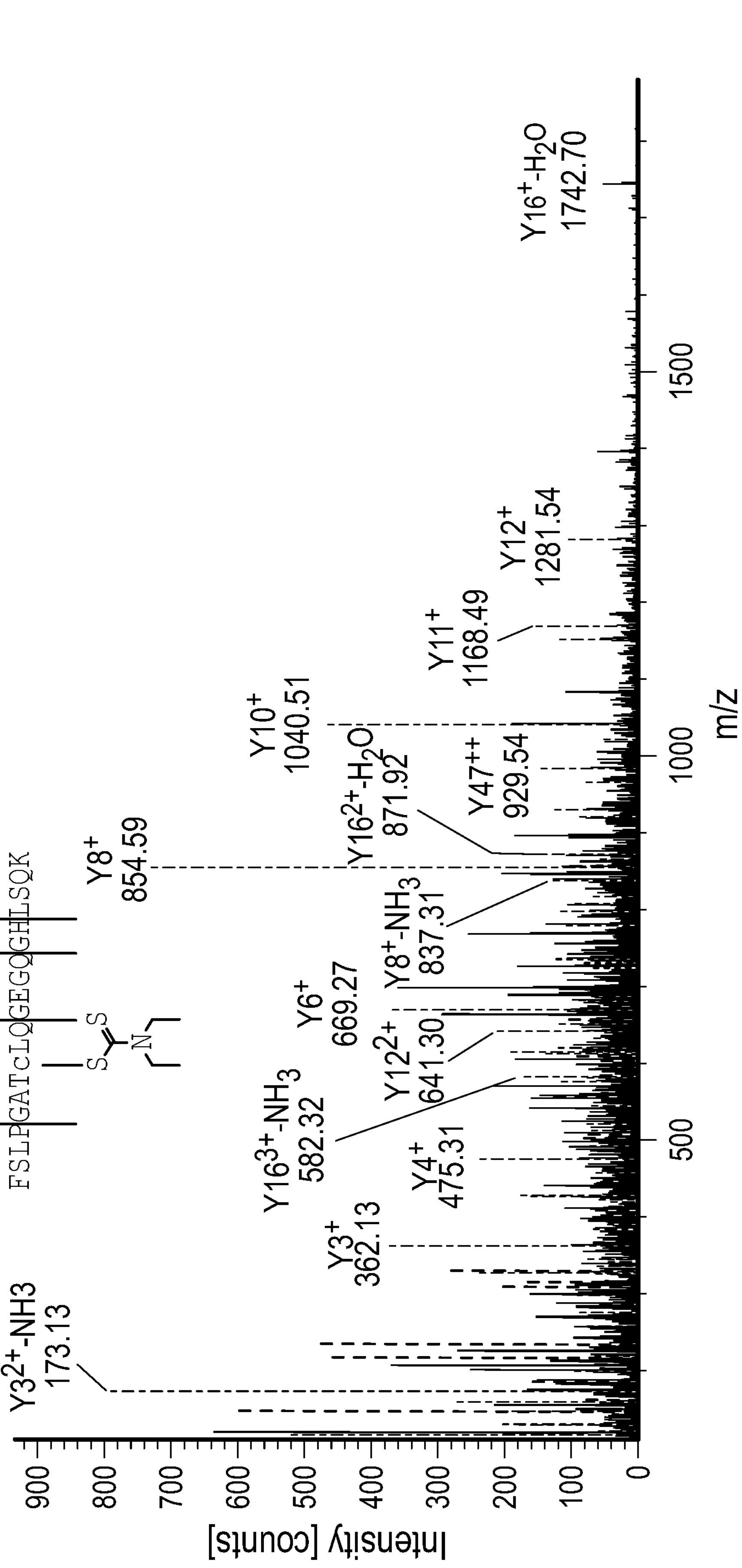

FSLPGATcLQGEGQGHLSQK
Y16+
Y10+
Y6+
Y4+
Y3 2+-NH3 173.13
Y3+ 362.13
Y4+ 475.31
Y16 3+-NH3 582.32
Y12 2+ 641.30
Y6+ 669.27
Y8+-NH3 837.31
Y8+ 854.59
Y16 2+-H2O 871.92
Y47++ 929.54
Y10+ 1040.51
Y11+ 1168.49
Y12+ 1281.54
Y16+-H2O 1742.70
Intensity [counts]
m/z

FIG. 28

```
                                                  .50                                                 .100
GSDMA3    1 .MPVFEDVTRALVRELN.PRGDLTPLDSLIDFKHFRPFCLVLRKRKSTLFWGARYVRTDYTLLDLLEPGSSPSDLTDSGNFSFKNMLDVQVQGLVEVPKT..VKVKGTAGLSQS..STLEVQ
hGSDMA    1 .MTMFENVTRALARQLN.PRGDLTPLDSLIDFKRFHPFCLVLRKRKSTLFWGARYVRTDYTLLDVLEPGSSPSDPTDTGNFGFKNMLDTRVEGDVDVPKT..VKVKGTAGLSQN..STLEVQ
mGSDMD    1 MPSAFEKVVKNVIKEVSGSRGDLIPVDSLRNSTSFRPYCLLNRKFSSSRFWKPRYSCVNLSIKDILFPSAPEPEPECFGSFKVSDVVDGNIQGRVMLSGMGEGKISGGAAVSDSSSASMNVC
hGSDMD    1 MGSAFERVVRRVVQELD.HGGEFIPVTSLQSSTGFQPYCLVVRKPSSSWFWKPRYKCVNLSIKDILEPDAAEPDVQRGRSFHFYDAMDGQIQGSVELAAPGQAKIAGGAAVSDSSSTSMNVY
                                      Cys38.          .50                                                .100

                                  .150                                                  .200
GSDMA3  117 TLSVAPSALENLKKERKLS.ADHSFLNEMRYHEKNLYVVMEAVEAKQEVTVEQTG..NANAIFSLPSLALLG..LQGSLNNNKAVTIPKGCVLAYRVRLLRVFLFNLWDIPYICNDSMQTFP
hGSDMA  117 TLSVAPKALETLQ.ERKLA.ADHPFLKEMQDQGENLYVVMEVVETVQEVTLERAGKAE..ACFSLPFFAPLG..LQGSINHKEAVTIPKGCVLAFRVRQLMVKGKDEWDIPHICNDNMQTFP
mGSDMD  123 ILRVTQKTWETMQHERHLQQPENKILQQLRSRGDDLFVVTEVLQTKEEVQITEVHSQEGSGQFTLPGALCLKGEGKGHQSRKKMVTIPAGSILAFRVAQL..LIGSKWDILLVSDEKQRTFE
hGSDMD  122 SLSVDPNTWQTLLHERHLRQPEHKVLQQLRSRGDNVYVVTEVLQTQKEVEVTRTHKREGSGRFSLPGATCLQGEGQGHLSQKKTVTIPSGSTLAFRVAQL..VIDSDLDVLLFPDKKQRTFQ
                              .150                                 .Cys191   .200
```

FIG. 29

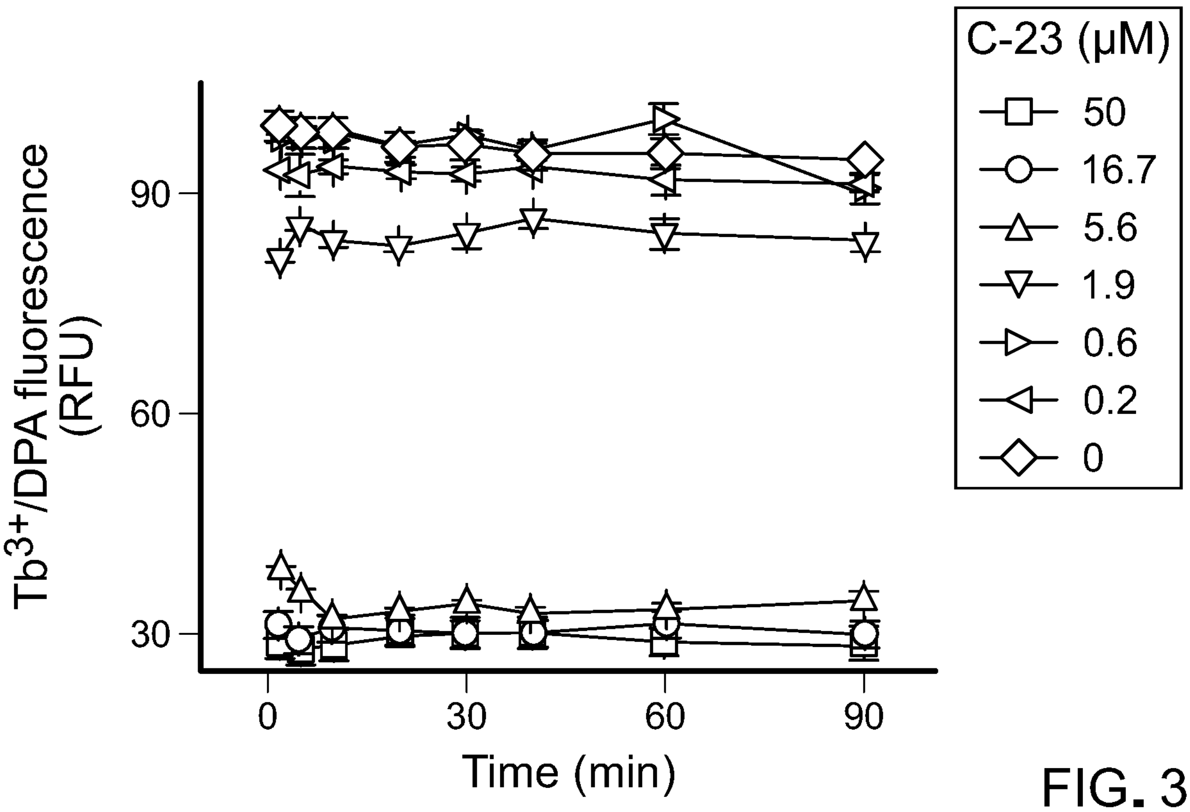

C-23 (μM)
50
16.7
5.6
1.9
0.6
0.2
0
Tb3+/DPA fluorescence (RFU)
90
60
30
0
30
60
90
Time (min)

FIG. 30

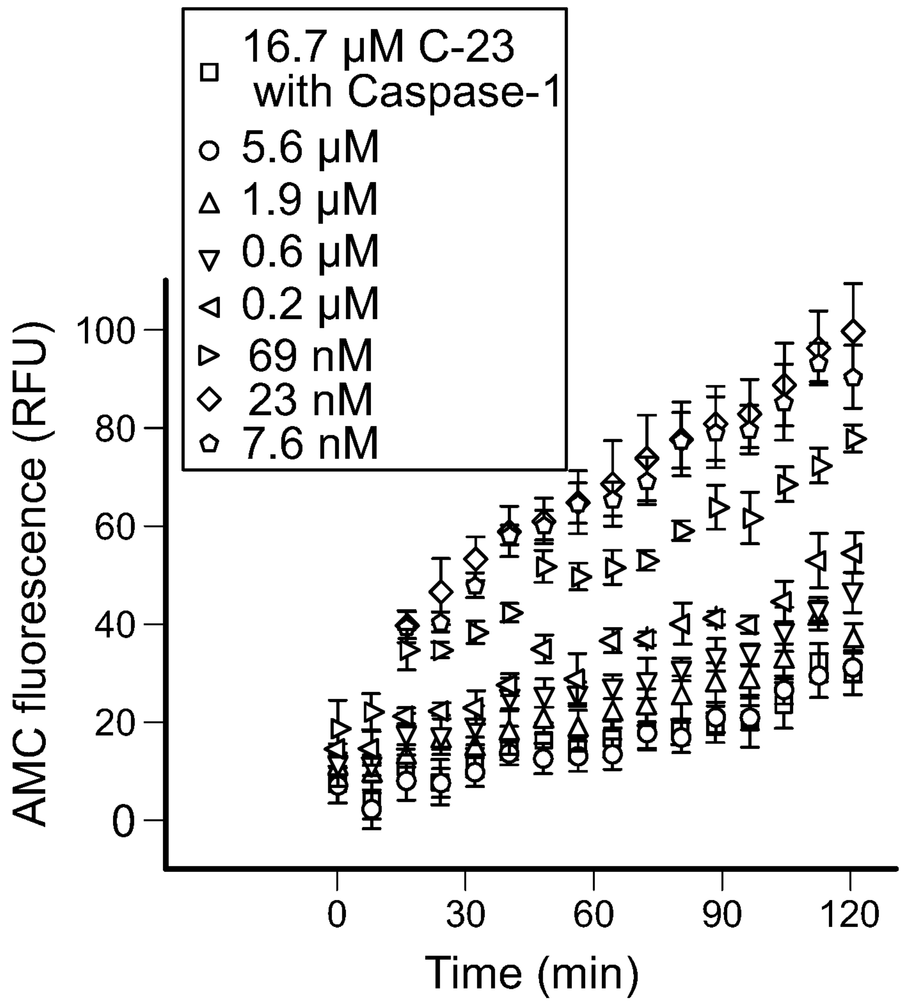

16.7 μM C-23 with Caspase-1
5.6 μM
1.9 μM
0.6 μM
0.2 μM
69 nM
23 nM
7.6 nM
AMC fluorescence (RFU)
100
80
60
40
20
0
0
30
60
90
120
Time (min)

FIG. 31

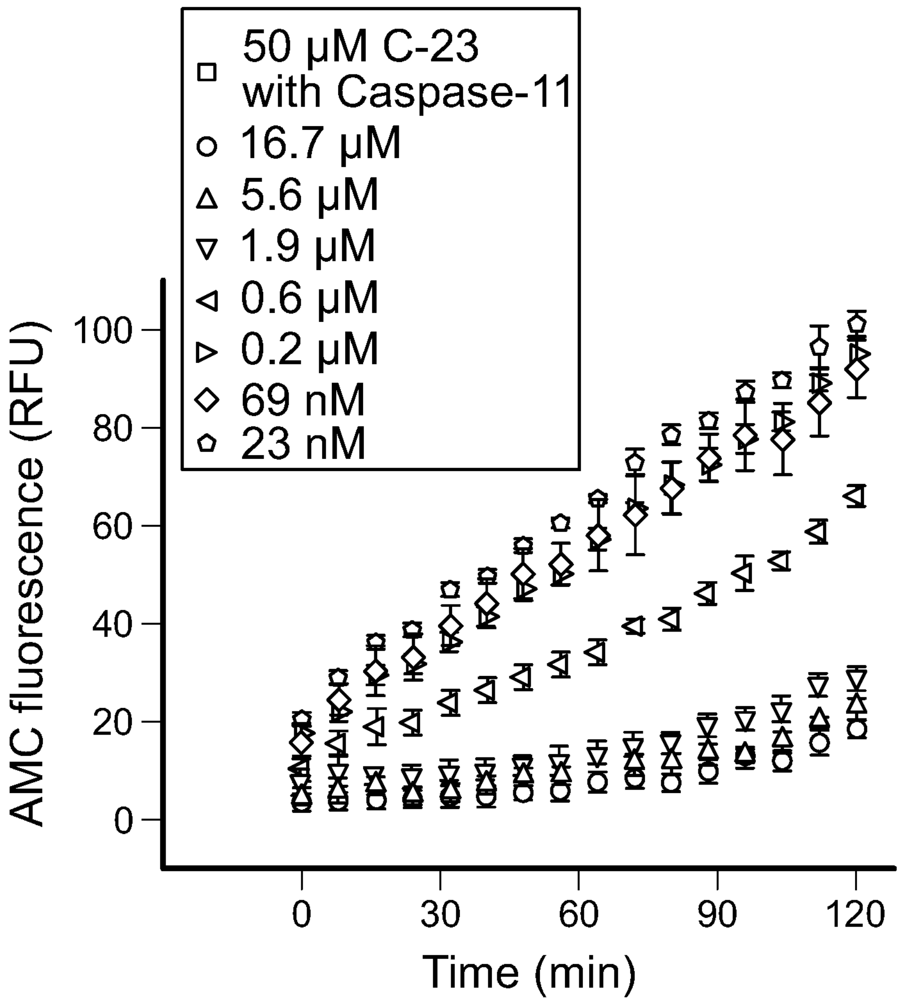

50 μM C-23 with Caspase-11
16.7 μM
5.6 μM
1.9 μM
0.6 μM
0.2 μM
69 nM
23 nM
AMC fluorescence (RFU)
0
20
40
60
80
100
0
30
60
90
120
Time (min)

FIG. 32

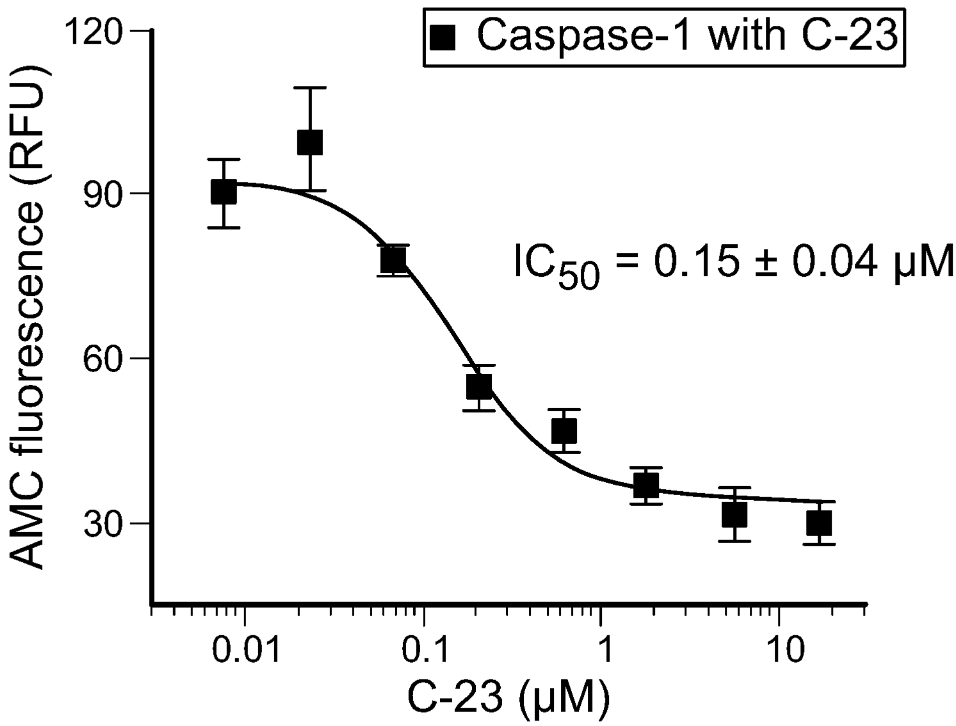

Caspase-1 with C-23
IC_{50} = 0.15 ± 0.04 μM
AMC fluorescence (RFU)
30
60
90
120
0.01
0.1
1
10
C-23 (μM)

FIG. 33

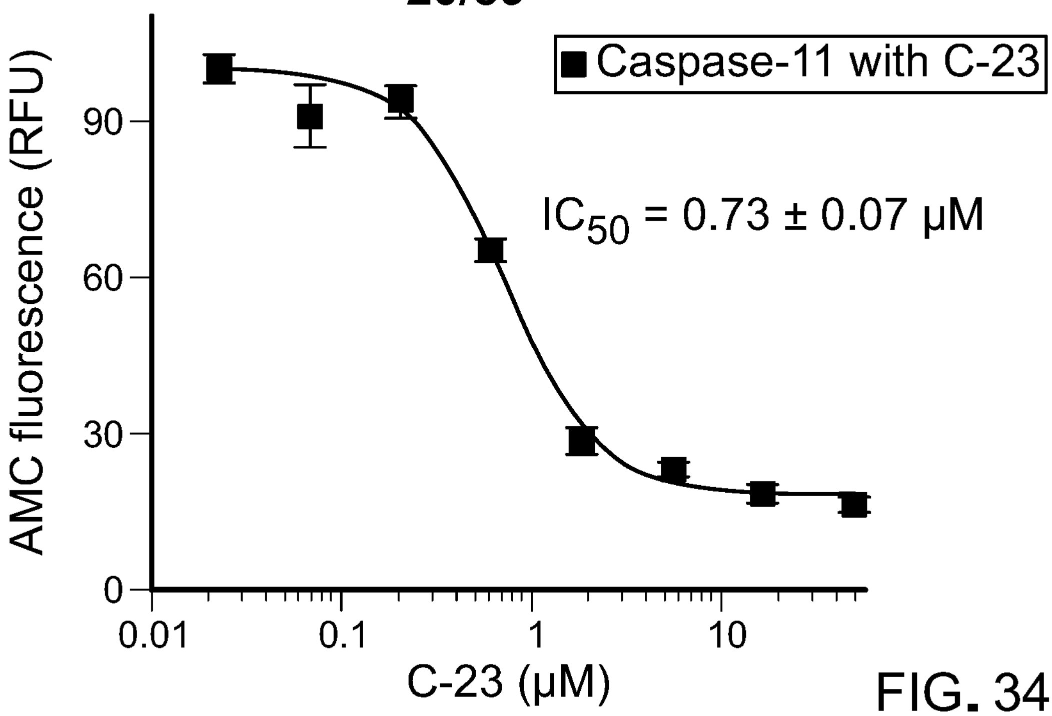

Caspase-11 with C-23
IC50 = 0.73 ± 0.07 μM
AMC fluorescence (RFU)
0
30
60
90
0.01
0.1
1
10
C-23 (μM)

FIG. 34

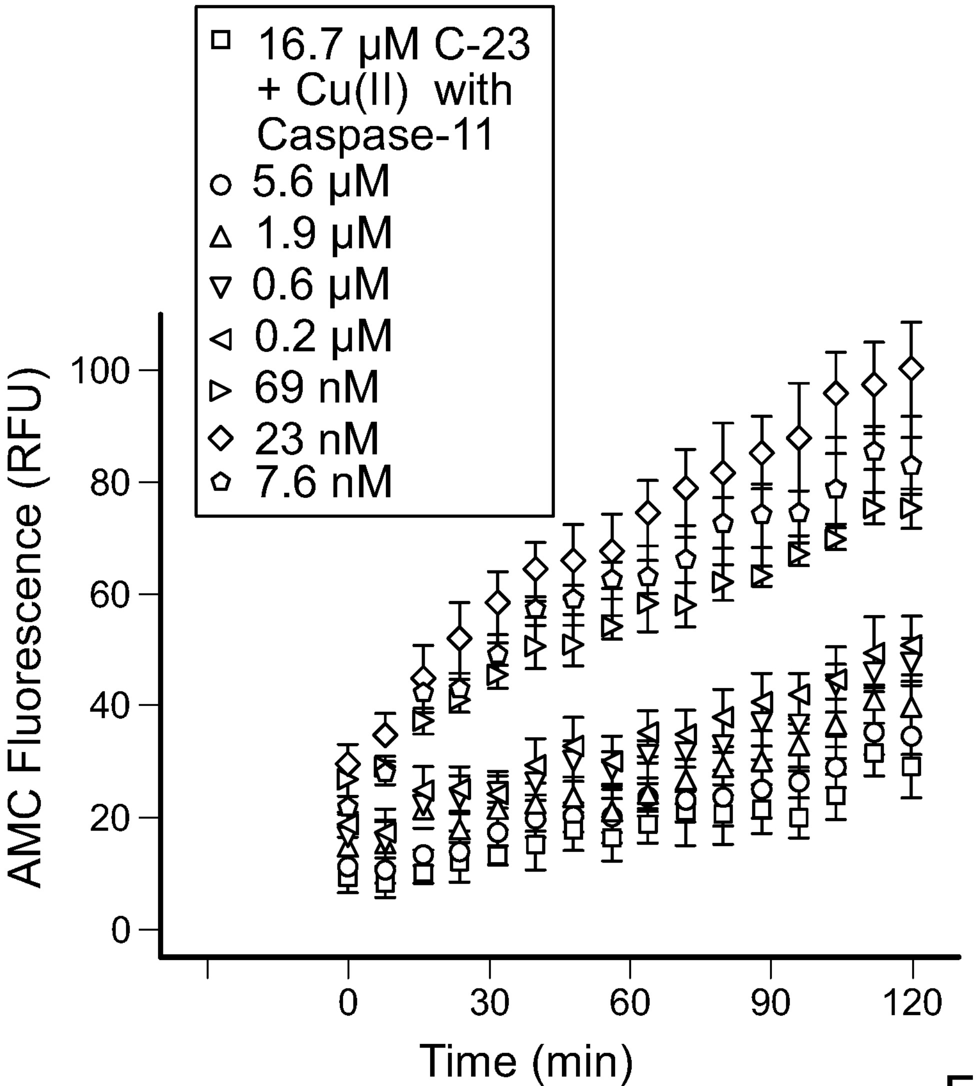

16.7 μM C-23 + Cu(II) with Caspase-11
5.6 μM
1.9 μM
0.6 μM
0.2 μM
69 nM
23 nM
7.6 nM
AMC Fluorescence (RFU)
0
20
40
60
80
100
0
30
60
90
120
Time (min)

FIG. 35

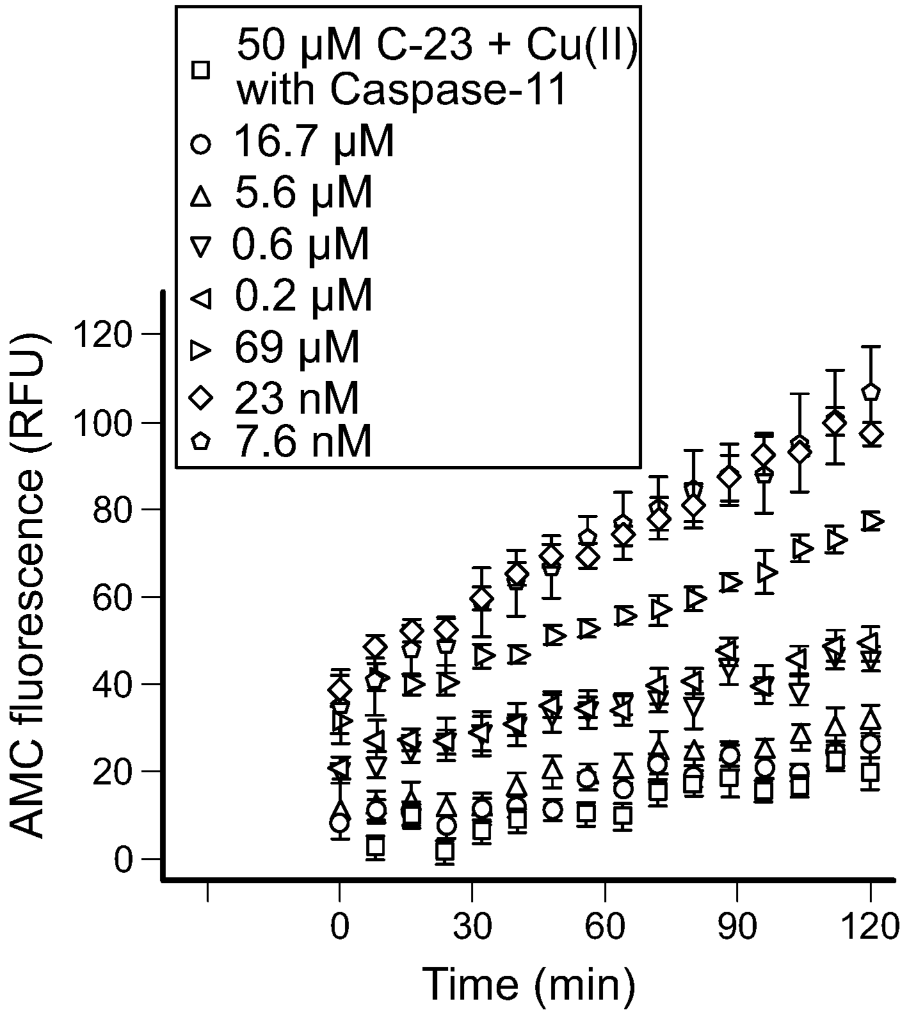

50 μM C-23 + Cu(II) with Caspase-11
16.7 μM
5.6 μM
0.6 μM
0.2 μM
69 μM
23 nM
7.6 nM
AMC fluorescence (RFU)
0
20
40
60
80
100
120
0
30
60
90
120
Time (min)

FIG. 36

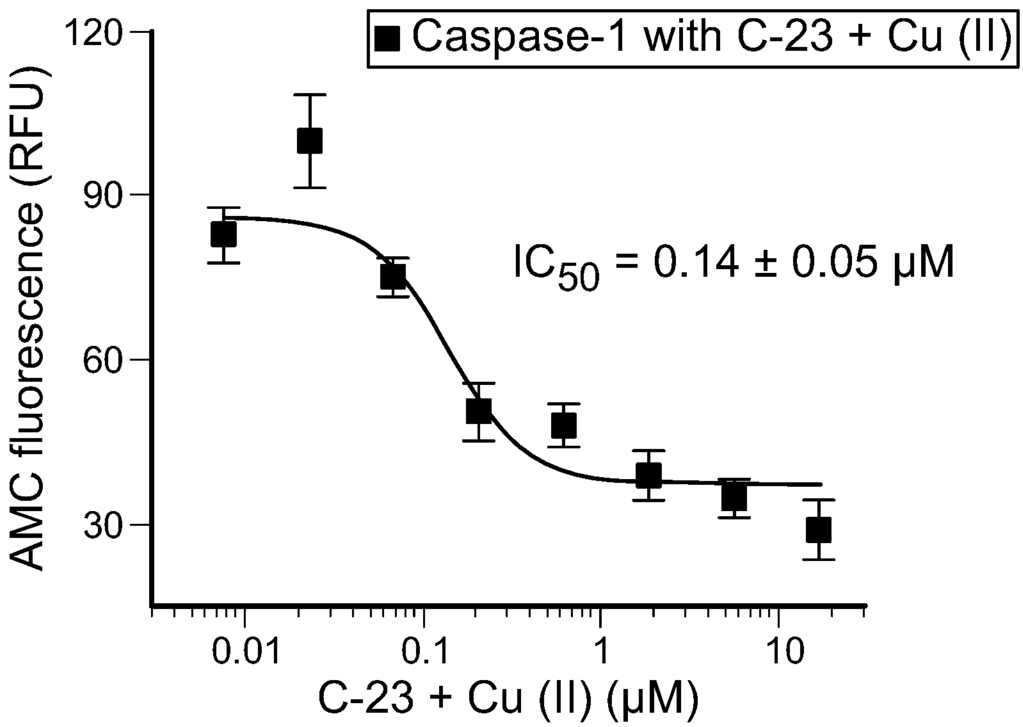

Caspase-1 with C-23 + Cu (II)
IC50 = 0.14 ± 0.05 μM
AMC fluorescence (RFU)
30
60
90
120
0.01
0.1
1
10
C-23 + Cu (II) (μM)

FIG. 37

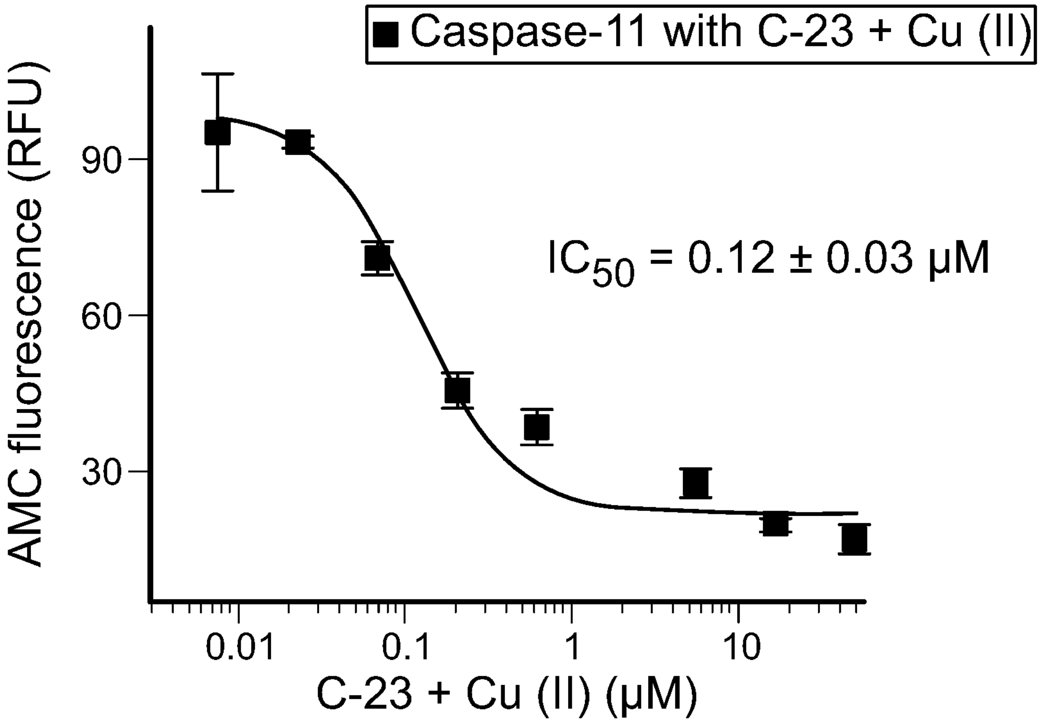
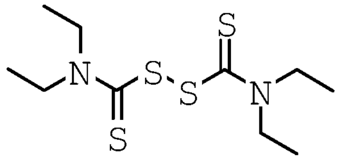
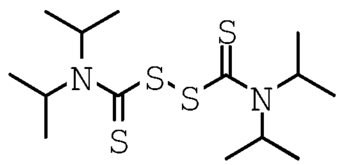

Caspase-11 with C-23 + Cu (II)
AMC fluorescence (RFU)
90
60
30
IC_{50} = 0.12 ± 0.03 μM
0.01
0.1
1
10
C-23 + Cu (II) (μM)

FIG. 38

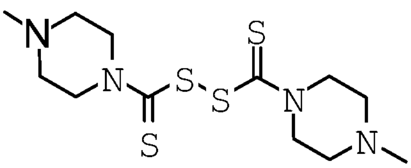
C-23



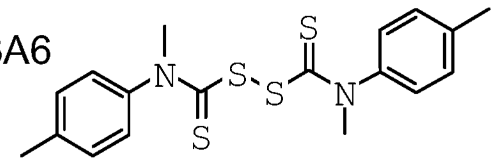
C-23A2



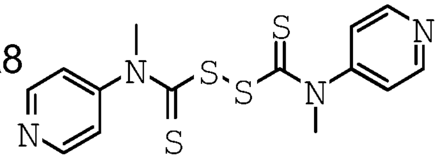
C-23A4



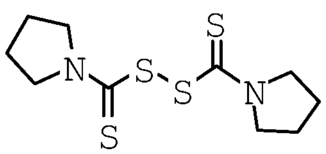
C-23A6



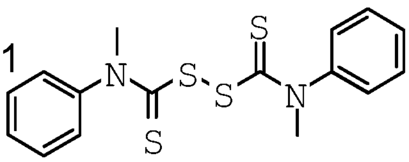
C-23A8



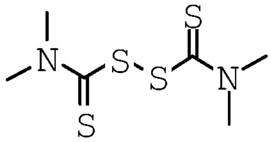
C-23A10



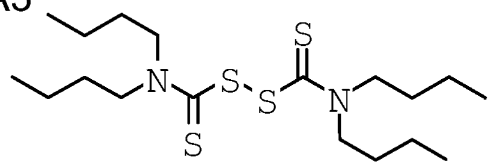
C-23A11



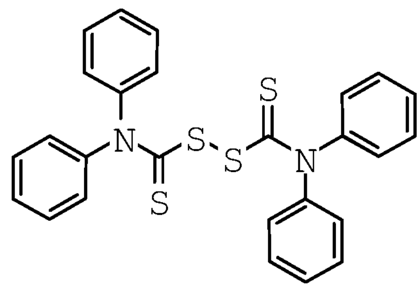
C-23A1



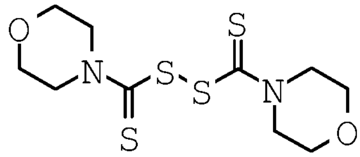
C-23A3



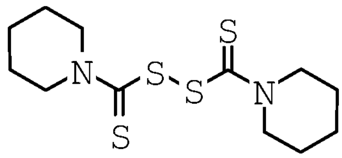
C-23A5



C-23A7



C-23A9



C-23A12

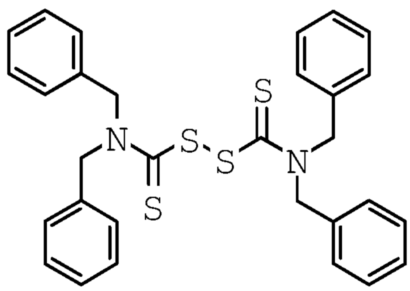
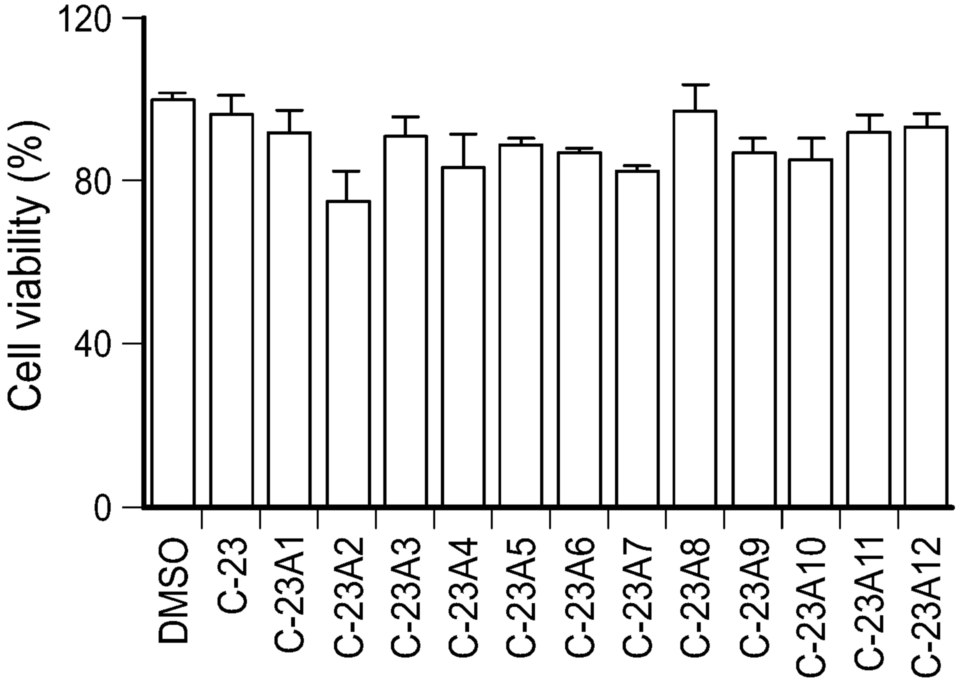
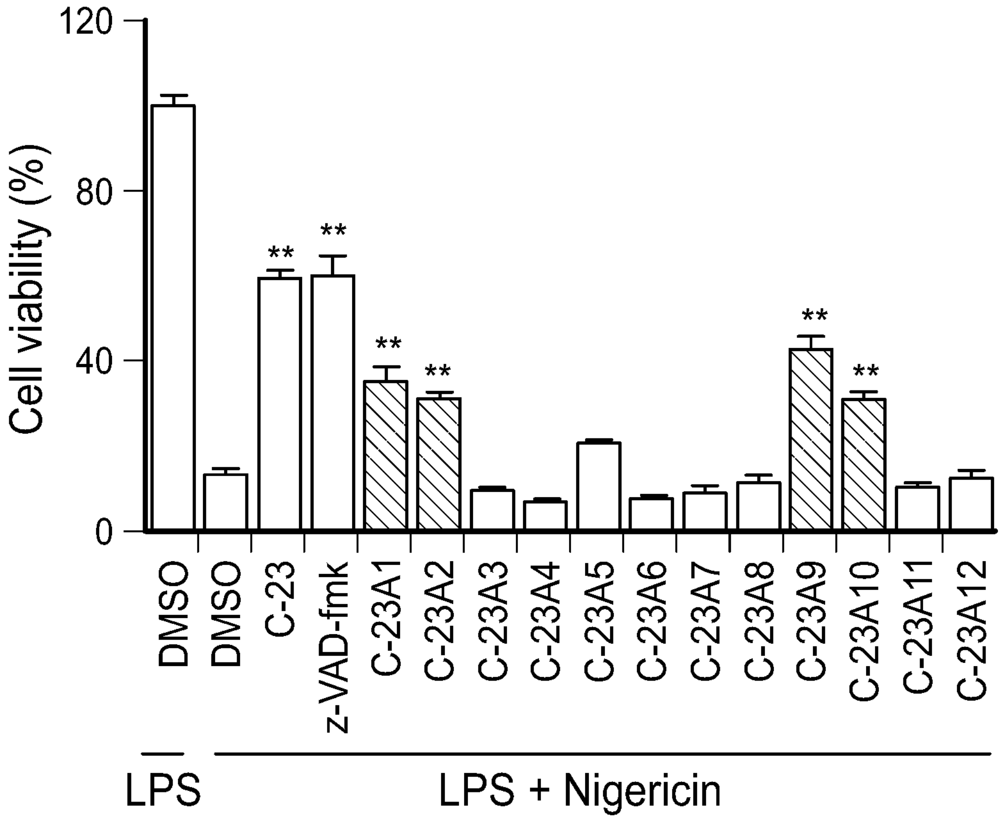


FIG. 39



120
80
40
0
Cell viability (%)
DMSO
C-23
C-23A1
C-23A2
C-23A3
C-23A4
C-23A5
C-23A6
C-23A7
C-23A8
C-23A9
C-23A10
C-23A11
C-23A12

FIG. 40



120
80
40
0
Cell viability (%)
**
DMSO
DMSO
C-23
z-VAD-fmk
C-23A1
C-23A2
C-23A3
C-23A4
C-23A5
C-23A6
C-23A7
C-23A8
C-23A9
C-23A10
C-23A11
C-23A12
LPS
LPS + Nigericin

FIG. 41

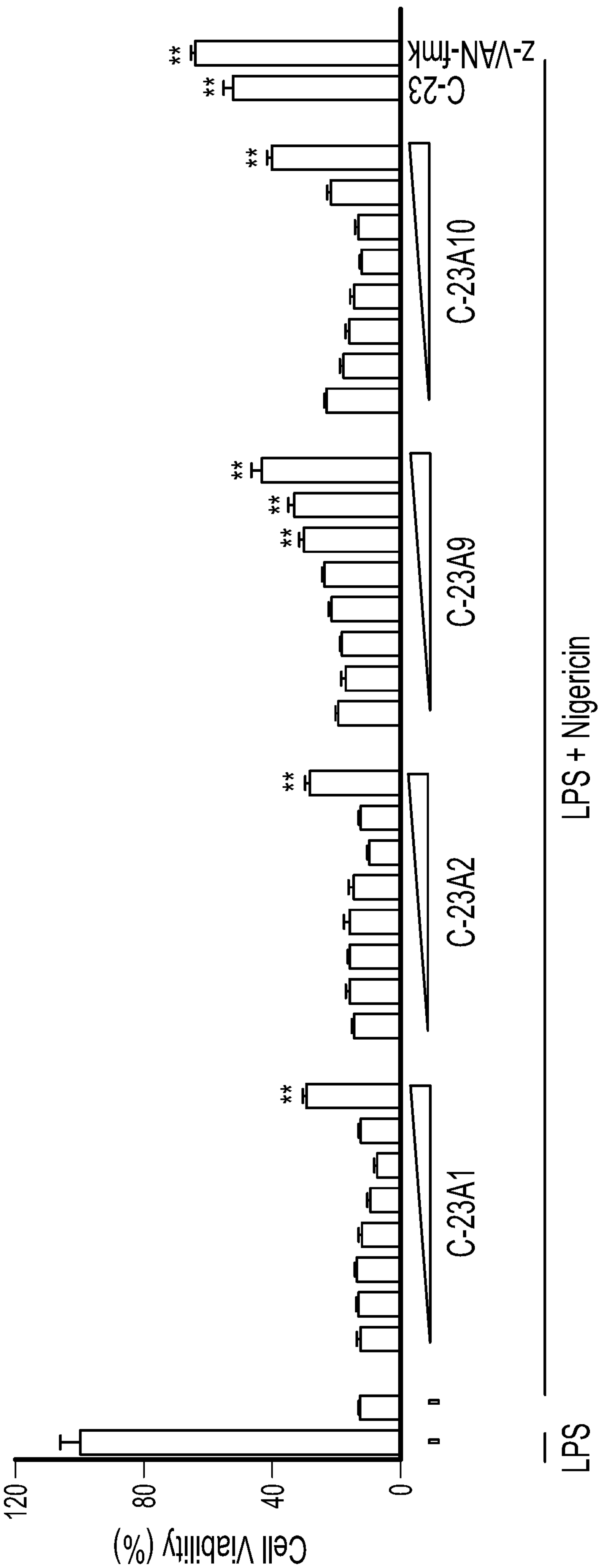
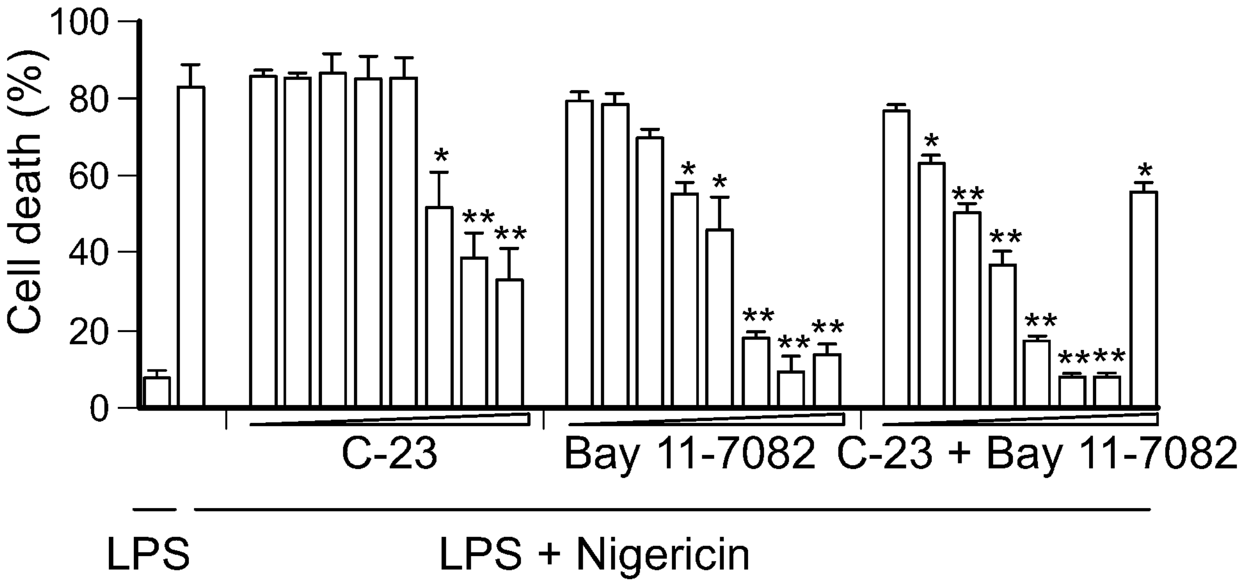
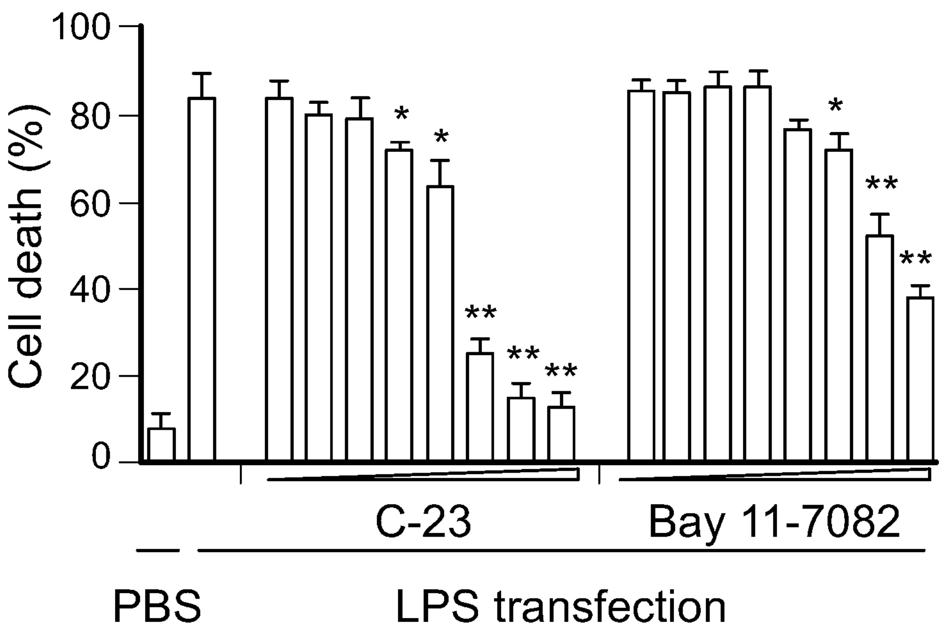

Cell Viability (%)
0
40
80
120
LPS
C-23A1
C-23A2
C-23A9
C-23A10
C-23
z-VAN-fmk
LPS + Nigericin
**

FIG. 42



100
80
60
40
20
0
Cell death (%)
*
**
**
*
*
**
**
**
*
**
**
**
****
*
C-23
Bay 11-7082
C-23 + Bay 11-7082
LPS
LPS + Nigericin

FIG. 43



100
80
60
40
20
0
Cell death (%)
*
*
**
**
**
*
**
**
C-23
Bay 11-7082
PBS
LPS transfection

FIG. 44

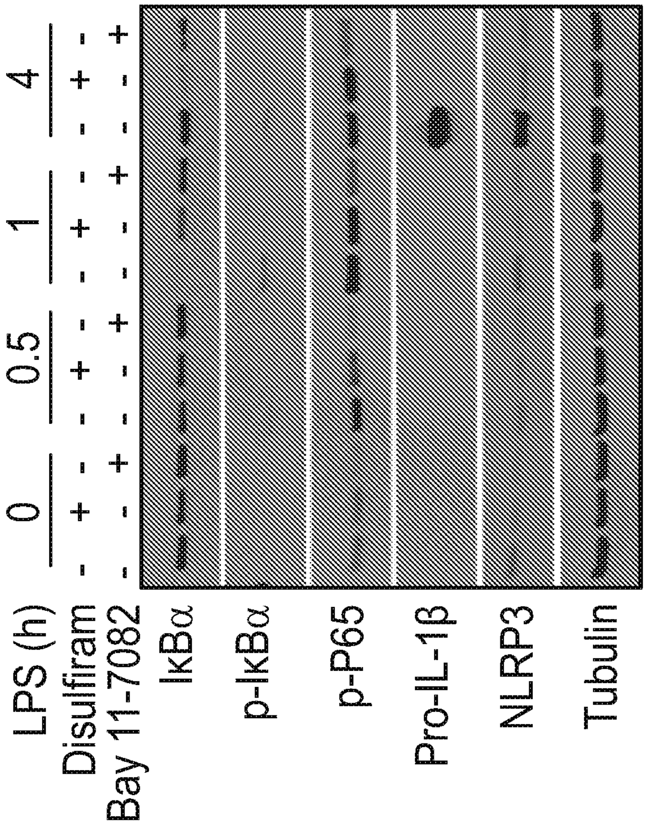

LPS (h)
0
0.5
1
4
Disulfiram
Bay 11-7082
IκBα
p-IκBα
p-P65
Pro-IL-1β
NLRP3
Tubulin

FIG. 45

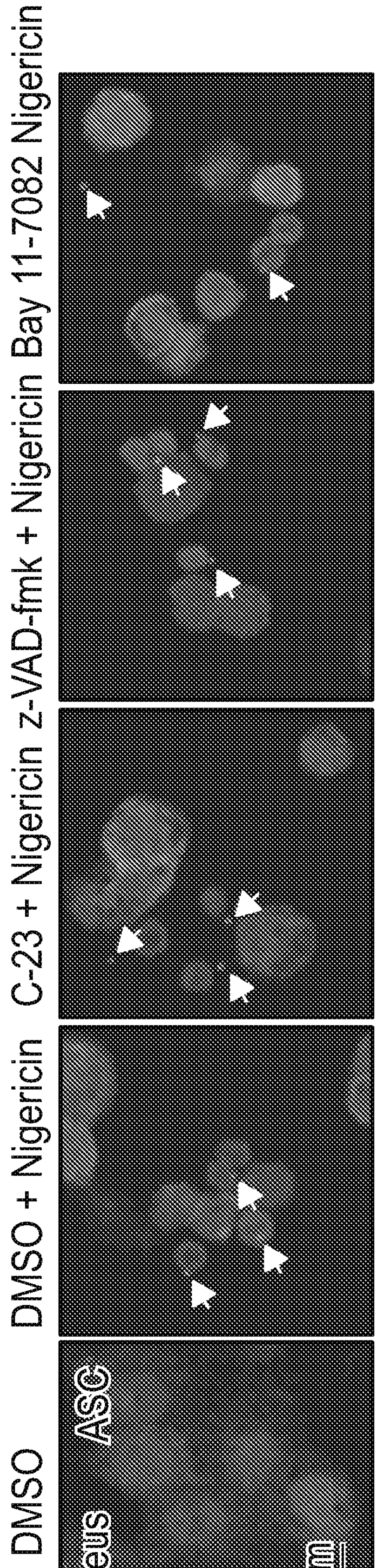

DMSO
DMSO + Nigericin
C-23 + Nigericin
z-VAD-fmk + Nigericin
Bay 11-7082 Nigericin
Nucleus
ASC
20μm

FIG. 46

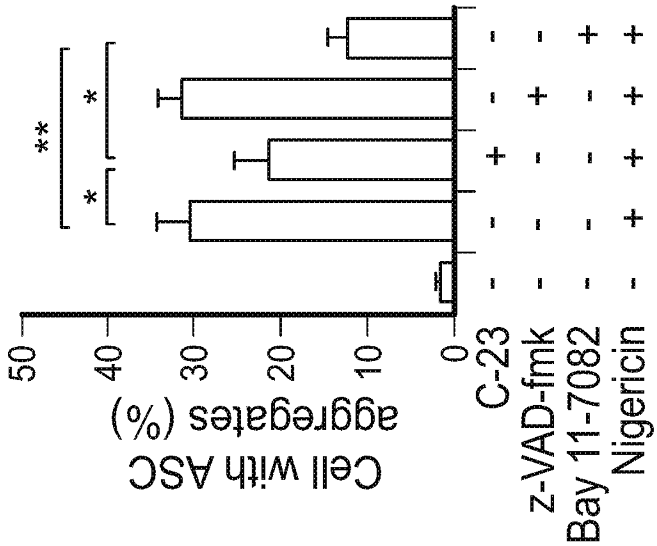

Cell with ASC aggregates (%)
0
10
20
30
40
50
*
**
*
C-23 - - + - - -
z-VAD-fmk - - - + -
Bay 11-7082 - - - - +
Nigericin - + + + +

FIG. 47

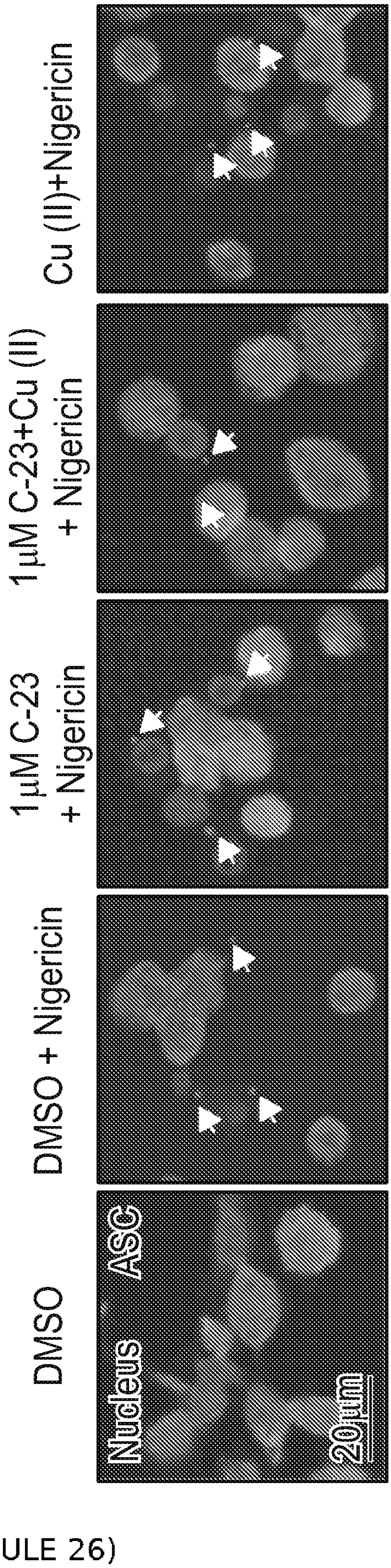

DMSO
DMSO + Nigericin
1μM C-23 + Nigericin
1μM C-23+Cu (II) + Nigericin
Cu (II)+Nigericin
Nucleus
ASC
20μm

FIG. 48

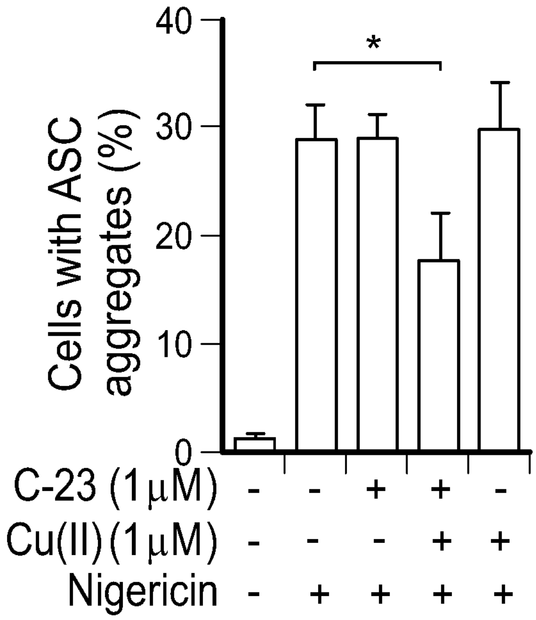

Cells with ASC aggregates (%)
40
30
20
10
0
*
C-23 (1μM) - - + + -
Cu(II) (1μM) - - - + +
Nigericin - + + + +

FIG. 49

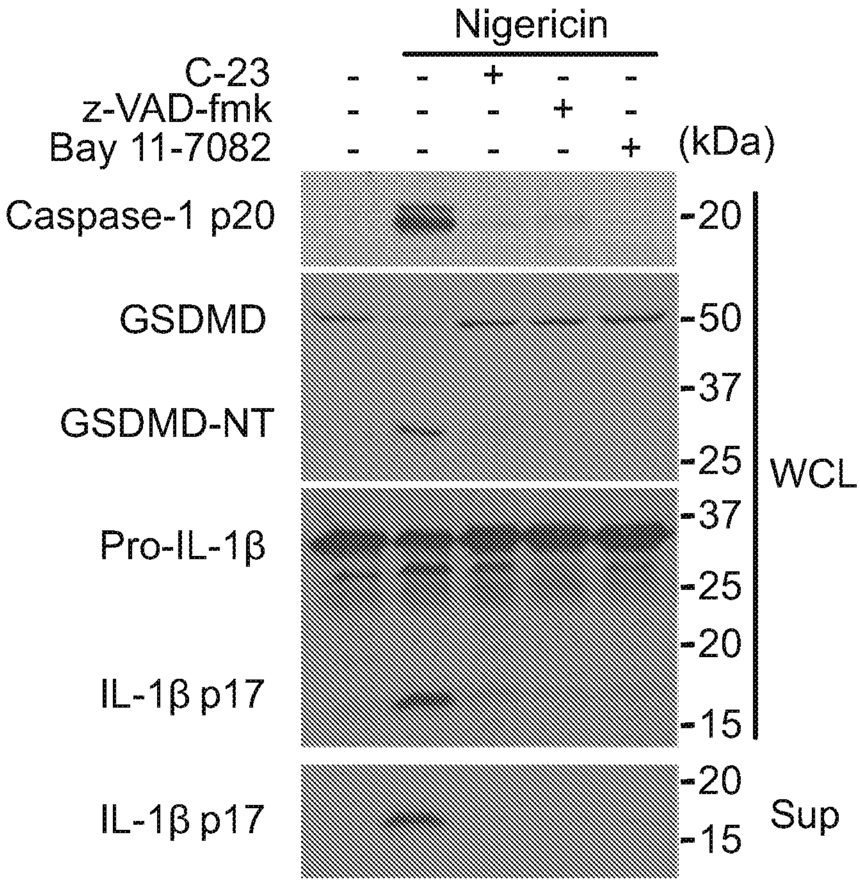

Nigericin
C-23 - - + - -
z-VAD-fmk - - - + -
Bay 11-7082 - - - - +
(kDa)
Caspase-1 p20
-20
GSDMD
-50
-37
GSDMD-NT
-25
WCL
Pro-IL-1β
-37
-25
-20
IL-1β p17
-15
IL-1β p17
-20
-15
Sup

FIG. 50

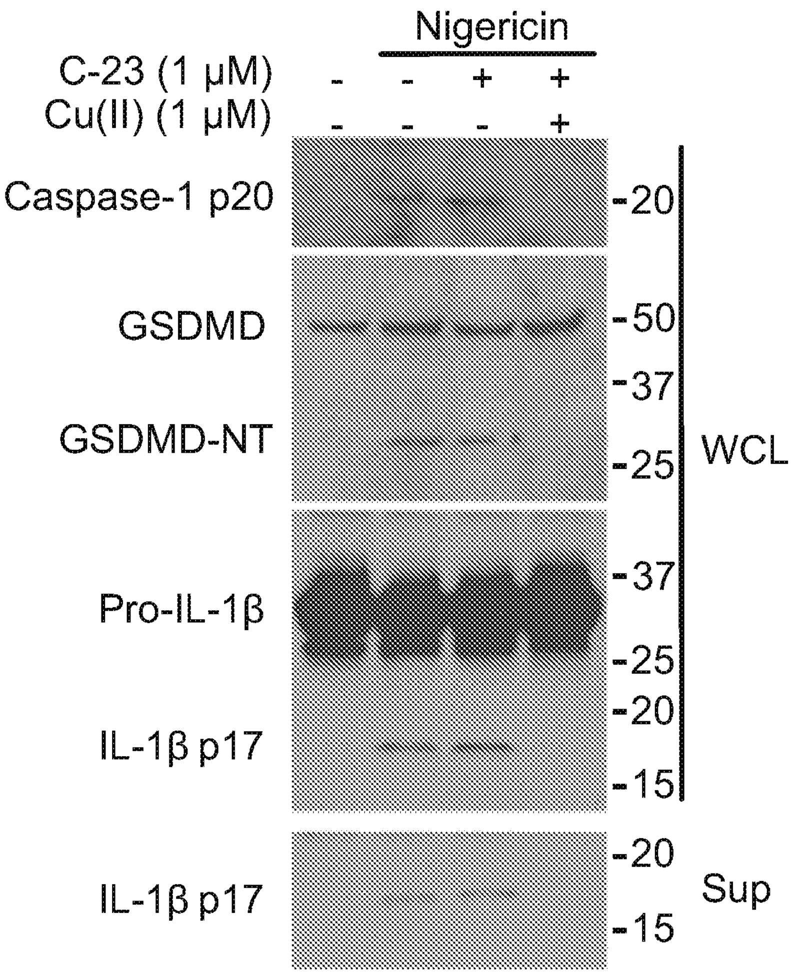

Nigericin
C-23 (1 μM) - - + +
Cu(II) (1 μM) - - - +
Caspase-1 p20
-20
GSDMD
-50
-37
GSDMD-NT
-25
WCL
Pro-IL-1β
-37
-25
-20
IL-1β p17
-15
IL-1β p17
-20
-15
Sup

FIG. 51

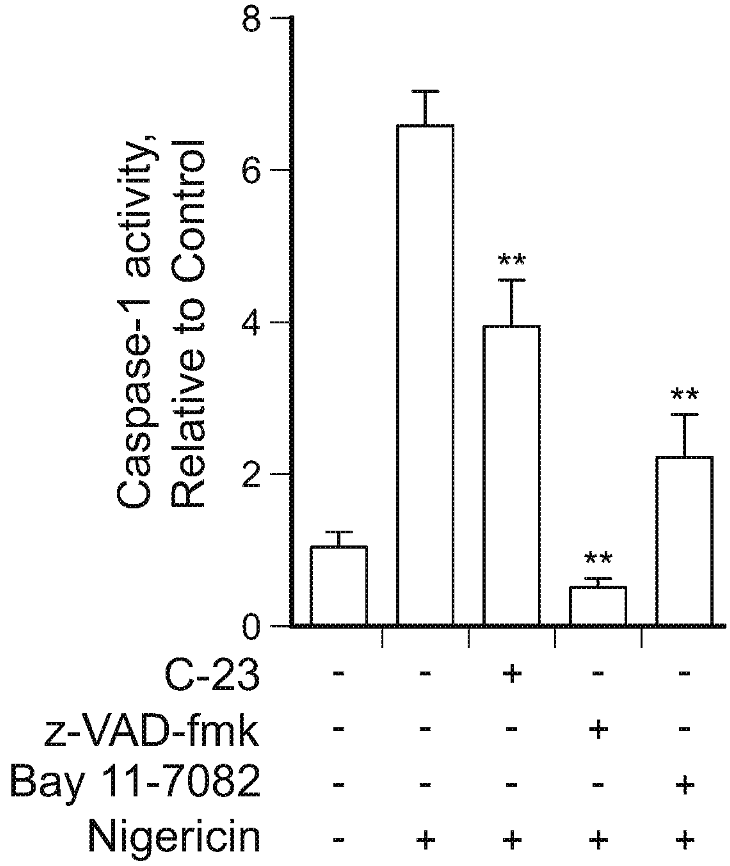

Caspase-1 activity, Relative to Control
8
6
4
2
0
**
**
**
C-23 - - + - -
z-VAD-fmk - - - + -
Bay 11-7082 - - - - +
Nigericin - + + + +

FIG. 52

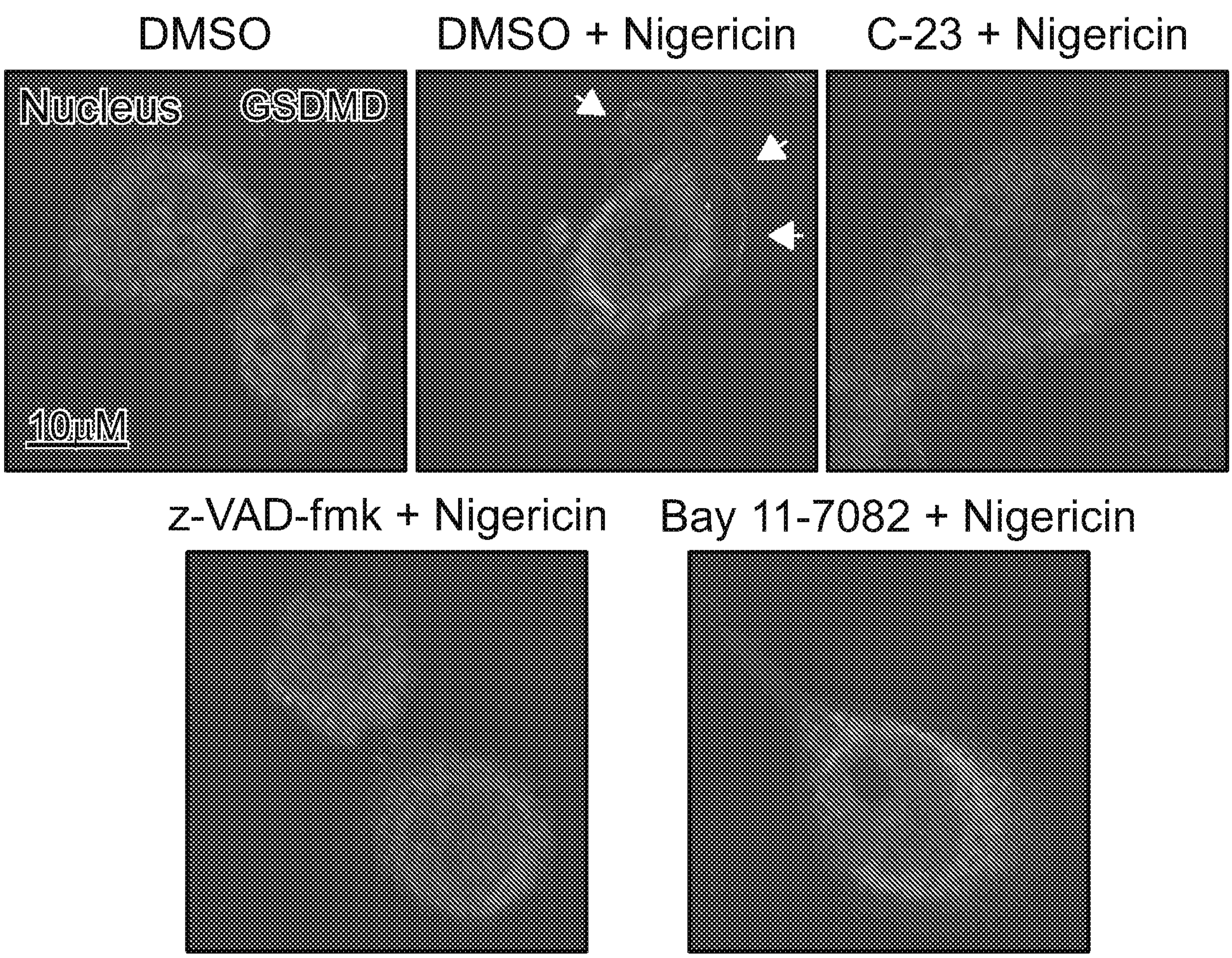

DMSO
DMSO + Nigericin
C-23 + Nigericin
Nucleus
GSDMD
10μM
z-VAD-fmk + Nigericin
Bay 11-7082 + Nigericin

FIG. 53

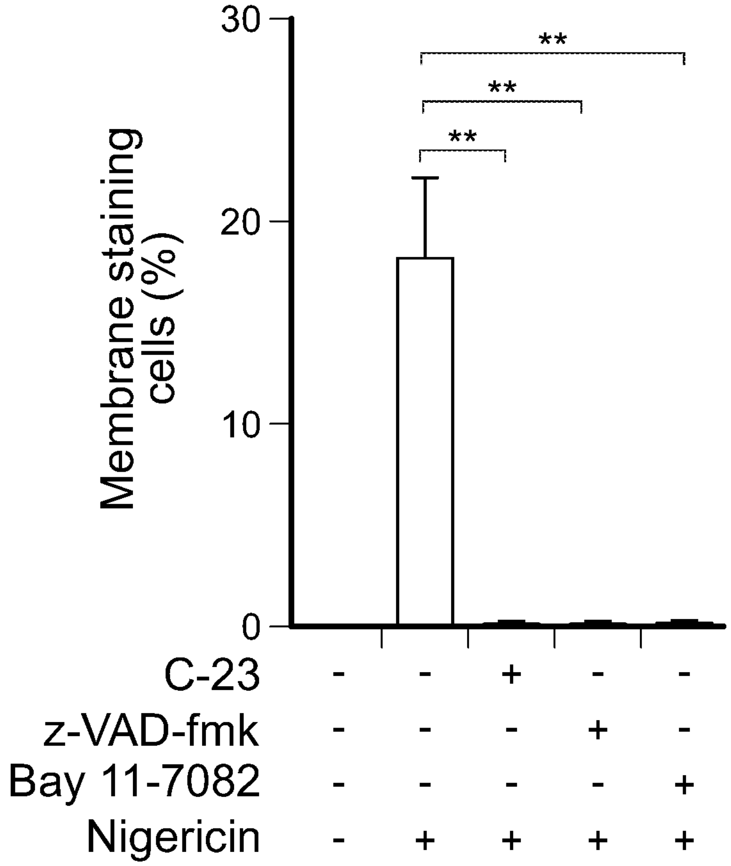

30
20
10
0
Membrane staining cells (%)
**
**
**
C-23 - - + - -
z-VAD-fmk - - - + -
Bay 11-7082 - - - - +
Nigericin - + + + +

FIG. 54

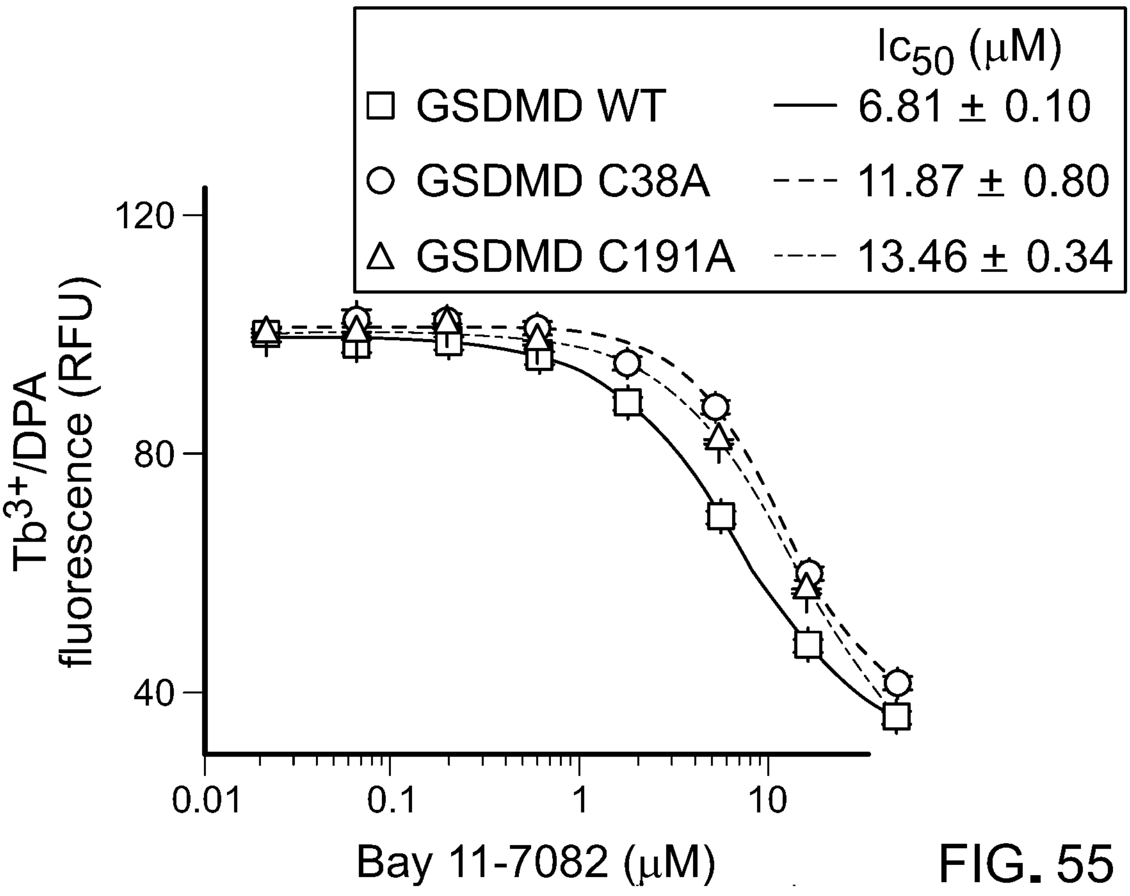

Ic50 (μM)
GSDMD WT 6.81 ± 0.10
GSDMD C38A 11.87 ± 0.80
GSDMD C191A 13.46 ± 0.34
120
80
40
Tb3+/DPA fluorescence (RFU)
0.01
0.1
1
10
Bay 11-7082 (μM)

FIG. 55

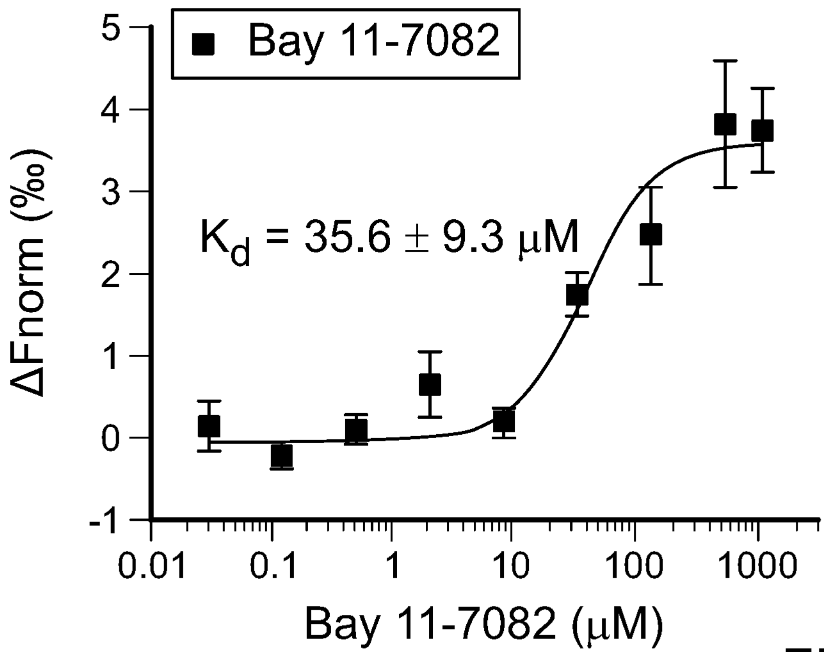

Bay 11-7082
5
4
3
2
1
0
-1
ΔFnorm (‰)
K_d = 35.6 ± 9.3 μM
0.01
0.1
1
10
100
1000
Bay 11-7082 (μM)

FIG. 56

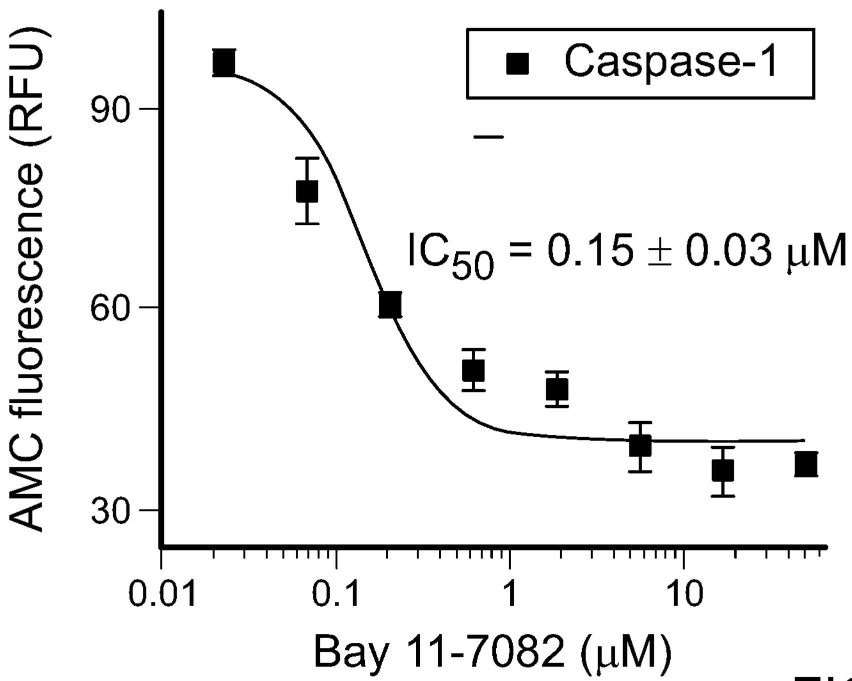

Caspase-1
90
60
30
AMC fluorescence (RFU)
IC_{50} = 0.15 ± 0.03 μM
0.01
0.1
1
10
Bay 11-7082 (μM)

FIG. 57

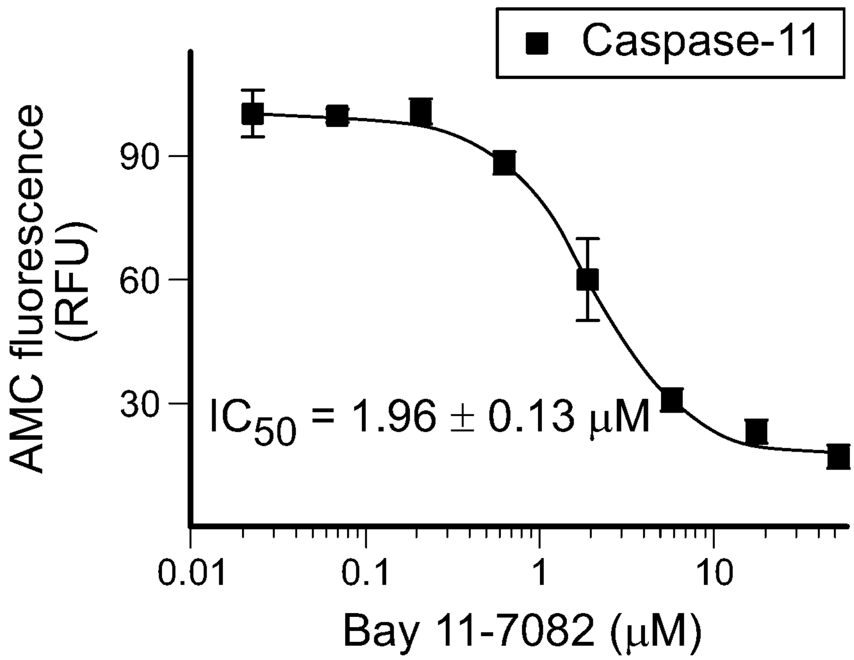

Caspase-11
AMC fluorescence (RFU)
90
60
30
IC50 = 1.96 ± 0.13 μM
0.01
0.1
1
10
Bay 11-7082 (μM)

FIG. 58

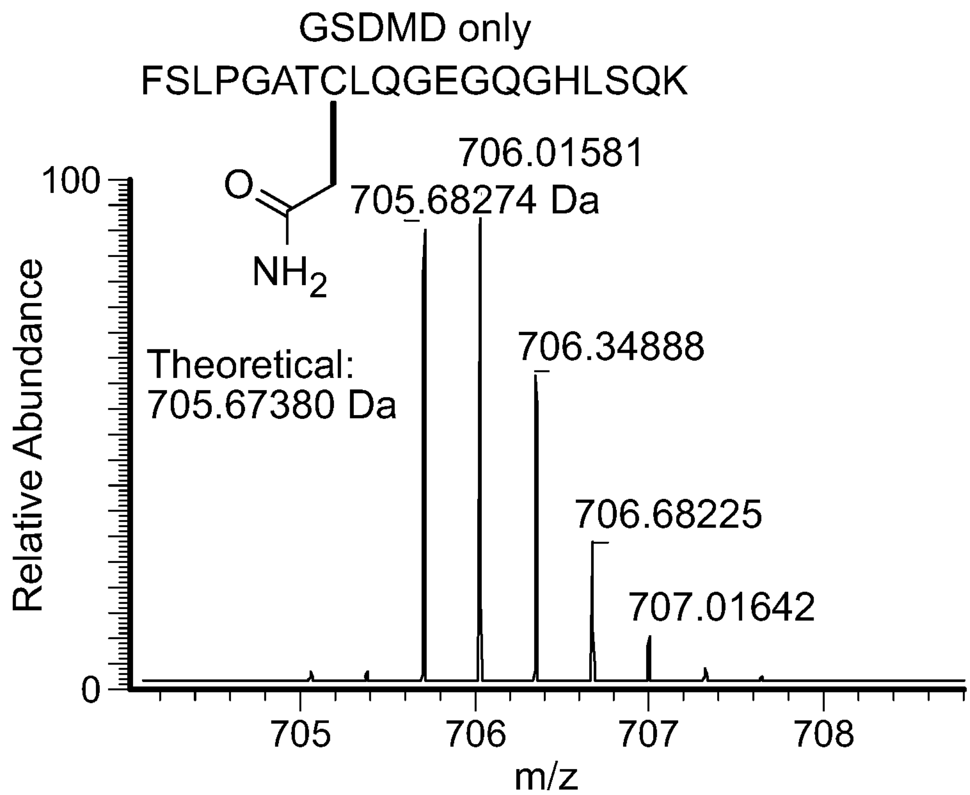

GSDMD only
FSLPGATCLQGEGQGHLSQK
O
NH2
100
0
Relative Abundance
Theoretical:
705.67380 Da
706.01581
705.68274 Da
706.34888
706.68225
707.01642
705
706
707
708
m/z

FIG. 59

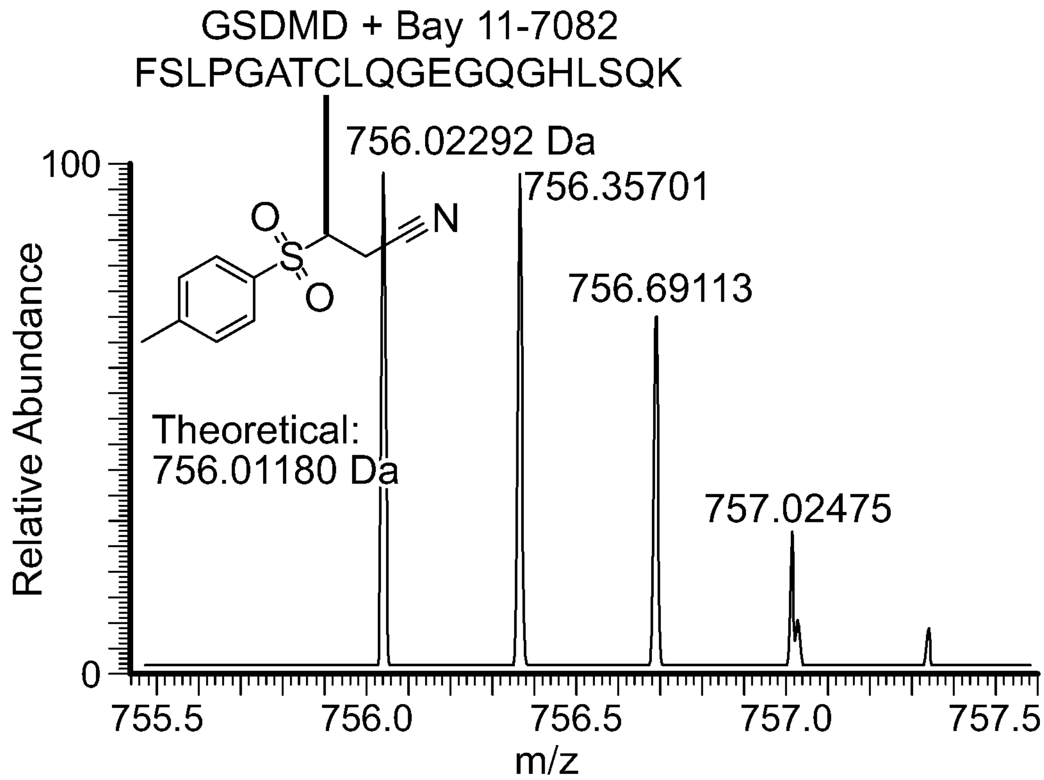

GSDMD + Bay 11-7082
FSLPGATCLQGEGQGHLSQK
756.02292 Da
756.35701
756.69113
757.02475
Theoretical:
756.01180 Da
Relative Abundance
100
0
755.5
756.0
756.5
757.0
757.5
m/z

FIG. 60

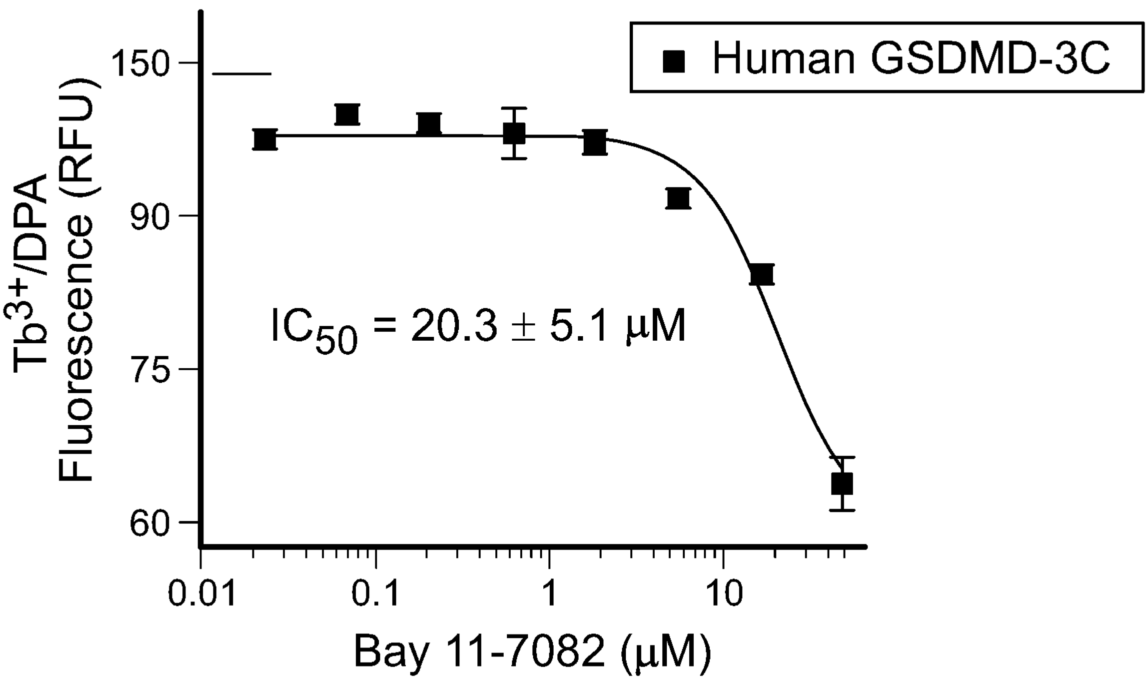
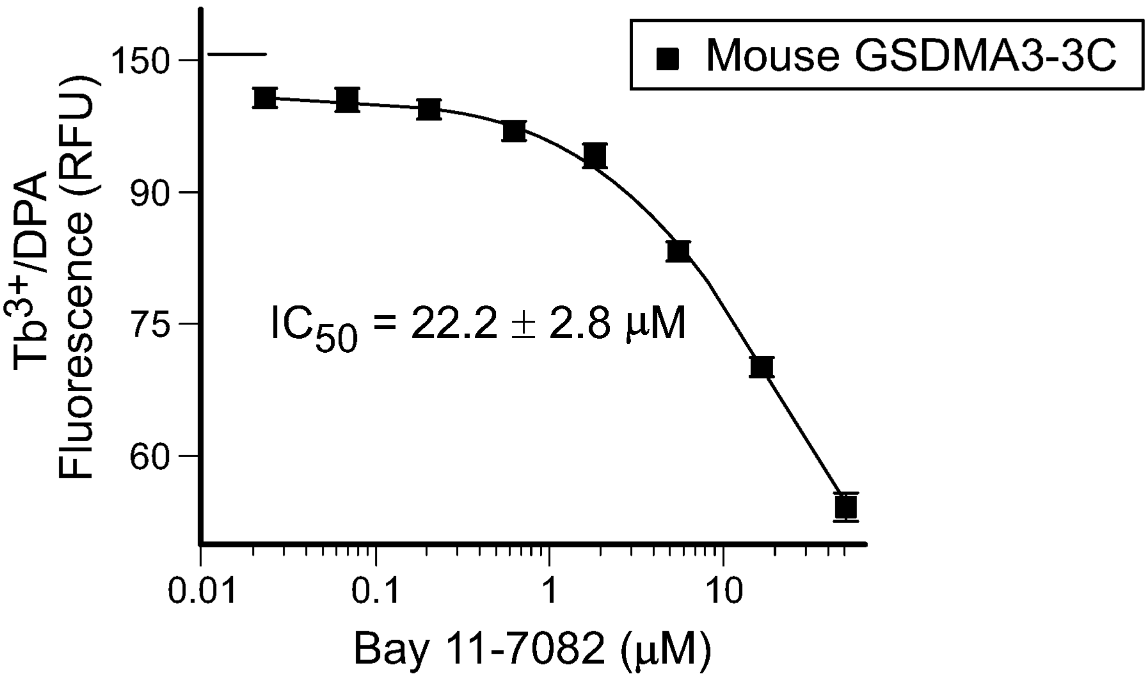
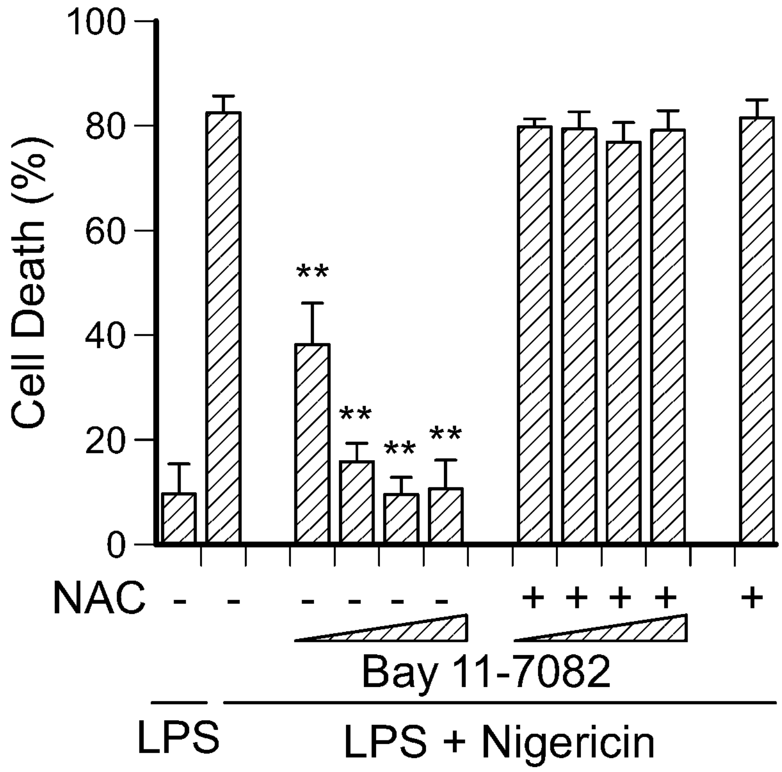

Human GSDMD-3C
Tb3+/DPA Fluorescence (RFU)
150
90
75
60
IC50 = 20.3 ± 5.1 μM
0.01
0.1
1
10
Bay 11-7082 (μM)

FIG. 61



Mouse GSDMA3-3C
Tb3+/DPA Fluorescence (RFU)
150
90
75
60
IC50 = 22.2 ± 2.8 μM
0.01
0.1
1
10
Bay 11-7082 (μM)

FIG. 62



Cell Death (%)
100
80
60
40
20
0
**
NAC
- - - - - - + + + + +
Bay 11-7082
LPS
LPS + Nigericin

FIG. 63

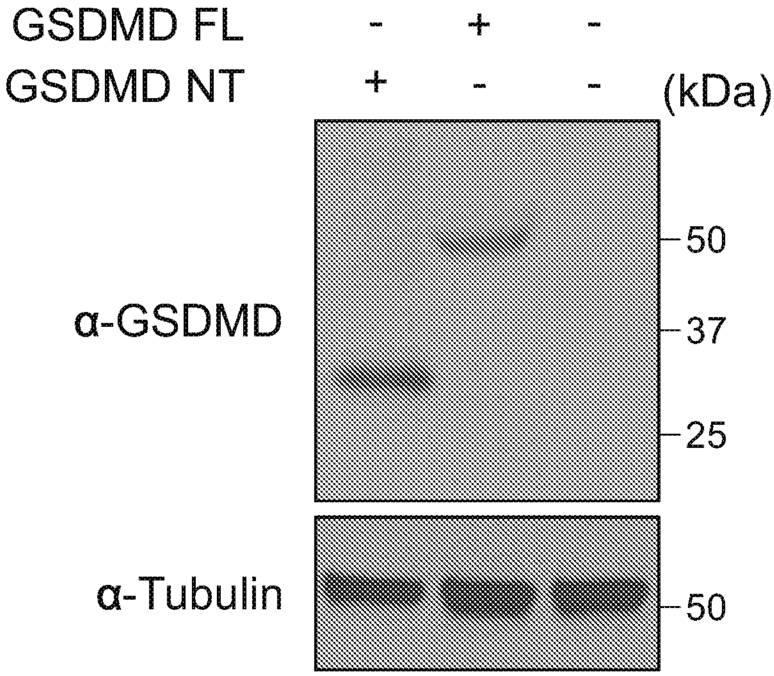

GSDMD FL - + -
GSDMD NT + - -
(kDa)
α-GSDMD
50
37
25
α-Tubulin
50

FIG. 64

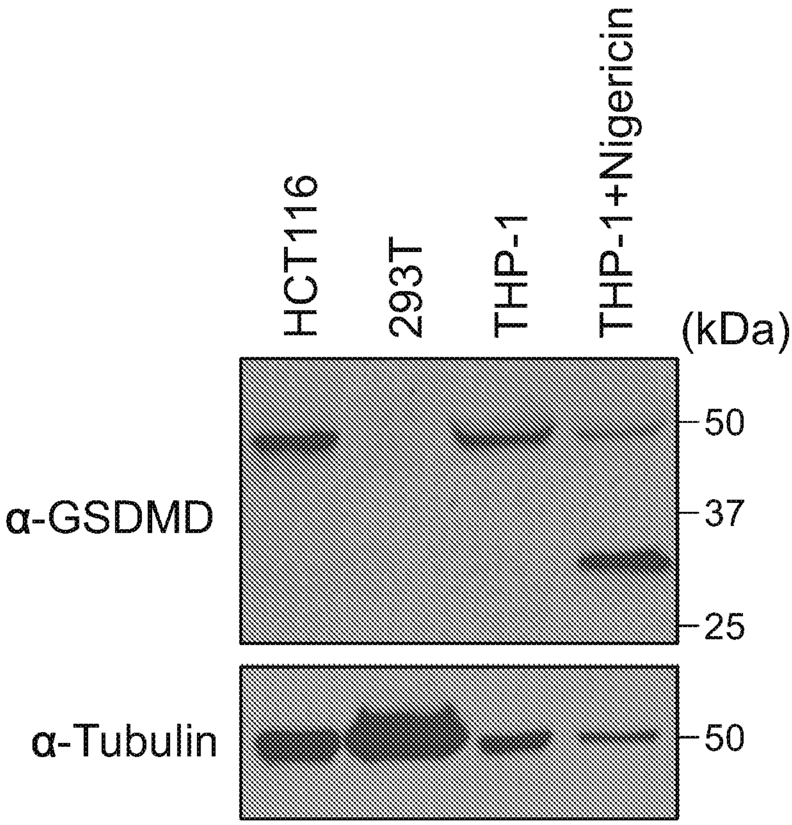

HCT116
293T
THP-1
THP-1+Nigericin
(kDa)
α-GSDMD
50
37
25
α-Tubulin
50

FIG. 65

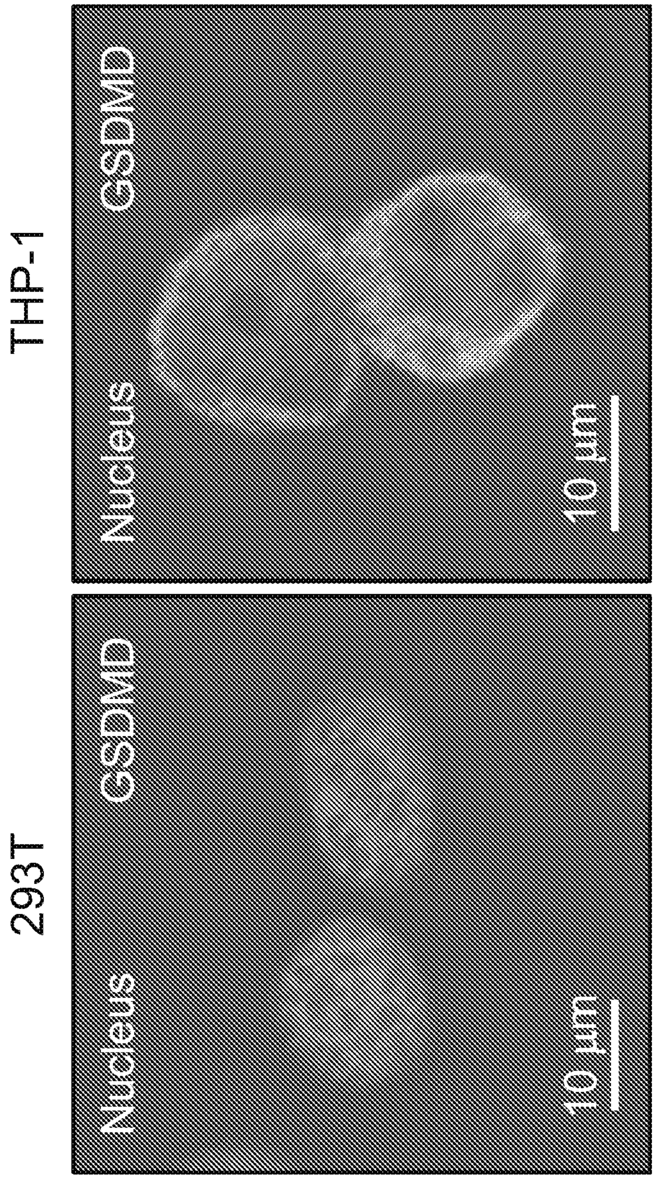


FIG. 66

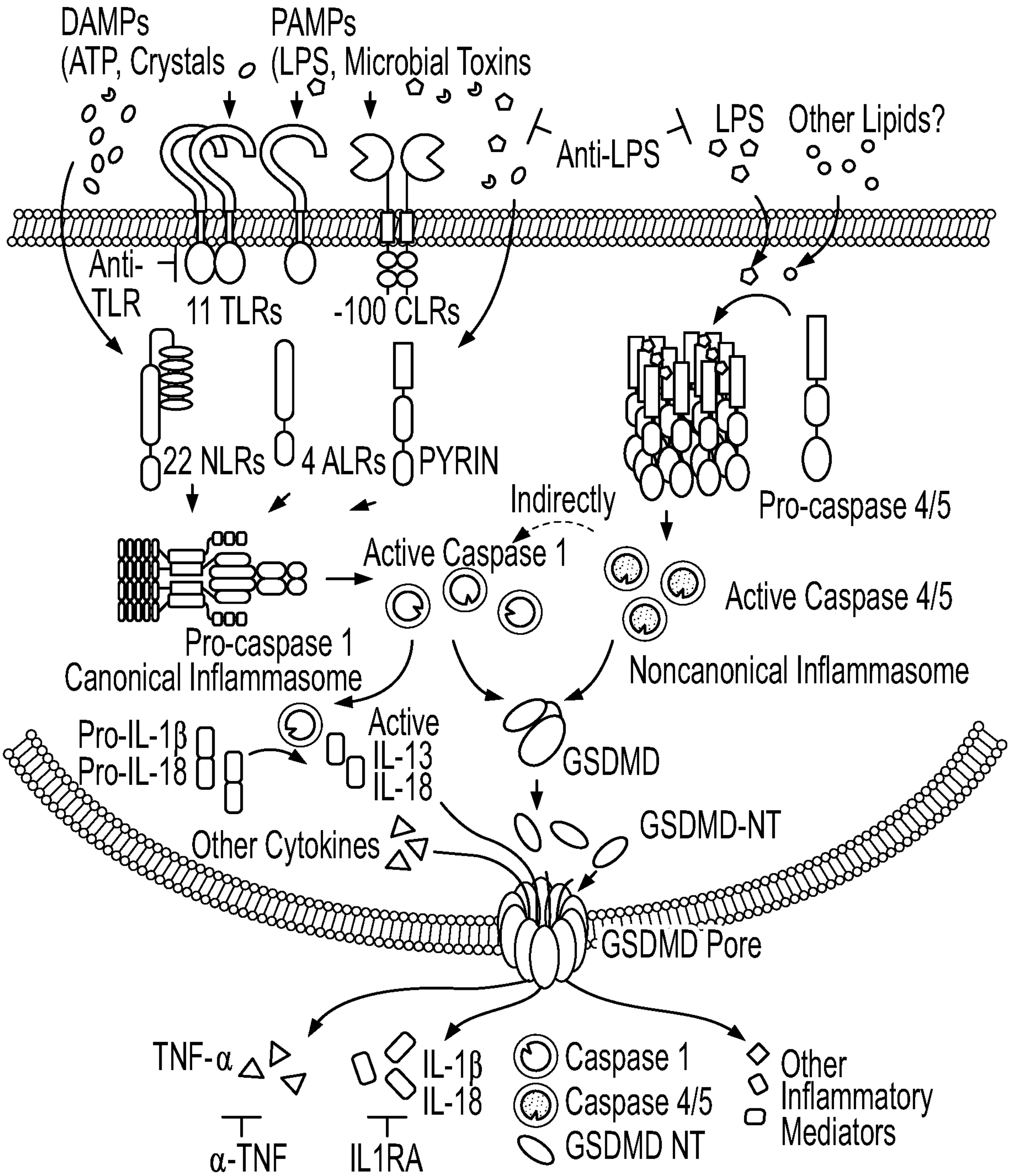

DAMPs
(ATP, Crystals
PAMPs
(LPS, Microbial Toxins
Anti-LPS
LPS
Other Lipids?
Anti-
TLR
11 TLRs
-100 CLRs
22 NLRs
4 ALRs
PYRIN
Indirectly
Pro-caspase 4/5
Active Caspase 1
Active Caspase 4/5
Pro-caspase 1
Canonical Inflammasome
Noncanonical Inflammasome
Pro-IL-1β
Pro-IL-18
Active
IL-13
IL-18
GSDMD
Other Cytokines
GSDMD-NT
GSDMD Pore
TNF-α
IL-1β
IL-18
Caspase 1
Caspase 4/5
GSDMD NT
Other
Inflammatory
Mediators
α-TNF
IL1RA

FIG. 67

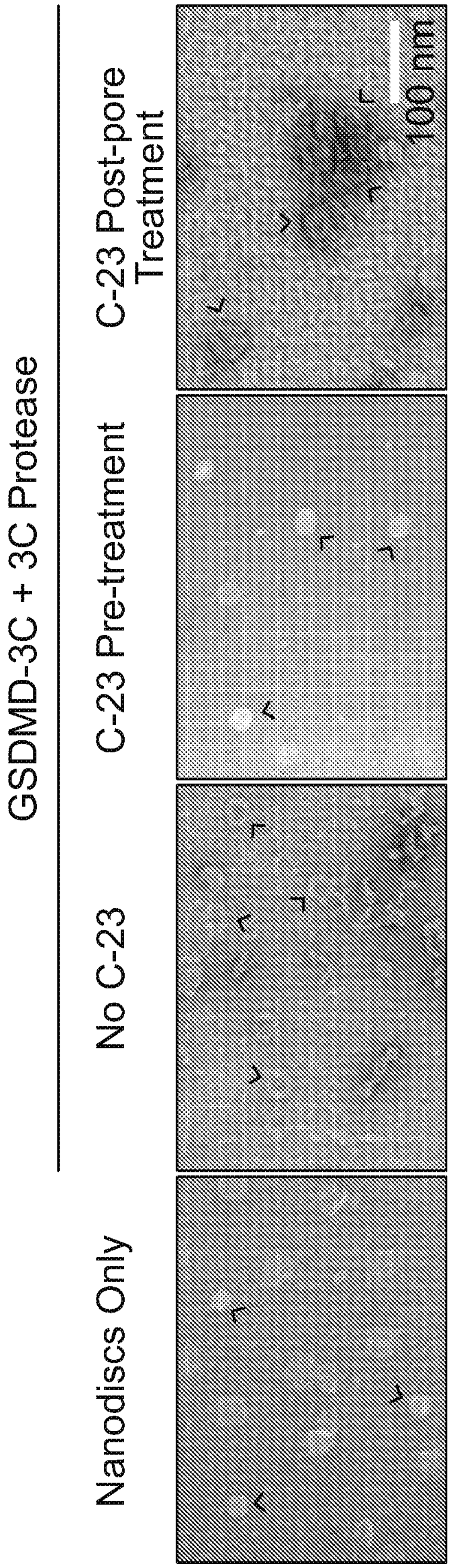

GSDMD-3C + 3C Protease
C-23 Post-pore Treatment
C-23 Pre-treatment
No C-23
Nanodiscs Only
100 nm

FIG. 68

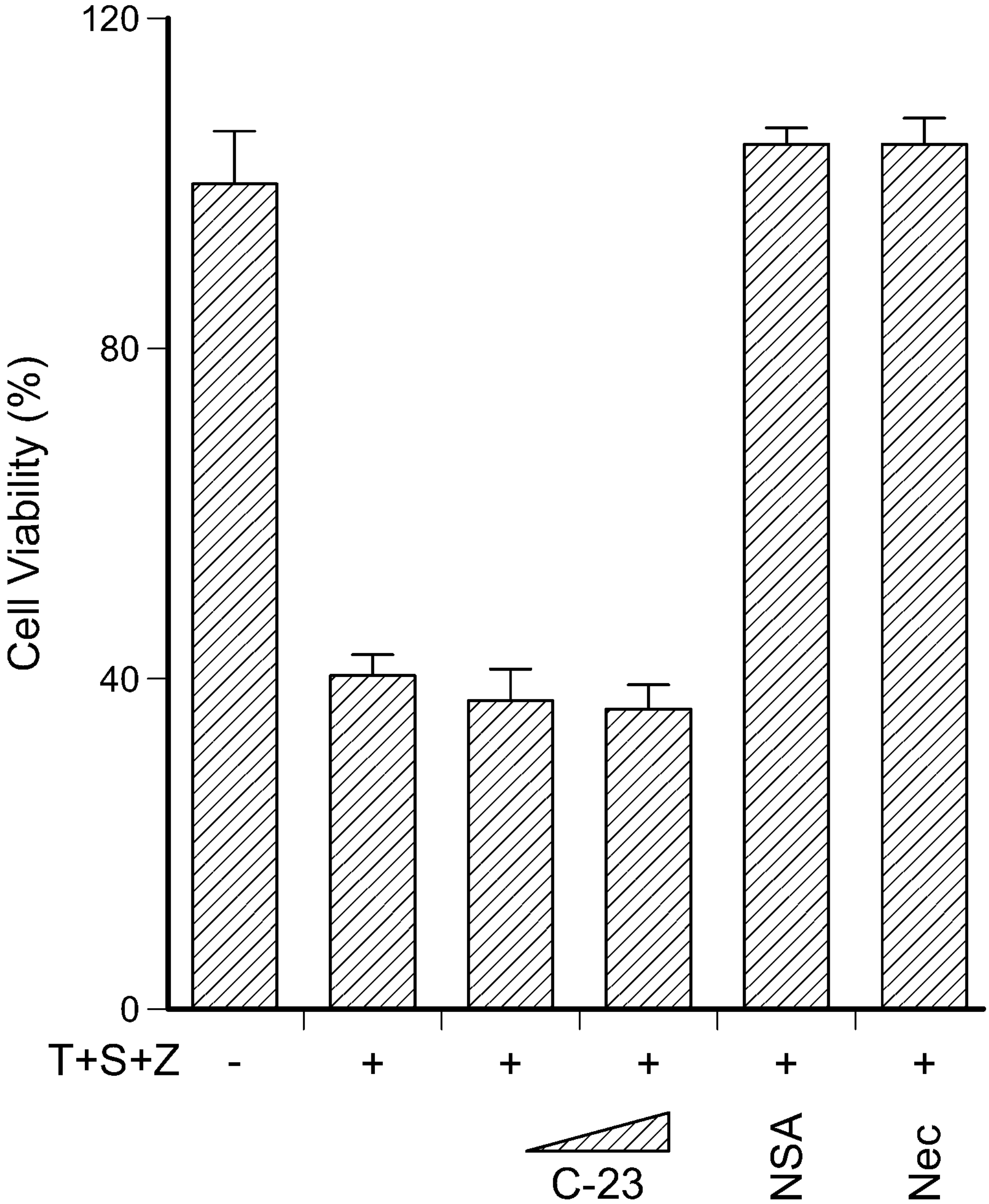
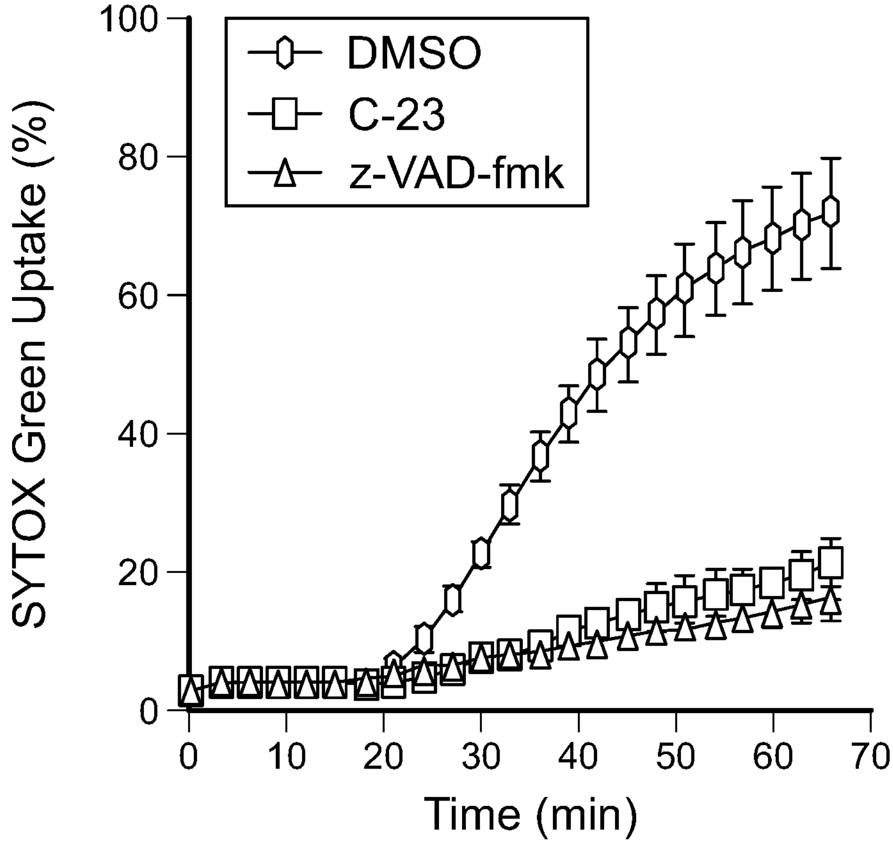
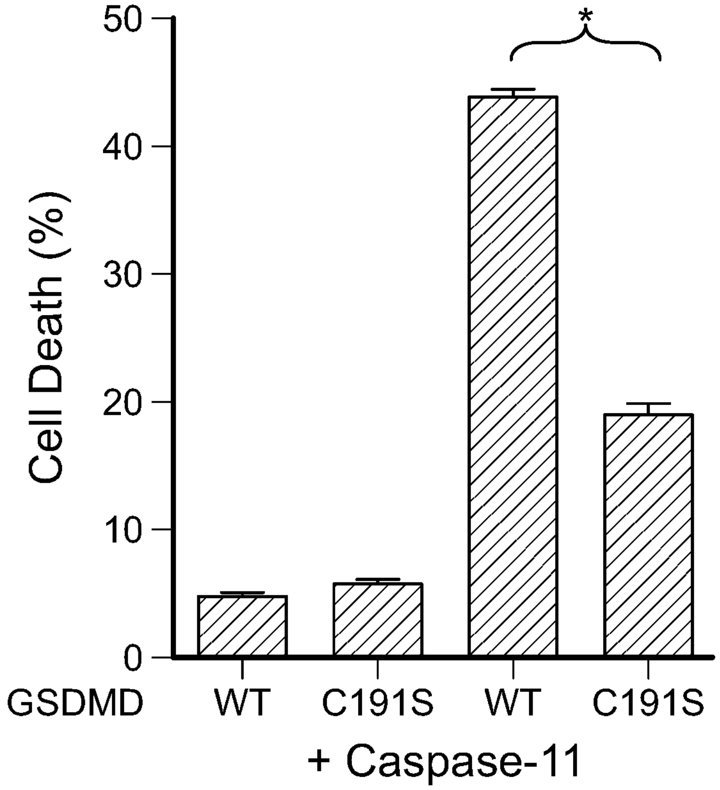


FIG. 69



100
80
60
40
20
0
SYTOX Green Uptake (%)
DMSO
C-23
z-VAD-fmk
0
10
20
30
40
50
60
70
Time (min)

FIG. 70



50
40
30
20
10
0
Cell Death (%)
*
GSDMD
WT
C191S
WT
C191S
+ Caspase-11

FIG. 71

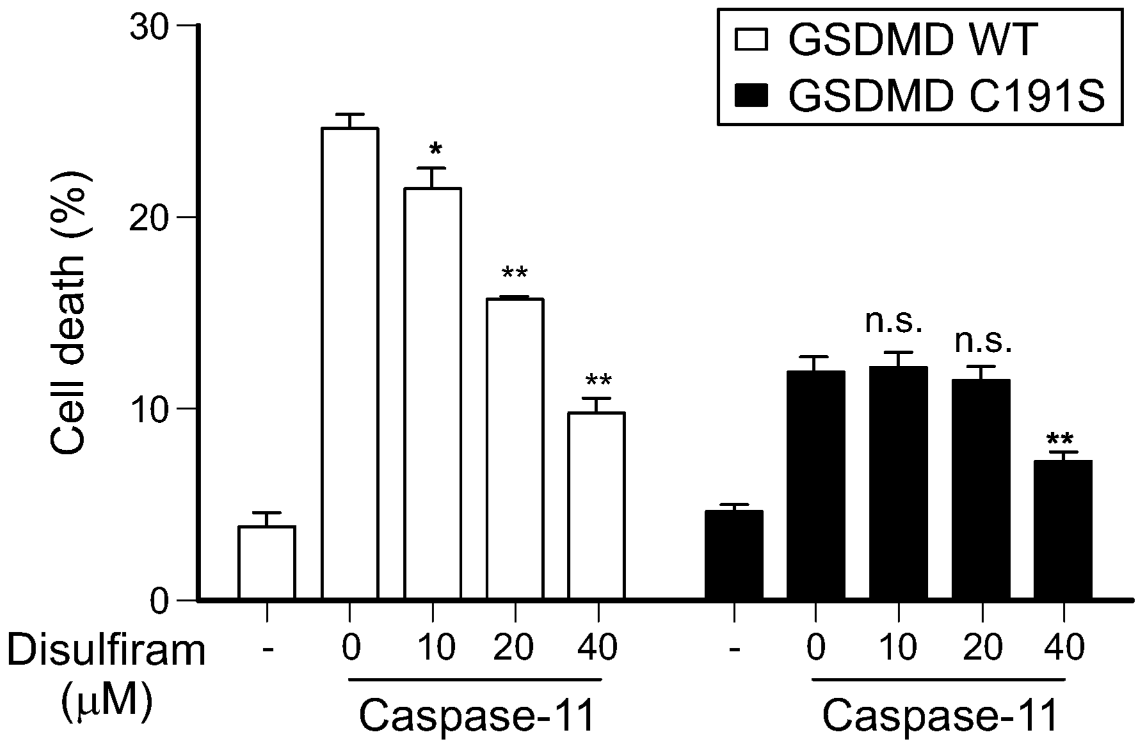

GSDMD WT
GSDMD C191S
Cell death (%)
30
20
10
0
*
**
**
n.s.
n.s.
**
Disulfiram (μM)
- 0 10 20 40
- 0 10 20 40
Caspase-11
Caspase-11

FIG. 72

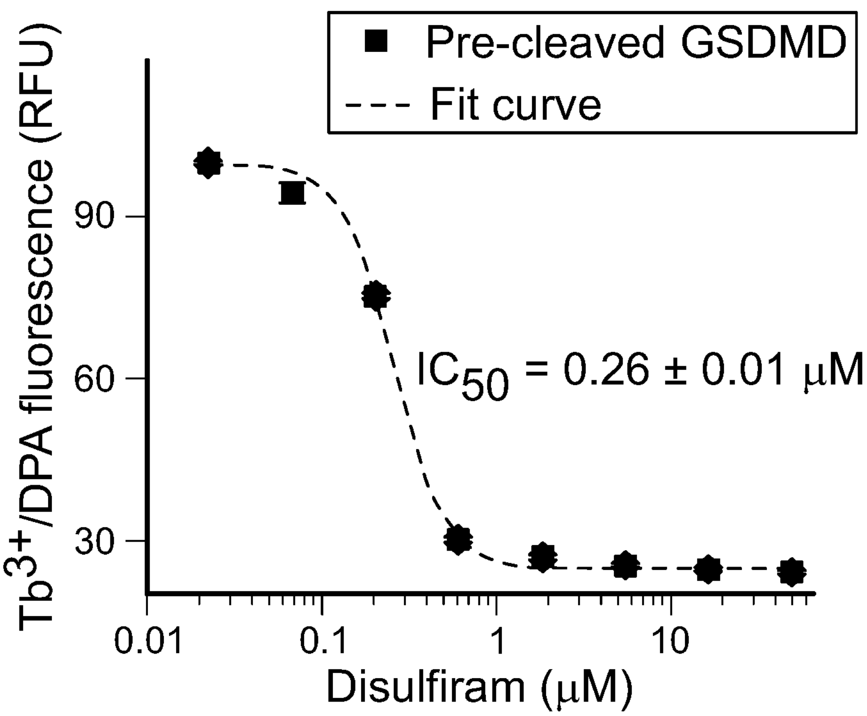

Pre-cleaved GSDMD
Fit curve
Tb3+/DPA fluorescence (RFU)
90
60
30
IC50 = 0.26 ± 0.01 μM
0.01
0.1
1
10
Disulfiram (μM)

FIG. 73

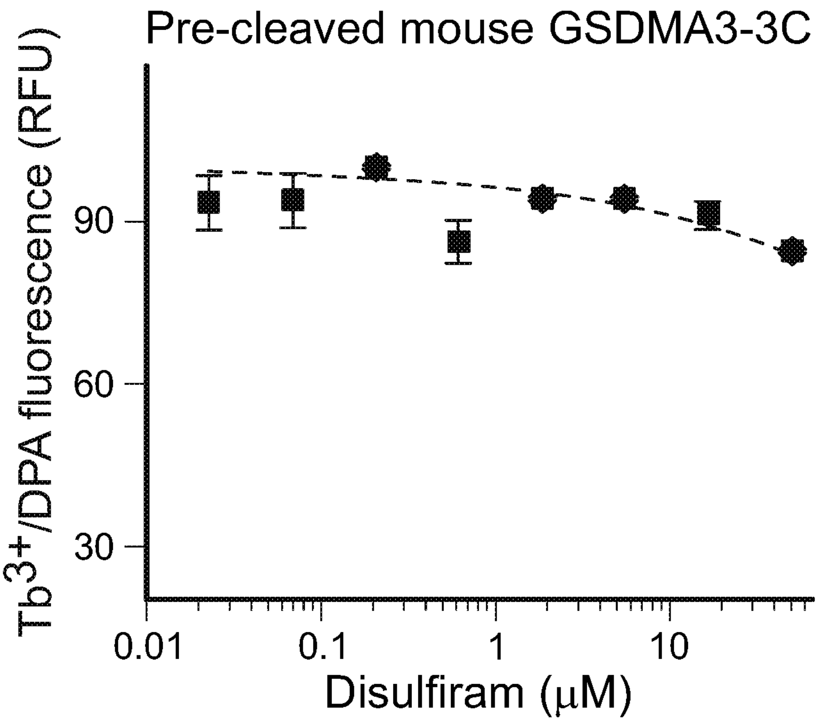

Pre-cleaved mouse GSDMA3-3C
Tb3+/DPA fluorescence (RFU)
90
60
30
0.01
0.1
1
10
Disulfiram (μM)

FIG. 74

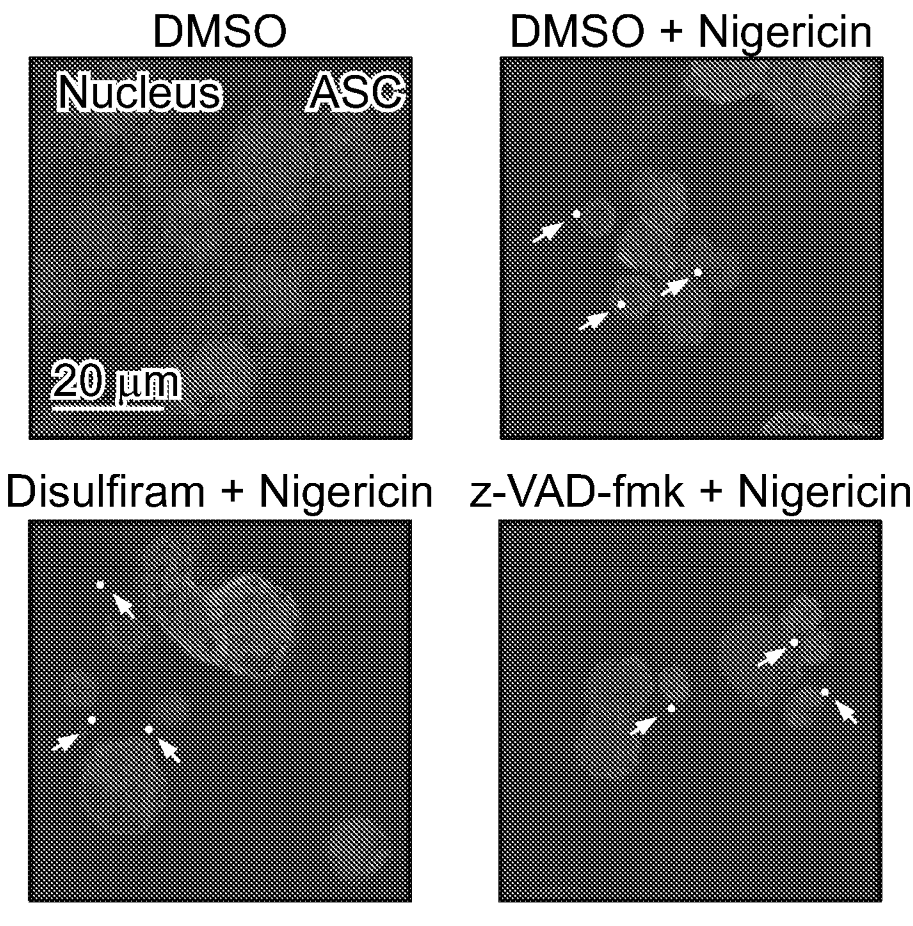
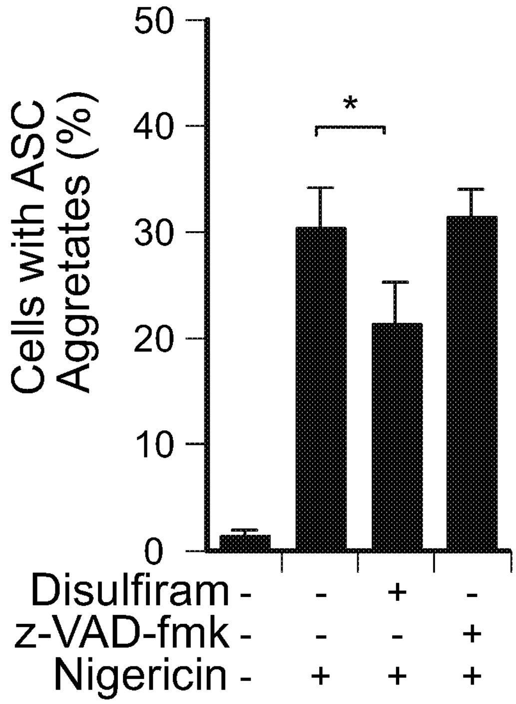
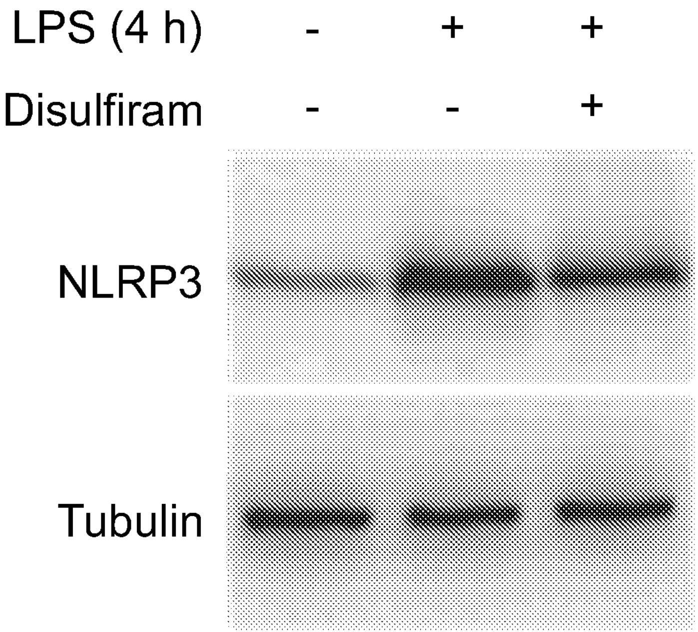

DMSO
DMSO + Nigericin
Nucleus
ASC
20 μm
Disulfiram + Nigericin
z-VAD-fmk + Nigericin

FIG. 75



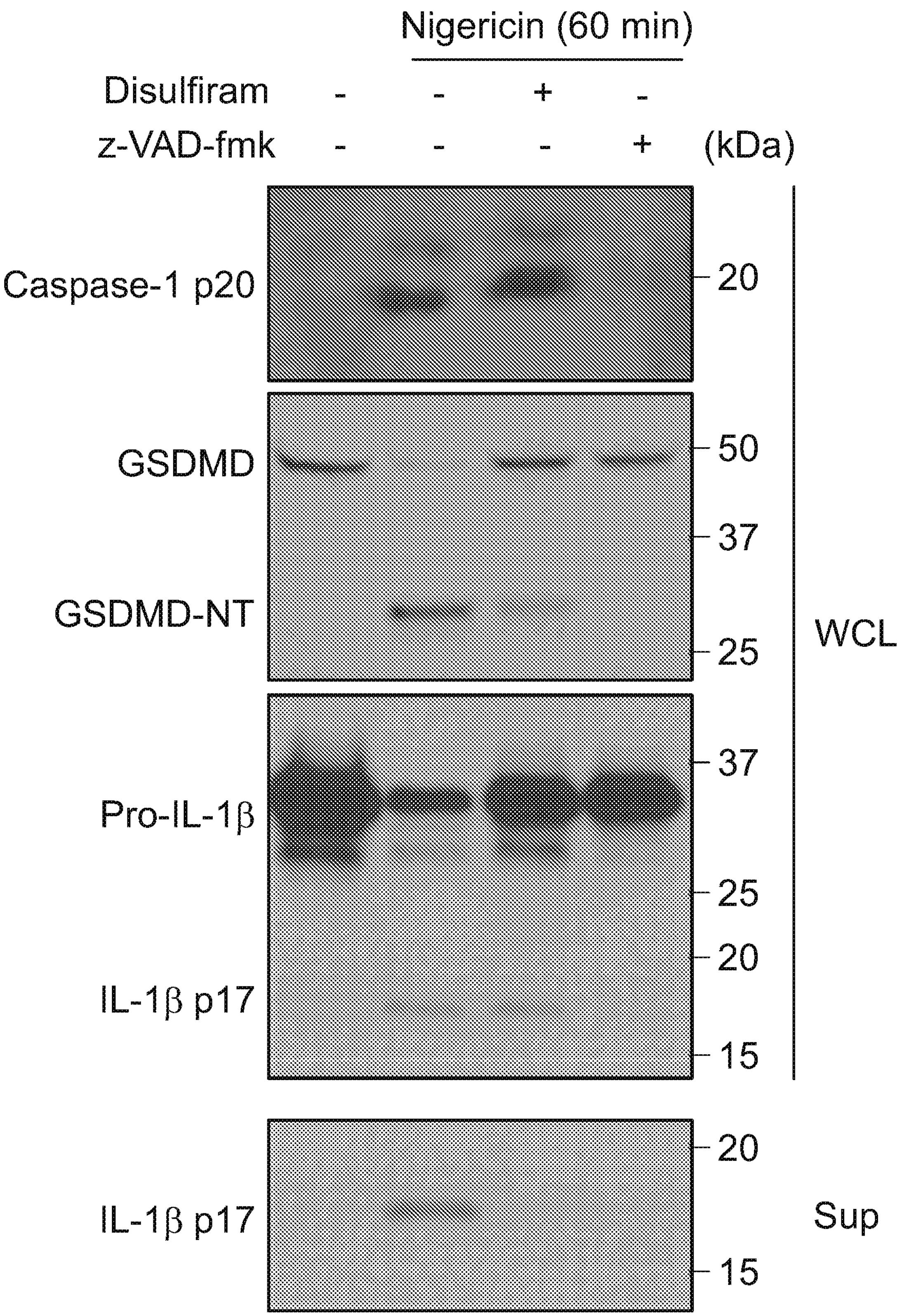
50
40
30
20
10
0
Cells with ASC Aggretates (%)
*
Disulfiram - - + -
z-VAD-fmk - - - +
Nigericin - + + +

FIG. 76



LPS (4 h) - + +
Disulfiram - - +
NLRP3
Tubulin

FIG. 77



Nigericin (60 min)
Disulfiram - - + -
z-VAD-fmk - - - +
(kDa)
Caspase-1 p20
20
GSDMD
50
37
GSDMD-NT
25
WCL
Pro-IL-1β
37
25
20
IL-1β p17
15
IL-1β p17
20
Sup
15

FIG. 78

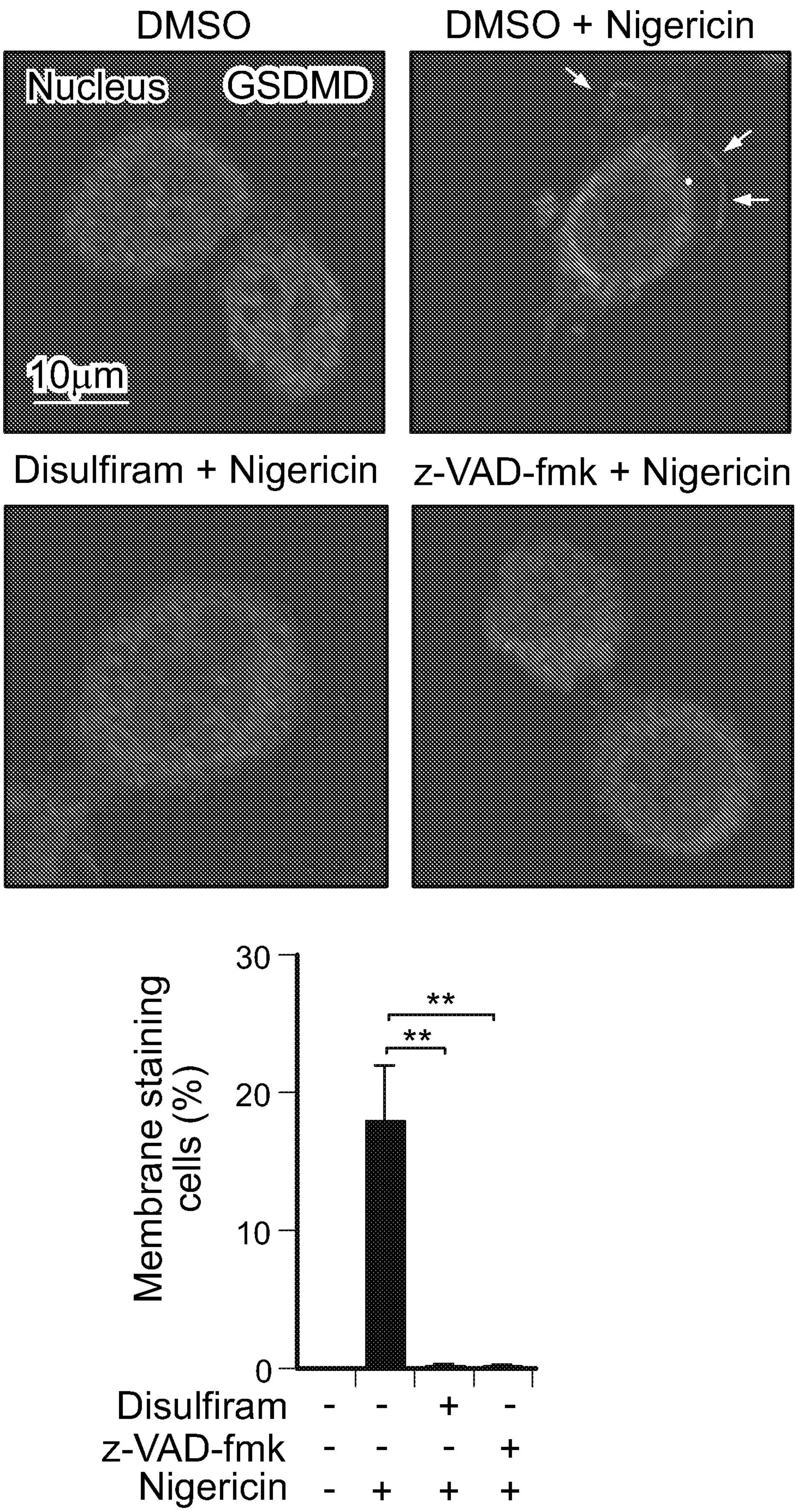

DMSO
DMSO + Nigericin
Nucleus
GSDMD
10μm
Disulfiram + Nigericin
z-VAD-fmk + Nigericin
Membrane staining cells (%)
30
20
10
0
**
**
Disulfiram - - + -
z-VAD-fmk - - - +
Nigericin - + + +

FIG. 79

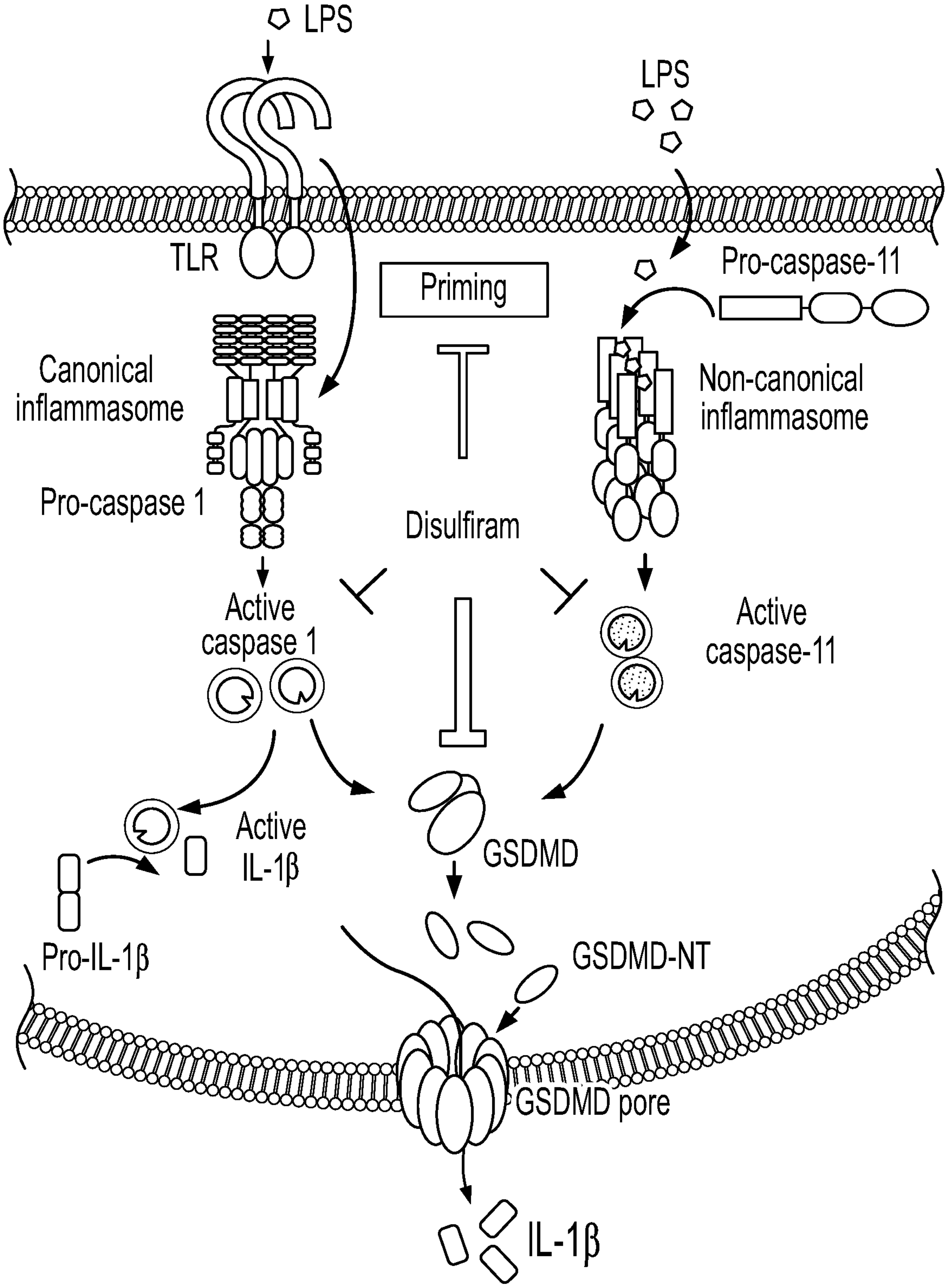

LPS
LPS
TLR
Priming
Pro-caspase-11
Canonical inflammasome
Non-canonical inflammasome
Pro-caspase 1
Disulfiram
Active caspase 1
Active caspase-11
Active IL-1β
GSDMD
Pro-IL-1β
GSDMD-NT
GSDMD pore
IL-1β

FIG. 80

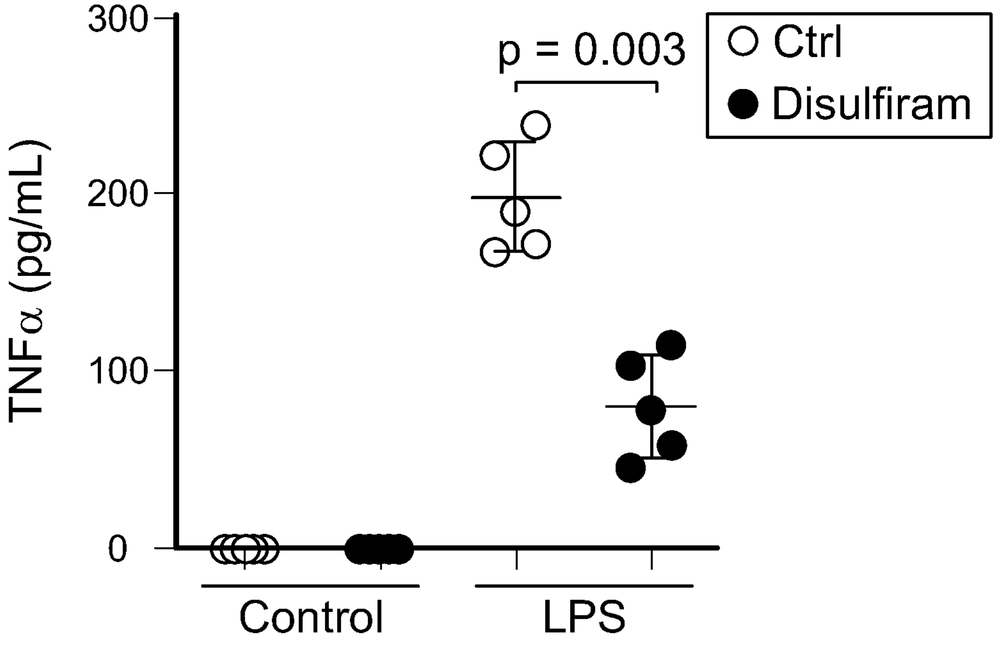

300
200
100
0
TNFα (pg/mL)
p = 0.003
Ctrl
Disulfiram
Control
LPS

FIG. 81

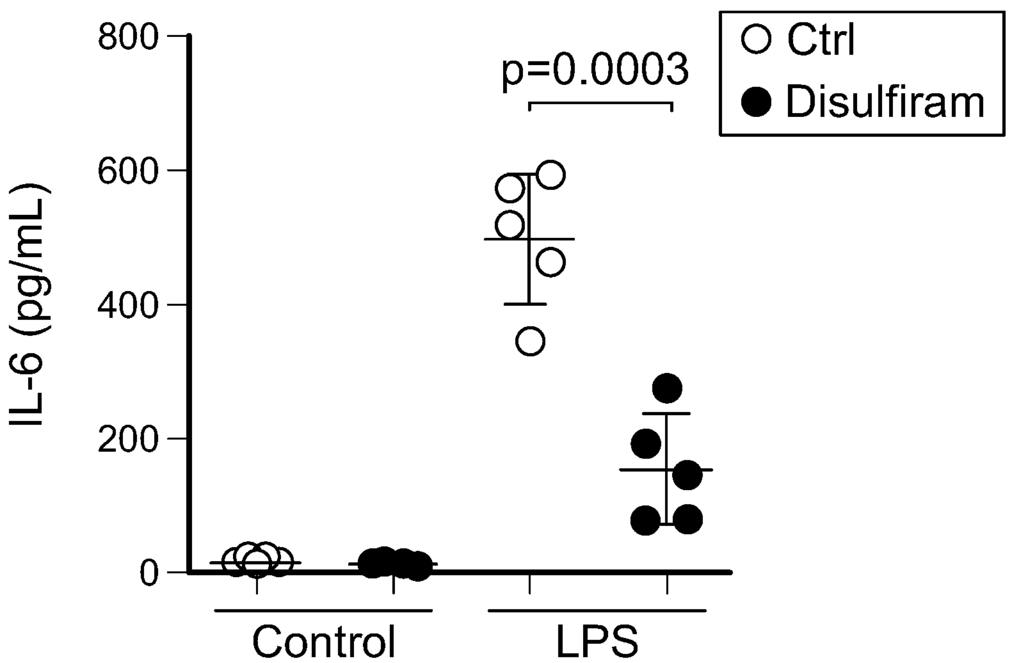
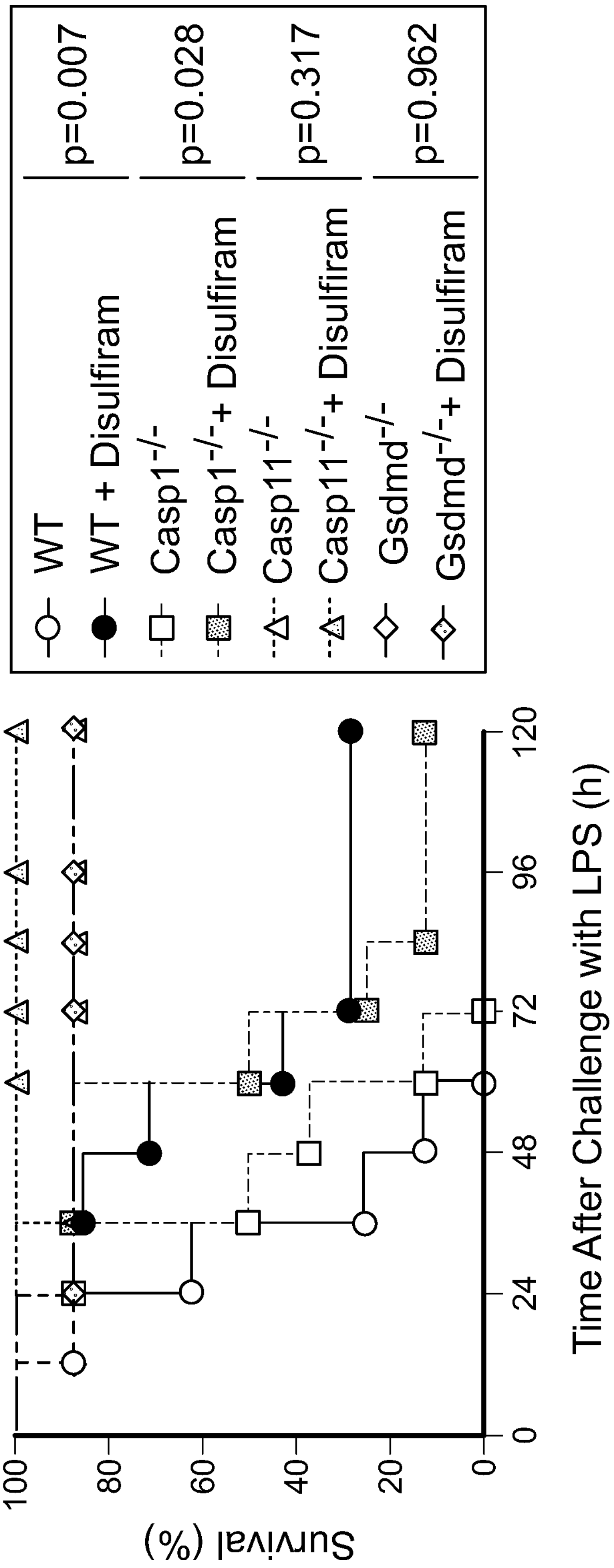
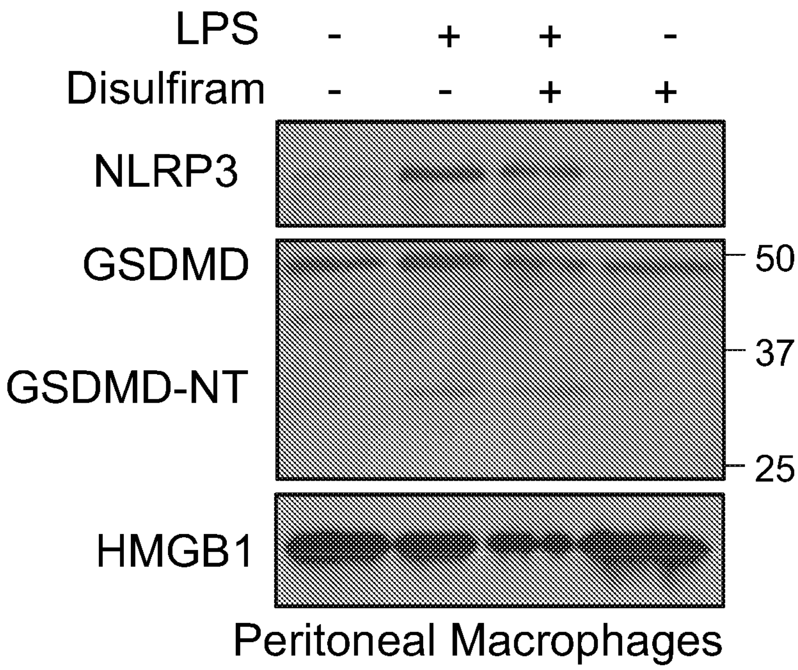

800
600
400
200
0
IL-6 (pg/mL)
p=0.0003
Ctrl
Disulfiram
Control
LPS

FIG. 82



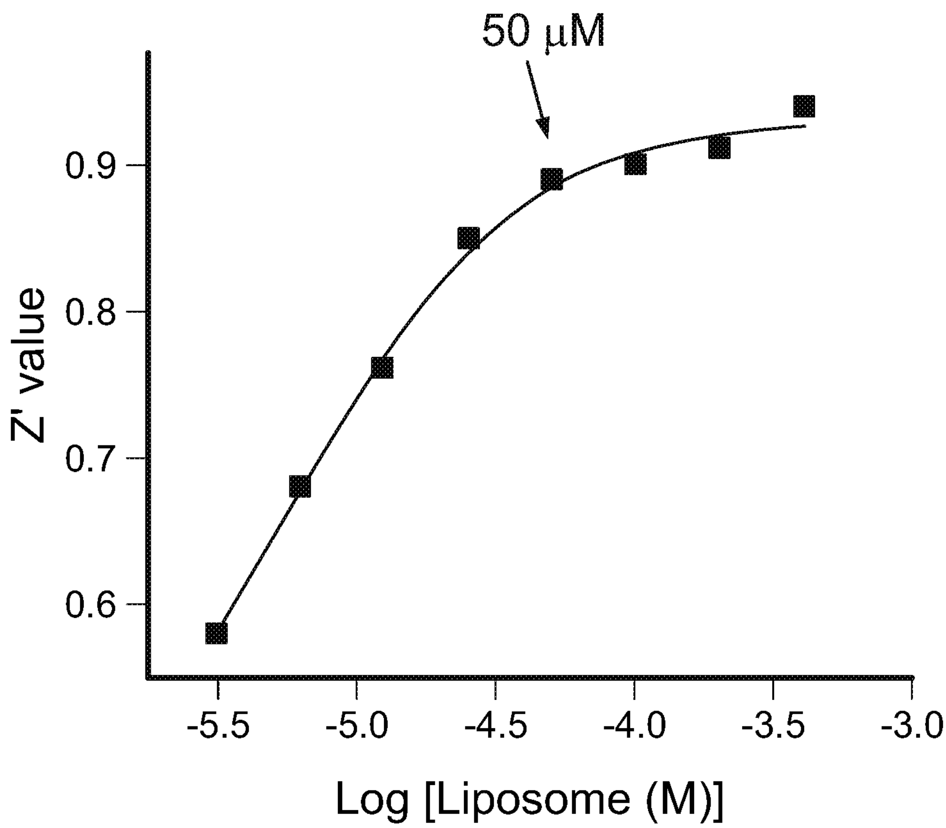
WT
WT + Disulfiram
p=0.007
Casp1$^{-/-}$
Casp1$^{-/-}$+ Disulfiram
p=0.028
Casp11$^{-/-}$
Casp11$^{-/-}$+ Disulfiram
p=0.317
Gsdmd$^{-/-}$
Gsdmd$^{-/-}$+ Disulfiram
p=0.962
Survival (%)
0
20
40
60
80
100
0
24
48
72
96
120
Time After Challenge with LPS (h)

FIG. 83



LPS - + + -
Disulfiram - - + +
NLRP3
GSDMD
GSDMD-NT
50
37
25
HMGB1
Peritoneal Macrophages

FIG. 84



50 μM
Z' value
0.9
0.8
0.7
0.6
-5.5
-5.0
-4.5
-4.0
-3.5
-3.0
Log [Liposome (M)]

FIG. 85

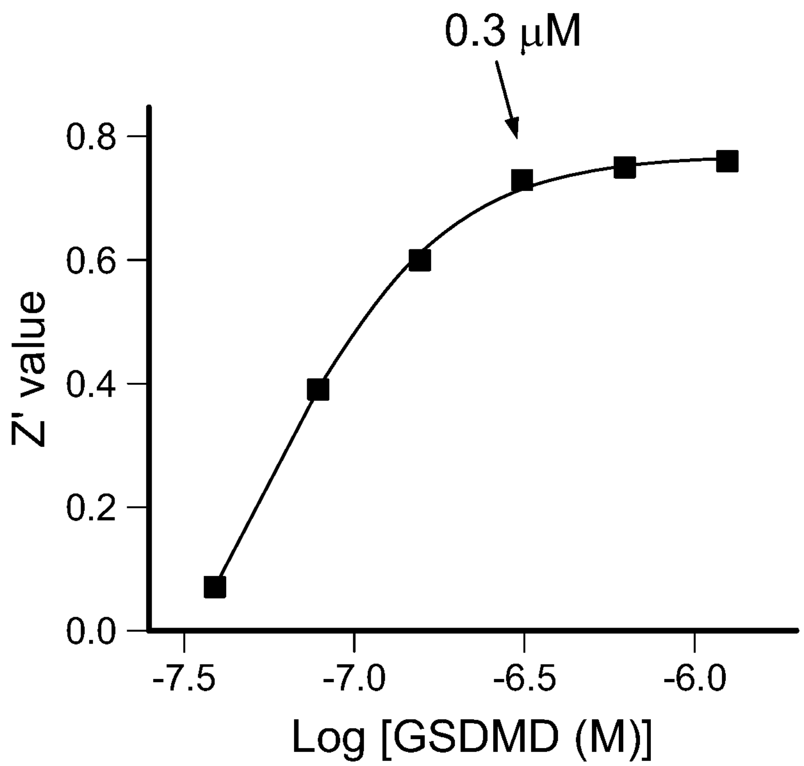

0.3 μM
Z' value
0.8
0.6
0.4
0.2
0.0
-7.5
-7.0
-6.5
-6.0
Log [GSDMD (M)]

FIG. 86

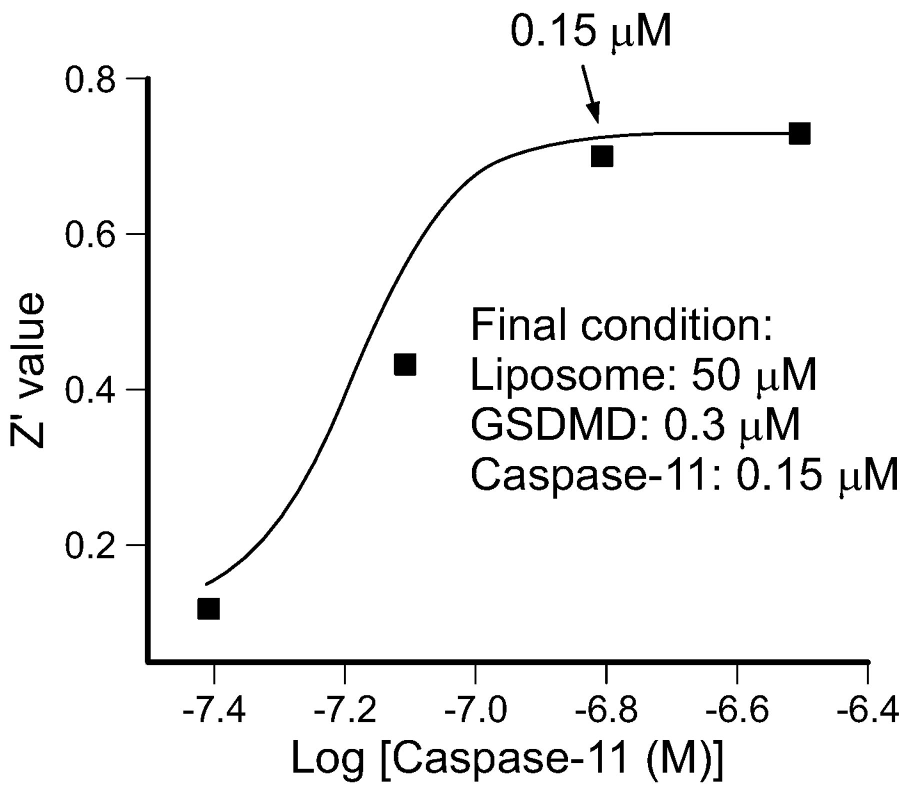

0.15 μM
Z' value
0.8
0.6
0.4
0.2
Final condition:
Liposome: 50 μM
GSDMD: 0.3 μM
Caspase-11: 0.15 μM
-7.4
-7.2
-7.0
-6.8
-6.6
-6.4
Log [Caspase-11 (M)]

FIG. 87

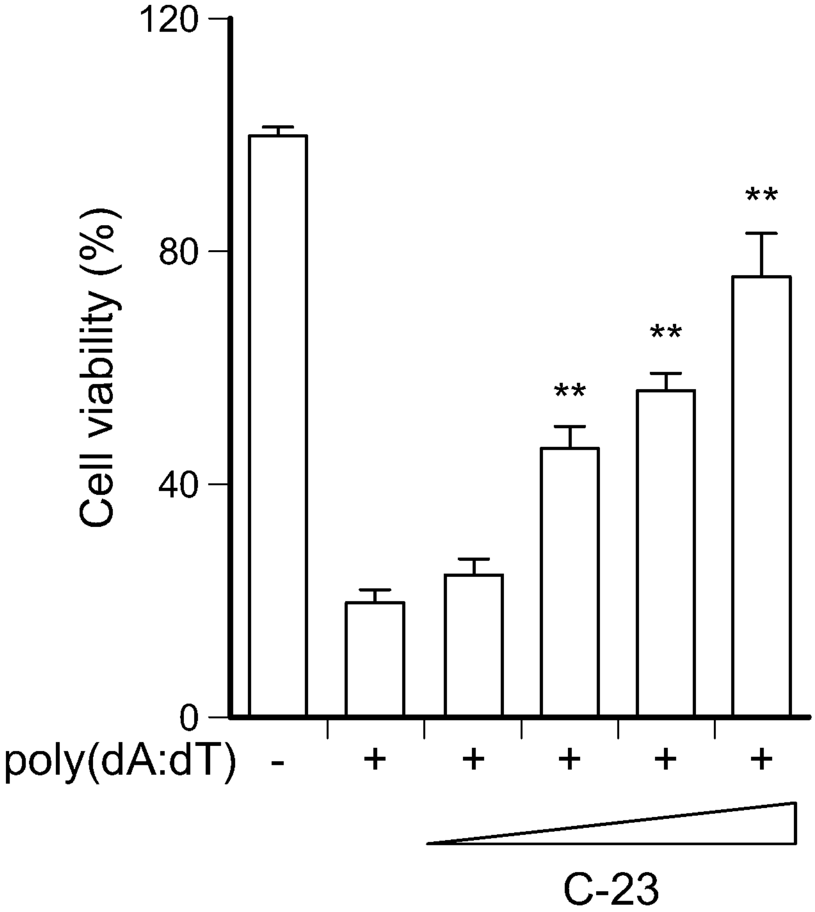


FIG. 88

```
                  .         .        .        .      .50
GSDMA3    1 .MPVFEDVTRALVRELN.PRGDLTPLDSLIDFKHFRPFCLVLRKRKSTLFWGARYVRTDYT
hGSDMA    1 .MTMFENVTRALARQLN.PRGDLTPLDSLIDFKRFHPFCLVLRKRKSTLFWGARYVRTDYT
mGSDMD    1 MPSAFEKVVKNVIKEVSGSRGDLIPVDSLRNSTSFRPYCLLNRKFSSSRFWKPRYSCVNLS
hGSDMD    1 MGSAFERVVRRVVQELD.HGGEFIPVTSLQSSTGFQPYCLVVRKPSSSWFWKPRYKCVNLS
                  .         .        .   Cys38.        .50      .

            .        .         .        .          .100      .
GSDMA3   60 LLDLLEPGSSPSDLTDSGNFSFKNMLDVQVQGLVEVPKT..VKVKGTAGLSQS..STLEVQ
hGSDMA   60 LLDVLEPGSSPSDPTDTGNFGFKNMLDTRVEGDVDVPKT..VKVKGTAGLSQN..STLEVQ
mGSDMD   62 IKDILFPSAPEPEPECFGSFKVSDVVDGNIQGRVMLSGMGEGKISGGAAVSDSSSASMNVC
hGSDMD   61 IKDILEPDAAEPDVQRGRSFHFYDAMDGQIQGSVELAAPGQAKIAGGAAVSDSSSTSMNVY
                  .        .         .         .100      .        .

                 .        .         .        .150        .        .
GSDMA3  117 TLSVAPSALENLKKERKLS.ADHSFLNEMRYHEKNLYVVMEAVEAKQEVTVEQTG..NANA
hGSDMA  117 TLSVAPKALETLQ.ERKLA.ADHPFLKEMQDQGENLYVVMEVVETVQEVTLERAGKAE..A
mGSDMD  123 ILRVTQKTWETMQHERHLQQPENKILQQLRSRGDDLFVVTEVLQTKEEVQITEVHSQEGSG
hGSDMD  122 SLSVDPNTWQTLLHERHLRQPEHKVLQQLRSRGDNVYVVTEVLQTQKEVEVTRTHKREGSG
                  .         .        .150        .         .        .

                .         .       .200         .        .        .
GSDMA3  175 IFSLPSLALLG..LQGSLNNNKAVTIPKGCVLAYRVRLLRVFLFNLWDIPYICNDSMQTFP
hGSDMA  174 CFSLPFFAPLG..LQGSINHKEAVTIPKGCVLAFRVRQLMVKGKDEWDIPHICNDNMQTFP
mGSDMD  184 QFTLPGALCLKGEGKGHQSRKKMVTIPAGSILAFRVAQL..LIGSKWDILLVSDEKQRTFE
hGSDMD  183 RFSLPGATCLQGEGQGHLSQKKTVTIPSGSTLAFRVAQL..VIDSDLDVLLFPDKKQRTFQ
                .Cys191   .200        .         .          .         .
```

FIG. 89

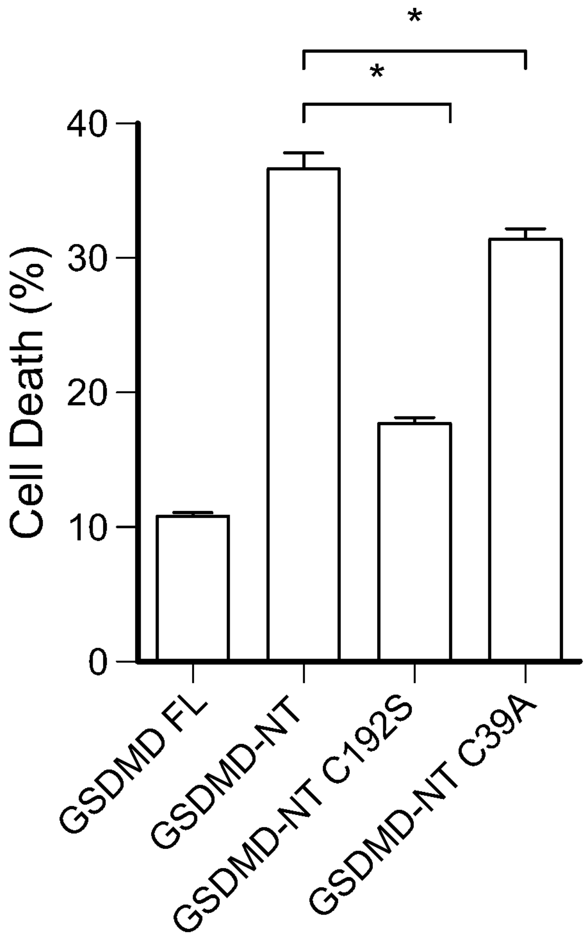

*
*
40
30
20
10
0
Cell Death (%)
GSDMD FL
GSDMD-NT
GSDMD-NT C192S
GSDMD-NT C39A

FIG. 90

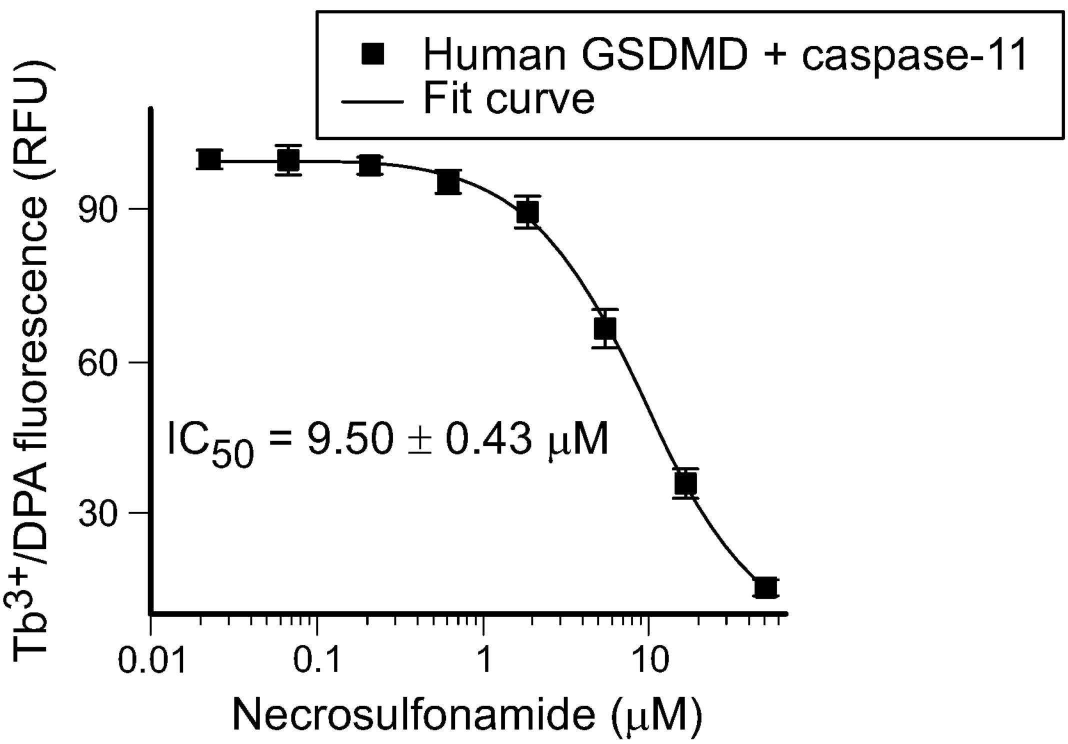
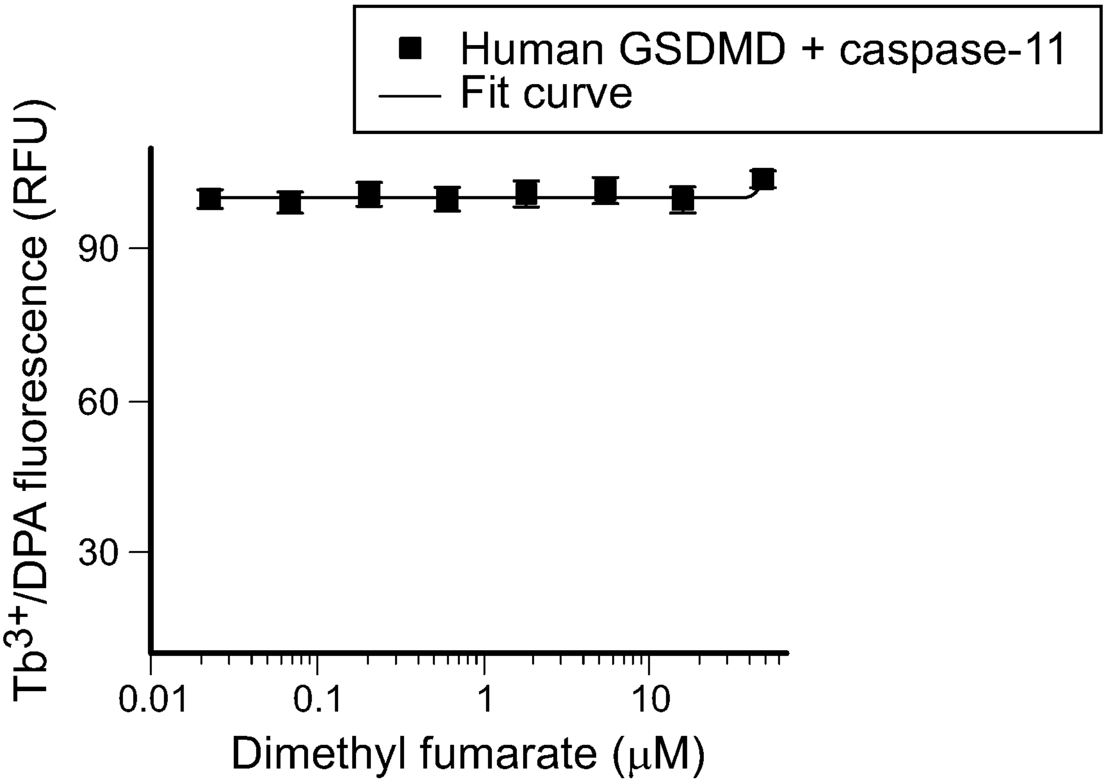
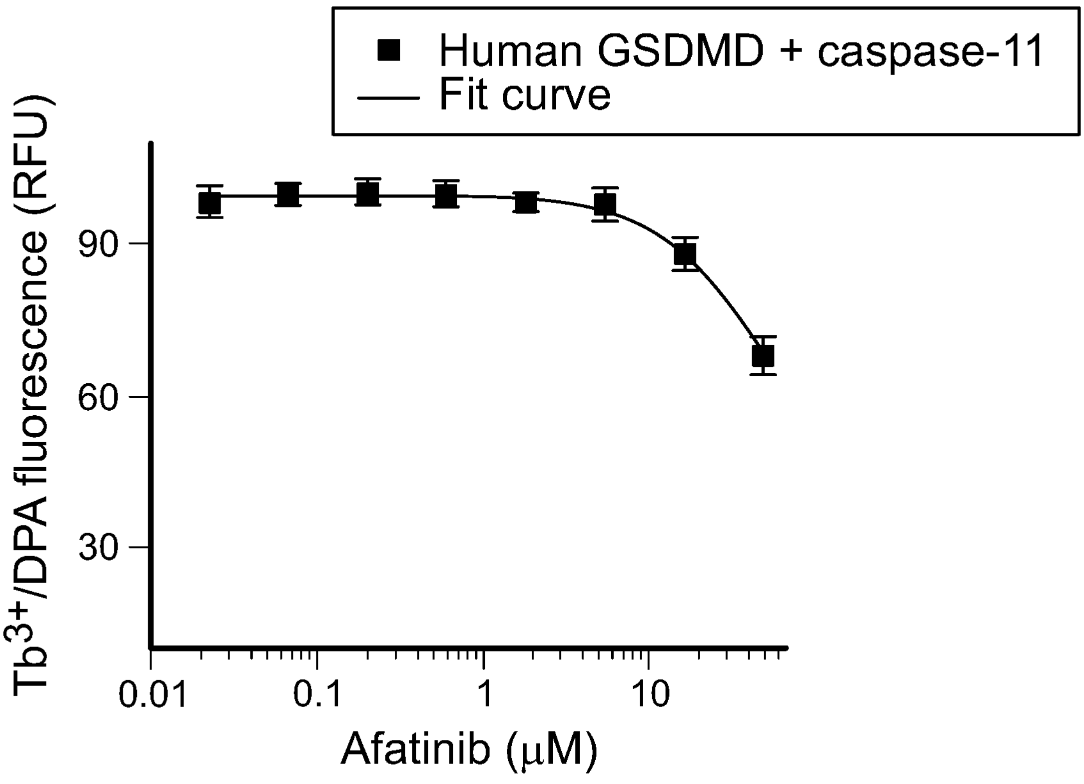

Human GSDMD + caspase-11
Fit curve
Tb3+/DPA fluorescence (RFU)
90
60
30
IC50 = 9.50 ± 0.43 μM
0.01
0.1
1
10
Necrosulfonamide (μM)

FIG. 91



Human GSDMD + caspase-11
Fit curve
Tb3+/DPA fluorescence (RFU)
90
60
30
0.01
0.1
1
10
Dimethyl fumarate (μM)

FIG. 92



Human GSDMD + caspase-11
Fit curve
Tb3+/DPA fluorescence (RFU)
90
60
30
0.01
0.1
1
10
Afatinib (μM)

FIG. 93

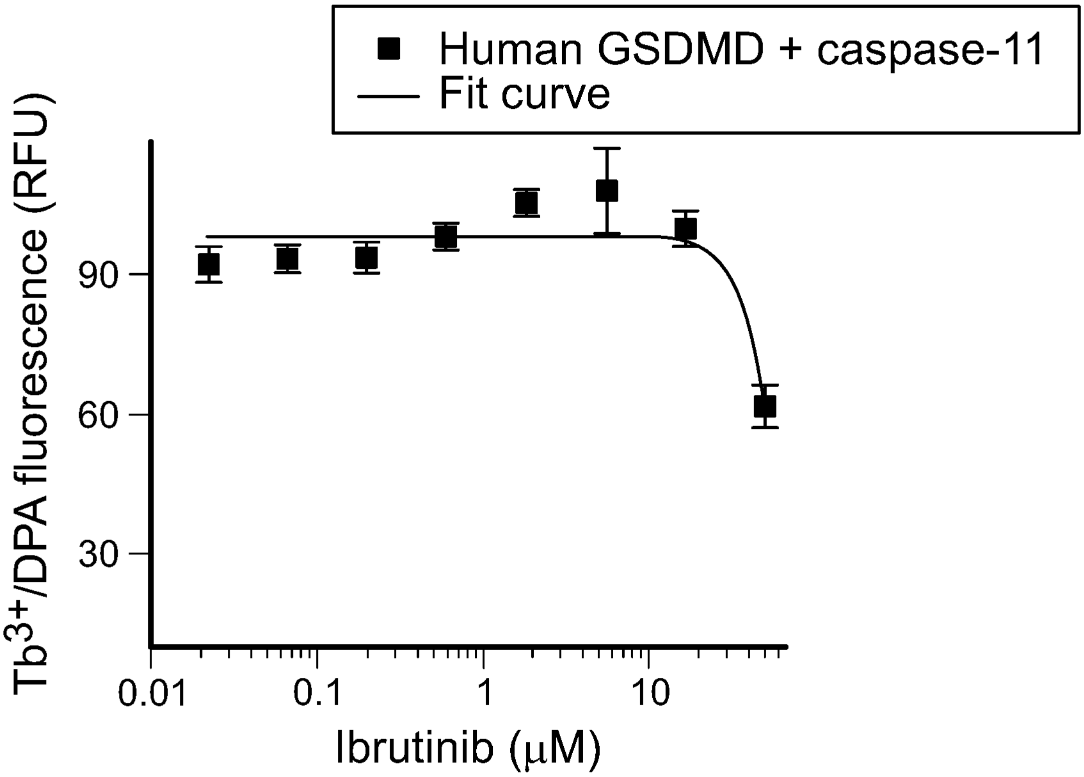

Human GSDMD + caspase-11
Fit curve
Tb3+/DPA fluorescence (RFU)
90
60
30
0.01
0.1
1
10
Ibrutinib (μM)

FIG. 94

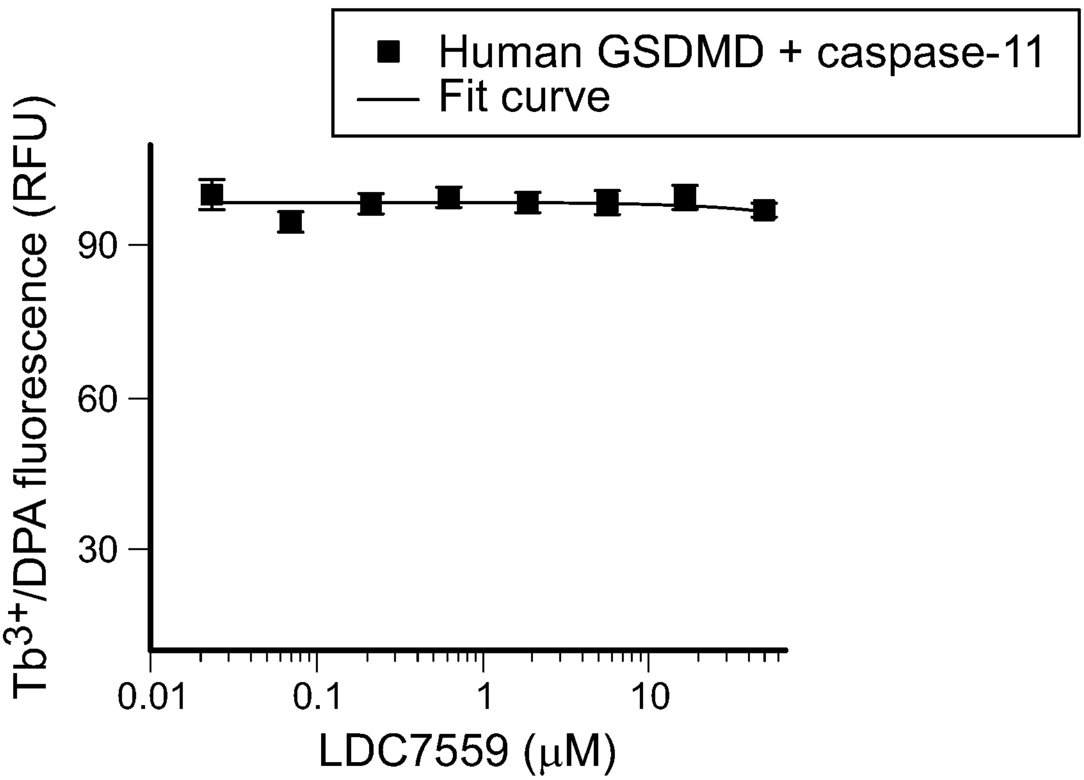

Human GSDMD + caspase-11
Fit curve
Tb3+/DPA fluorescence (RFU)
90
60
30
0.01
0.1
1
10
LDC7559 (μM)

FIG. 95

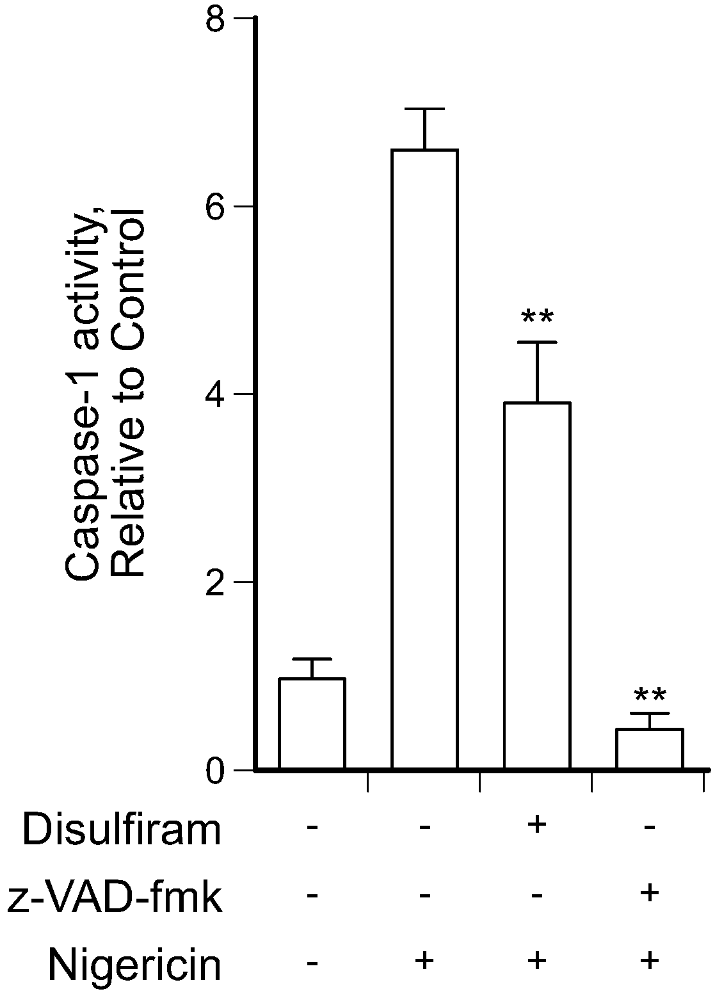

Caspase-1 activity, Relative to Control
8
6
4
2
0
**
**
Disulfiram - - + -
z-VAD-fmk - - - +
Nigericin - + + +

FIG. 96

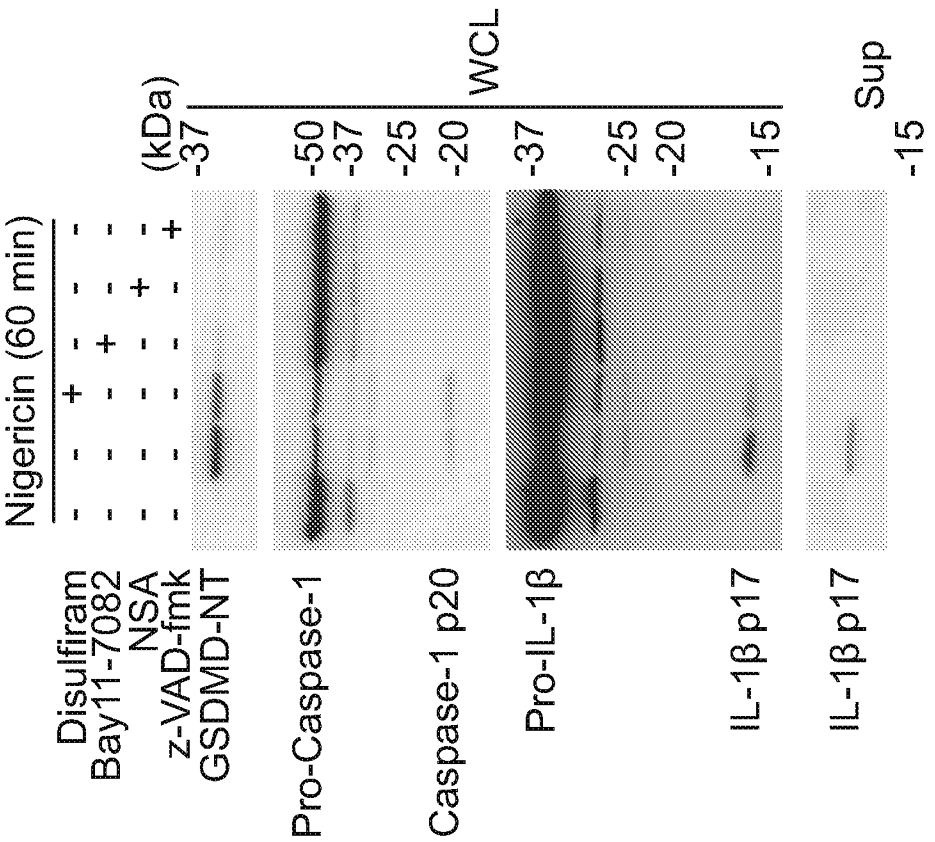

Nigericin (60 min)
Disulfiram
Bay11-7082
NSA
z-VAD-fmk
GSDMD-NT
Pro-Caspase-1
Caspase-1 p20
Pro-IL-1β
IL-1β p17
IL-1β p17
(kDa)
-37
-50
-37
-25
-20
-37
-25
-20
-15
-15
WCL
Sup

FIG. 98

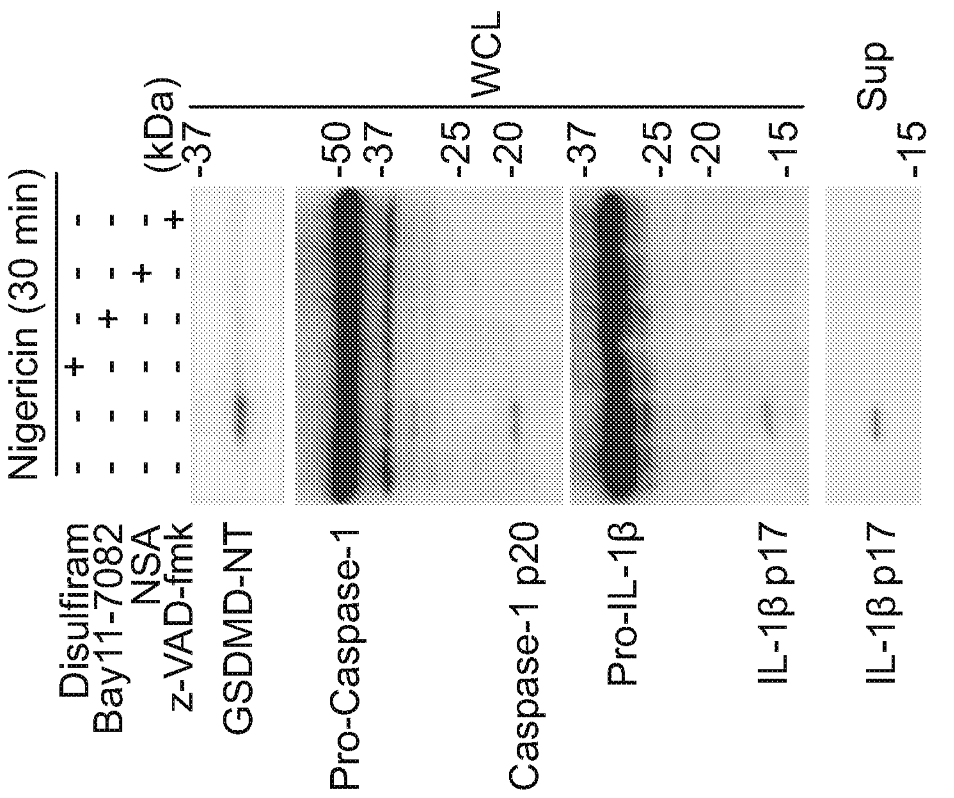

Nigericin (30 min)
Disulfiram
Bay11-7082
NSA
z-VAD-fmk
GSDMD-NT
Pro-Caspase-1
Caspase-1 p20
Pro-IL-1β
IL-1β p17
IL-1β p17
(kDa)
-37
-50
-37
-25
-20
-37
-25
-20
-15
-15
WCL
Sup

FIG. 97