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(54) **Title:** S-NITROSYLATION OF GLUCOSYLATING TOXINS AND USES THEREFOR

(57) **Abstract:** Provided herein are methods for ameliorating the pathophysiology cysteine protease exotoxin comprising the step of administering to said individual an effective dose of an S-nitrosylating agent and an inositol phosphate or analog thereof.



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PATENT APPLICATION

FOR

S-NITROSYLATION OF GLUCOSYLATING TOXINS AND USES THEREFOR

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PRIORITY CLAIM

[001] This application claims priority to U.S. Provisional Patent Application serial number 61/519,852 filed May 31, 2011, which is incorporated herein by reference in its entirety.

STATEMENT REGARDING FEDERALLY FUNDED RESEARCH

[002] This invention was made with government support under R01AI088748, N01AI30050, R01DK084509, K01DK076549, R21-DK078032-01, R01-HL059130, R01-HL091876, R01HL095463, HL075443-06A and 1UL1RR029876-01 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND

[003] The present invention relates generally to the fields of microbiology, pharmacology, and medicine. More specifically, the present invention relates to allosteric therapeutics for microbial infections and associated glucosylating toxins.

[004] *Clostridium difficile* (*C. difficile*) infection (CDI) is the most prevalent cause of hospital-acquired infectious diarrhea and life-threatening colitis worldwide (Kelly et al., *N Engl J Med* 359:1932-1940 (2008)). Two large exotoxins, TcdA (308 kDa) and TcdB (270 kDa), are secreted from the majority of *C. difficile* bacterial strains that cause disease in humans, and there is little ambiguity that these toxins are pathogenic since toxin-deficient strains are avirulent (Savidge et al., *Gastroenterology* 125:413-20 (2003); Lyras et al. *Nature*

458:1176-79 (2009); Kuehne et al., *Nature* 467:711-13 (2010)). The clostridial glucosylating toxins and the multifunctional autoprocessing repeats-in-toxins (MARTX) share a common virulence mechanism for cell entry that represents a potential target for therapeutic intervention (Fullner et al., *Infect Immun* 75: 5079-84 (2007); Sheahan et al., *EMBO J* 26: 2552-61 (2007); Egerer et al., *J Biol Chem* 282: 25314-21 (2007); Pei et al., *Protein Science* 18: 856-62 (2009)). Cellular internalization of these exotoxins is dependent on cytosolic inositol hexakisphosphate (InsP₆) allosteric cofactor, which activates an autocatalytic cysteine protease domain to facilitate toxin self-cleavage (Reineke et al., *Nature* 446:415-19 (2007); Prochazkova et al., *J Biol Chem* 283: 23656-64 (2008); Lupardus et al., *Science* 322:265-68 (2008); Egerer et al., *J Biol Chem* 284:3389-95 (2009)). Intracellular release of the smaller N-terminus glucosyltransferase effector domain results in the mono-O glucosylation of small GTPases of the Rho family, including RhoA, Rad, and Cdc42 (Popoff et al., *Biochim Biophys Acta*. 88:797-812 (2009)). Glucosylation of Rho proteins inhibits their molecular switch function, thus blocking Rho GTPase-dependent signaling in intestinal epithelial cells, leading to alterations in the actin cytoskeleton, fluid secretion, acute inflammation, and necrosis of the colonic mucosa.

[005] Host defense mechanisms that might be employed to protect against the clostridial glucosylating toxins are not well defined, although *C. difficile* toxins are potent inducers of nitric oxide (NO), which is known to be protective against these toxins (Ng et al., *Gastroenterology* April 13 (2010)). However, the precise molecular mechanism for the protective effects of NO remains unknown. Some diverse signaling cascades associated with NO production are attributed to S-nitrosothiol (SNO) species that act via covalent modification of specific cysteine residues in target molecules (S-nitrosylation)(Hess, et al., *Nature Rev* 6:150-166 (2005)) and aberrant S-nitrosylation may play a role in disease-etiology (MW et al., *Trends Mol Med* 15: 391-404 (2009)).

[006] There is a recognized need in the art for alternative therapies for infections by toxins such as *Clostridium difficile*. Specifically, the prior art is deficient in treatments for glucosylating toxin infections. The present invention fulfills this long-standing need and desire in the art.

SUMMARY

[007] Certain embodiments are directed to S-nitrosylation of pathologic cysteine proteases, such as autocatalytic exotoxins (e.g., CGTs, gingipains, CPD_{MARTX}, and CPD_{adh}). In certain aspects, the methods and compositions described herein provide protection against or ameliorate the effects of cellular intoxication by pathogenic cysteine proteases. Studies using *C. difficile*, provide evidence for cross-talk between inositol phosphate cofactors and NO signaling. In certain aspects, compositions and methods described herein use inositol phosphates to enhance S-nitrosothiol action as a therapeutic for pathologies associated with various pathogenic cysteine proteases. Pathologies include bacterial induced colitis and inflammation. In certain aspects, the methods and composition described herein provide for a therapeutic allostery against pathologic cysteine proteases, such as the clostridial small molecule-activated glucosylating toxins (CGTs).

[008] Pathogenic cysteine proteases are autocatalytic polypeptides that contain a cysteine protease domain (CPD). Cleavage of the polypeptide releases one or more polypeptide domains that result in one or more pathologic conditions in a subject (e.g., toxic shock, diarrhea, colitis, inflammation, and the like). Amino acids 576 to 756 of SEQ ID NO:1 (*C. difficile* TcdA) provide one example of a CPD, other similar domains can be identified by using various search and analysis algorithms. Other target polypeptides can have a CPD domain that is 30, 40, 50, 60, 70, 80, 90, 95, or 100% identical to the CPD of TcdA.

[009] Certain embodiments are directed to methods for ameliorating the pathophysiology of a microbial cysteine protease exotoxin in a subject comprising, administering to the subject an effective dose of an S-nitrosylating agent and an inositol phosphate or analog thereof. In certain aspects, the exotoxin is a *Clostridium* exotoxin, such as a *Clostridium difficile*, *Clostridium sordellii*, *Clostridium novyi*, *Clostridium botulinum*, *Clostridium perfringens*, or *Clostridium tetani* exotoxin. In a further aspect the exotoxin can be *Vibrio cholera* RTX, gingipain, CPD_{MARTX}, or CDP_{adh} exotoxin.

[010] In certain embodiments the inositol phosphate can be a hexakisphosphate, inositol pyrophosphate or analog thereof. The inositol phosphate or analog thereof can be

administered in an amount of about 1, 10, 20, 30, 40, 50, 60, 70, 80, 90 μ M to about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 mM, including all values and ranges there between.

[011] S-nitrosylation is a post-translational modification of a protein that is mediated by reaction with nitric oxide. As used herein the term “nitrosylation” refers to the addition of NO to a thiol group (SH), oxygen, carbon or nitrogen by chemical means. An “S-nitrosylating agent” refers to a compound that can function in vivo to react with protein thiol groups, transferring a NO group to the thiol to form an S-nitrosothiol. Suitable nitrosylating agents are disclosed in Feelisch and Stamler, “Donors of Nitrogen Oxides”, Methods in Nitric Oxide Research edited by Feelisch and Stamler, (John Wiley & Sons) (1996), the entire teachings of which are hereby incorporated into this application by reference. S-nitrosylating agents include nitrosylators that directly nitrosylate (e.g., GSNO), potentiators that potentiate nitrosylation (e.g., N-acetyl cysteine (NAC)), and nitric oxide generators that produce nitrosylators (nitrite plus GSH or NAC), as well as esters and/or salts thereof. S-nitrosylating agents include acidic nitrite, alkyl nitrate, nitrosyl chloride, ethyl nitrite, amyl nitrite, glutathione (GSH), S-nitrosogluthathione (GSNO), S-nitrosocysteinyl glycine, S-nitrosocysteine, N-acetyl cysteine, S-nitroso-N-acetyl cysteine, nitroglycerine, nitroprusside, nitric oxide, S-nitrosohemoglobin, S-nitrosoalbumin, S-nitroso-N-acetylpenicillamine, S-nitrosocysteine, S-nitroso-gamma-methyl-L-homocysteine, S-nitroso-L-homocysteine, S-nitroso-gamma-thio-L-leucine and S-nitroso- δ -thio-L-leucine and S-nitrosoalbumin. In certain aspects, the S-nitrosylating agent is a phytocheletin, i.e., a glutathione oligomer. In certain aspects, the S-nitrosylating agent comprises (a) a nitrate and (b) glutathione and/or N-acetylcysteine. In a further aspect the nitrate is an organic nitrate, such as ethyl nitrite or amyl nitrite. In certain aspects, the S-nitrosylating agent is administered in an amount of about 1, 10, 20, 30, 40, 50, 60, 70, 80, 90 μ M to about 0.1, 0.5, 1, 2, 3, 4, 5 mM, including all values and ranges there between.

[012] Certain embodiments are directed to methods of treating a *Clostridium* infection in a subject comprising administering to the subject an effective dose of an S-nitrosylating agent and an inositol phosphate or analog thereof.

[013] Other embodiments are directed to methods of reducing or preventing the effects of a pathogenic cysteine protease in a subject. In certain aspects, the subject has an infection,

wherein the infection results in the production of pathogenic cysteine proteases. In a further aspect the cysteine protease is a glucosylating toxin. Certain embodiments are directed to methods of treating a subject that include administering to a subject an effective amount of an inositol phosphate and an S-nitrosylating agent, wherein a pathogenic cysteine protease is inactivated by S-nitrosylation.

[014] Further embodiments are directed to methods of treating a subject having an infection that produces a glucosylating toxin, comprising the steps of administering to a subject an effective dose of (a) an agent that causes or increases S-nitrosylation of the toxin and (b) an inositol phosphate or analog thereof.

[015] Certain embodiments are directed to pharmaceutical compositions comprising an S-nitrosylating agent and an inositol phosphate or analog thereof. In certain aspects, the inositol phosphate is a hexakisphosphate, or inositol pyrophosphate. In a further aspect, the S-nitrosylating agent is S-nitrosoglutathione, nitrite, glutathione, N-acetylcysteine, a GSNO reductase inhibitor, N-acetylcysteine, and/or phytocheletins.

[016] In certain aspects, the S-nitrosylating agent and the inositol phosphate or analog thereof are comprised in the same pharmaceutical composition. In certain aspects, the composition is formulated for delivery to the gastrointestinal tract. In a further aspect, the composition can be formulated for delivery of the active ingredients to the mouth, throat, esophagus, stomach, small intestine, ileum, jejunum, duodenum, large intestine, cecum, ascending colon, transverse colon, descending colon, sigmoid colon, or rectum.

[017] In certain aspects, a pharmaceutical composition is a sustained release composition comprising an agent that causes or increases S-nitrosylation of a glucosylating toxin; and an inositol phosphate. Most preferably, the composition is formulated for release in gastrointestinal tract, e.g., the mouth, esophagus, stomach, the small intestine, the ileum, the jejunum, the duodenum, the large intestine, the cecum, the ascending colon, the transverse colon, the descending colon, the sigmoid colon or the rectum. In one preferred aspect, the agent is S-nitrosoglutathione (GSNO).

[018] In one embodiment of the present invention, there is provided a method of treating infection in an individual associated with a glucosylating toxin, comprising the step of

administering to said individual an effective dose of an agent that causes or increases S-nitrosylation of said toxin. In one preferred aspect, the agent is S-nitrosoglutathione. The S-nitrosoglutathione may be administered in an effective concentration depending upon the result desired, for example, of about 1 μ M to about 1 mM. Preferably, this agent activates or enhances local GSNO concentrations.

[019] In one aspect, the toxin is from a member of the genus *Clostridium*. Representative species in the genus *Clostridium* include but are not limited to *Clostridium difficile*, *Clostridium sordellii*, *Clostridium novyi*, *Clostridium botulinum*, *Clostridium perfringens* and *Clostridium tetani*. In another aspect, the toxin is *Vibrio cholerae* CPD_{RTX}, the gingipains and structurally similar cysteine regulated domains belonging to the CPD_{MARTX} and CPD_{adh} superfamilies.

[020] The compositions and methods described herein can be used to neutralize a toxin either *in vitro* or *in vivo*. For example, the toxins, or the bacteria producing such toxins, are contacted with one or more of the compositions described herein. Effectiveness may be determined by an assay to determine if the toxin cleavage product is present.

[021] As such, the present invention also provides methods for treating an infection, such as but not limited to *Clostridium difficile* infection in a subject. The compounds provided may be administered one or more times to a subject in need of such treatment. Dosage formulations may comprise conventional non-toxic, physiologically or pharmaceutically acceptable carriers or vehicles suitable for the method of administration. These compounds or pharmaceutical compositions thereof may be administered independently one or more times to achieve, maintain or improve upon a pharmacologic or therapeutic effect derived from these compounds or other agents suitable for *C. difficile* infection being treated. It is well within the skill of an artisan to determine dosage or whether a suitable dosage comprises a single administered dose or multiple administered doses. An appropriate dosage depends on the subject's health, the progression or remission or at risk status of the infection, the route of administration and the formulation used. Preferably, these compounds may be administered in an oral formulation, although the scope of the invention does not limit administration to an oral route. Moreover, the methods and compositions provided herein are applicable to conditions not classified as infections.

[022] As used herein, the term "contacting" refers to any suitable method of bringing compound, e.g., inositol phosphate, into contact with a *Clostridium* toxin or the bacterial cell producing the same. *In vitro* or *ex vivo* this is achieved by exposing the compound to the toxin and/or bacteria in a suitable medium. For *in vivo* applications, any known method of administration is suitable.

[023] As used herein, the term "subject" refers to any recipient or individual administered a composition as described herein that are effective as therapeutics or inhibitors against a pathologic condition associated with cysteine proteases.

[024] Other embodiments of the invention are discussed throughout this application. Any embodiment discussed with respect to one aspect of the invention applies to other aspects of the invention as well and vice versa. Each embodiment described herein is understood to be embodiments of the invention that are applicable to all aspects of the invention. It is contemplated that any embodiment discussed herein can be implemented with respect to any method or composition of the invention, and vice versa. Furthermore, compositions and kits of the invention can be used to achieve methods of the invention.

[025] The use of the word "a" or "an" when used in conjunction with the term "comprising" in the claims and/or the specification may mean "one," but it is also consistent with the meaning of "one or more," "at least one," and "one or more than one."

[026] Throughout this application, the term "about" is used to indicate that a value includes the standard deviation of error for the device or method being employed to determine the value.

[027] The use of the term "or" in the claims is used to mean "and/or" unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and "and/or."

[028] As used in this specification and claim(s), the words "comprising" (and any form of comprising, such as "comprise" and "comprises"), "having" (and any form of having, such as "have" and "has"), "including" (and any form of including, such as "includes" and

"include") or "containing" (and any form of containing, such as "contains" and "contain") are inclusive or open-ended and do not exclude additional, unrecited elements or method steps.

[029] Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating specific embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

DESCRIPTION OF THE DRAWINGS

[030] The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of the specification embodiments presented herein.

[031] **FIGS. 1A-1H.** *C. difficile* toxins are among the proteins S-nitrosylated in vivo. FIGs. 1A-1C: A murine toxigenic ileal-loop model was used to demonstrate TcdA-induced pathology (FIG. 1B) compared with vehicle (veh) control (FIG. 1A) and TcdA-SNO (FIG. 1C) treatment groups (scale bar = 50µm). Analysis of tissue sections revealed that TcdA (10 µg) induced epithelial cell damage, neutrophil tissue infiltration and edema of the intestinal mucosa were reduced in TcdA-SNO treated animals. FIG. 1D: Increased tissue GSNO levels correlate with elevated iNOS expression in ileal-loops exposed to TcdA for 4 hrs. FIGs 1E and 1F: SNO-immunofluorescence showing abundant epithelial S-nitrosylation in human colitis (FIG. 1F) but not in histologically normal colon (FIG. 1E) where SNOs are largely confined to lamina propria cells (arrows illustrate brush border membrane; SNOs; DAPI nuclear counter-stain; scale bar = 20 µm). This finding confirms that SNO-proteins are increased in human colitis. FIG. 1G: Cysteine saturation fluorescence assay demonstrates in situ S-nitrosylation of TcdA in intoxicated ileal loops identified by mass spectrometry. FIG. 1H: Biotin-switch assay using a C-terminus specific anti-TcdA monoclonal antibody (clone A1H3) demonstrates exotoxin S-nitrosylation in tissue lysates from TcdA exposed ileal loops, but not in vehicle controls. Treatment of toxin exposed mucosal extracts with DTT (1 mM) prior to the biotin-switch eliminated TcdA-SNO.

[032] **FIGs. 2A-2I.** S-nitrosylation inhibits *C. difficile* toxin activity. FIGs 2A-2B: Anti-SNO immunofluorescence showing that Caco-2 cells transfected with pCMV6-eNOS express higher levels of membrane-associated SNO-proteins that co-localize with anti-zonula occludens (ZO-1) immunoreactivity (Scale bar = 10 μ M). FIG. 2C: eNOS expression is specific to pCMV60-eNOS transfected Caco-2 cells. The transfection efficiency of a pCMV6-gfp vector in Caco-2 cells was approximately 50%. FIG. 2D: Anti-SNO blot (non-reducing conditions) shows that TcdA is preferentially S-nitrosylated in pCMV60-eNOS transfected Caco-2 cells and S-nitrosylation is diminished following membrane permeable GSH-ethyl ester (GSH-EE) treatment. Low levels of toxin S-nitrosylation is also evident in vector control cells, reflecting lower endogenous SNO. Equal protein loading is demonstrated by GAPDH labeling. FIGs. 2E-2F: TcdB induced significantly less cell rounding in vector control (FIG. 2E) vs. eNOS (FIG. 2F) transfected Caco-2 cells (71.1 ± 14.9 vs. 26.5 ± 8.2 %, respectively; + SEM, n = 3; $p < 0.05$, Mann-Whitney U-test on ranks) (Scale bar = 25 μ M). FIG. 2G: MTT cytotoxicity assay showing significant cytoprotection of pCMV6-eNOS expressing Caco-2 cells against TcdB (3.7 nM for a 10 min exposure). This protection is reversed by GSH-EE and by L-NAME treatment ($p < 0.05$, compared to vector control (*)) and eNOS (#) transfected cells respectively; Mann-Whitney U-test on ranks). FIG. 2H: Toxin-induced Rac1 glucosylation in vector and eNOS transfected Caco-2 cells. Cells were exposed to a 10 min TcdB pulse (3.7nM) and cellular lysates were examined for non-glucosylated and total Rac1 after 60 min. eNOS-Caco-2 cells showed significantly less toxin-induced Rac1 glucosylation as compared with vector-controls (65.8 ± 11.4 vs. 34.3 ± 10.7 %, respectively; n = 3; $p < 0.05$, Mann-Whitney U-test on ranks). FIG. 2I: Toxin-induced Rac1 glucosylation in wild type (Wt) and iNOS deficient murine peritoneal macrophages. Macrophages were first activated with 20 ng IL1- γ for 24 hrs prior to toxin exposure. Cells were exposed to a 10 min TcdB pulse (3.7 nM) and cell lysates were examined for non-glucosylated and total Rac1 after 60 min. iNOS deficient macrophages showed significantly more Rac1 glucosylation as compared with wild type cells (56.5 ± 11.2 vs. 31.3 ± 5.2 %, respectively; + SEM, n = 3; $p < 0.05$, Mann-Whitney U-test on ranks).

[033] **FIGs. 3A-3D.** S-nitrosylated toxins in *C. difficile* patient stool samples. FIG. 3A: Biotin-switch assay showing S-nitrosylated toxin (TcdA-SNO) labeled with IR800-streptavidin dye (right inset). MTT cell viability assays (550 nm) demonstrate that toxin S-

nitrosylation significantly attenuates TcdA-induced cytotoxicity in Caco-2 cells (*, $p < 0.05$; Mann-Whitney U-test on ranks). FIG. 3B: Patient stool (n=8) samples positive for TcdA (antibody A1H3), and confirmed by cytotoxicity assay. FIG. 3C: Cytotoxicity was assessed by mixing mRG1 cells with enhancing antibody A1H3, with or without coded human stool samples (examples shown are UTMB10 (positive) and UTMB14 (negative), 50 x dilution in PBS) in the presence or absence of neutralizing antibody (He et al., *J Microbiol Methods* 78:97-100 (2009)). FIG. 3D: Ratios of TcdA-SNO vs. total TcdA show a negative relationship between toxin S-nitrosylation and cytotoxicity. TcdA-SNO was first immunoprecipitated from stool samples using an anti-nitrosocysteiny antibody and samples were then probed for TcdA (antibody A1H3).

[034] **FIGS. 4A-4G.** Toxin S-nitrosylation is allosterically regulated by InsP₆ and InsP₇. FIG. 4A: N-terminus extended cysteine protease domain model for TcdB (based on TcdB (3PA8.pdb) and RTX (3FZY.pdb) crystal structures showing the flexible p-flap abutting the bound allosteric ligand InsP₆. FIG. 4B: S-nitrosylation of TcdA significantly inhibits InsP₆ binding and is reversed by UV irradiation, which cleaves the SNO bond. As a positive control, InsP₆ binding to deoxygenated hemoglobin, a well established means of allosteric modulation, confirms a binding affinity of >1.6 nM/mg protein. InsP₆ binding to hemoglobin is inhibited by primary amide-biotinylation which blocks access to the InsP₆ binding site. FIG. 4C: Simulated docking of InsP₇ binding to the allosteric pocket in TcdB using Autodock 4.0 shows several poses that closely match InsP₆ bound in the crystal structure (comparable results are determined for TcdA and RTX_{VC}). Calculated binding energies for InsP₆ and InsP₇ to TcdB are -21.60 and -23.01 kcal mol⁻¹, respectively. FIG. 4D: *In vitro* cleavage assays demonstrate that InsP₇ (100 μM) has a significantly greater activity for TcdB than InsP₆ ($p < 0.001$, Mann Whitney U-test for ranks, n=3). r-InsP7 ((rac1)-1(3)-PP-(2,3,4,5,6,)InsP₅) and m-InsP7 (D-myo-5-PP-(1,2,3,4,6)InsP₅) cleavage activities for TcdB were not significantly different. FIG. 4E: SNO-immunoblot showing that InsP₆ induces S-nitrosylation of TcdB. DTT (1mM) inhibits S-nitrosylation by InsP₆ (100 μl) (anti-TcdB labeling with the C-terminus targeting monoclonal antibody 5A8-E11). FIG. 4F: SNO-immunoblot showing that InsP₆ induced S-nitrosylation is markedly reduced in the TcdB Cys698Ser toxin mutant. FIG. 4G: InsP₆ induced TcdB autocleavage is dose-dependently inhibited by simultaneous addition of GSNO. FIG. 4H: SDS-PAGE showing unprocessed

(270 kDa) and processed TcdB cleavage products (207 & 63 kDa). TcdB autocleavage is inhibited by the simultaneous addition of GSNO (100 μ M), but is potentiated by GSH (100 μ M). No significant toxin autocleavage is evident in the presence of GSH or DTT alone. Real-time BiaCore toxin cleavage assays demonstrated that GSNO rapidly inhibits TcdB autocleavage.

[035] **FIGs. 5A-5G.** A novel microbial S-nitrosylation-catalytic motif. FIG. 5A: Surface rendering of the TcdB cysteine protease domain (3PA8.pdb) showing the exposed S-nitrosylation consensus motif E743-C698-H653. In silico docking of GSNO to this crystal structure using Autodock 4.0 demonstrates that the lowest energy cluster associates with the active site, where the S-NO bond aligns to the catalytic cysteine. FIG. 5B: Crystal structures of TcdA, RTX_{VC} and gingipain demonstrate that this S-nitrosylation motif is structurally conserved amongst a diverse array of microbial cysteine protease domains. FIG. 5C: InsP₆ induced S-nitrosylation of TcdB cysteine protease domain mutants. SNO-immunoblot shows S-nitrosylation results for TcdB His653Ala, Glu743Ala and Cys698Ser mutants in the presence of GSNO and InsP₆ (100 μ M; 10 min at 37°C). FIG. 5D: Analysis of the RTX_{VC} cysteine protease domain crystal structure with an intact elongated N-terminus (3FZY.pdb), demonstrates an extensive network of interconnecting hydrogen bonds within the active site (dotted lines). This interconnecting hydrogen bond network is conserved in TcdA and TcdB. FIG. 5E: Catalytic activity of TcdB cysteine protease domain mutants. SDS-PAGE showing InsP₆ induced cleavage (100 μ M for 60 min) of TcdB Cys698Ser, Glu743Ala, and His653Ala mutants. The Cys698Ser and His653Ala mutants are catalytically inactive, whereas self-cleavage is greatly enhanced in the Glu743Ala mutant. FIG. 5F: InsP₆ dose-response studies (10 min incubation, using an N-terminus specific TcdB VHH single chain antibody (JC12) for assay of cleavage activity) demonstrated that the Glu743Ala mutant is approximately two orders of magnitude (FIG. 5G) more sensitive to InsP₆ induced cleavage than the wild type toxin.

[036] **FIGs. 6A-6F.** GSNO based therapy for *Clostridium difficile* infection. FIG. 6A: Biotin-switch assay showing increased protein S-nitrosylation in Caco-2 cells incubated with GSNO (100 μ M for 30 min). FIG. 6B: Dose-response curves for GSNO inhibition of TcdB (3.7 nM; 10 min incubation) in the absence (closed circles) or presence of GSH (1 mM; open

circles) and InsP₆ (100 μ M; filled triangles). FIG. 6C: GSNO (10 mg/kg in 0.1 ml) inhibits TcdA (10 μ g) induced fluid secretion in murine ileal loops and this protective effect is enhanced by allosteric effector InsP₆ (1 mM; n>6 group; p<0.05 compared with vehicle control (*) and TcdA-vehicle (#), respectively; ANOVA on ranks). FIG. 6D: Exposing mouse ileal loops to TcdA (10 μ g) for 4 hrs induced a significant accumulation of gene transcripts for TNF- α and IL-1 γ . GSNO significantly attenuated this response and inhibition was potentiated by InsP₆ (1 mM; p<0.05 compared with vehicle control (*) and TcdA-vehicle (#) treated loops respectively; ANOVA on ranks). FIG. 6E: Kaplan-Merier survival plots of C57BL/6 mice inoculated intragastrically with 10⁶ *C. difficile* VPI 10463 and orally gavaged with GSNO (10 mg/kg/day); GSNO/InsP₆ (10 & 0.25 mg/kg/day, respectively), InsP₆ (0.25 mg/kg/day) or vancomycin (50 mg/kg/day; n=12 per group). GSNO/InsP₆ (10 & 0.25 mg/kg/day, respectively) was also delivered continuously by mini-osmotic pumps via an intracecal catheter. FIG. 6F: Intracecal GSNO/InsP₆ administration conferred significant protection from *C. difficile* infection, with survival rates greater than 80% (n=12/group; survival at Day 4; p<0.05 compared with vehicle control (*) and GSNO (#) respectively; ANOVA on ranks).

DESCRIPTION

[037] The emergence of a hypervirulent form of *C. difficile* (NAP1/027) has resulted in a global increase in *C. difficile* infection, due in part to the high levels of toxin produced by this strain (Kelly et al., *N Engl J Med* 359:1932-40 (2008); Savidge et al., *Gastroenterology* 125:413-20 (2003); Lyras et al. *Nature* 458: 1176-79 (2009); Kuehne et al., *Nature* 467:711-13 (2010)). Examples provided herein demonstrate that GSNO is an endogenous inhibitor of *C. difficile* infection, which acts in significant part by S-nitrosylation of toxin active site thiol. GSNO accumulation during *C. difficile* infection reaches levels that inactivate the toxin *in vitro* and *in vivo*, and GSNO can be used therapeutically to inhibit fulminant disease. GSNO is well tolerated in humans, and is a multifaceted protective agent that exhibits broad-spectrum anti-microbial activity (Hess et al., *Nature Rev* 6: 150-166 (2005); MW et al., *Trends Mol Med* 15: 391-404 (2009)). S-nitrosylating agents can be used as a basis of a new treatment for *C. difficile* infection.

[038] Hyper- or hypo-nitrosylation of specific proteins may represent disease-modifying events (Benhar et al., *Science* 320:1050-54 (2008)). A major challenge in NO therapeutics is to control the nitrosylation of specific protein targets that correlate best with pathophysiology. Recent *in vitro* studies have raised the idea that various allosteric effectors may play a role in conferring NO specificity (MW et al., *Trends Mol Med* 15: 391-404 (2009)). The present invention provides physiological context for this principle by showing that InsP₆ and InsP₇ are specificity-determinants of toxin cysteine protease S-nitrosylation. More generally, allosteric modulation of S-nitrosylation suggests new therapeutic approaches to regulating nitrosylation of disease-modifying molecular targets.

[039] The toxin cysteine protease structurally resembles the caspase protease domain family, which is also subject to S-nitrosylation (MW et al., *Trends Mol Med* 15: 391-404 (2009)). Notably, inhibition of pro-caspase 3 by S-nitrosylation entails both orthosteric (active site) and allosteric mechanisms (Matsumoto et al. *Science* 301: 657-61 (2003)). Pro-caspase 3 is maintained in the S-nitrosylated and inactive state in the mitochondria but not in the cytosol. Although the basis of maintained S-nitrosylation in the mitochondria is not known (Benhar et al., *Science* 320:1050-54 (2008)), plasma membrane/endosome compartments may provide privileged access to allosteric cofactors that would influence S-nitrosylation levels. S-nitrosylation is already known to suppress parasitic and viral pathogenesis by inhibiting cysteine protease-regulated metabolism and reproductive function (MW et al., *Trends Mol Med* 15: 391-404 (2009); Saura et al. *Immunity* 10: 21-28 (1999)). However, host S-nitrosylation has not been shown to directly inhibit microbial exotoxin activity, and the dual protective mechanism involving catalytic Cys inhibition and allosteric activation is unique. In particular cross-talk between inositol phosphates and NO has not previously been described. Given the recent emergence of an extensive array of new microbial proteins (CPD_{MARTX} and CPD_{adh}) that appear to require an active cysteine protease for host cell adhesion and entry (Pei et al., *Protein Science* 18: 856-62 (2009)), S-nitrosylation may represent a universal host defense mechanism.

I. Pathogenic Cysteine Proteases

[040] Various pathogens, such as those of the genus *Clostridium*, produce highly potent pathogenic cysteine proteases, such as clostridial glucosylating toxins (CGTs). CGTs are single-chain proteins subdivided into at least four functional domains (See Jank and Aktories,

Trends Microbiol. 16:222-29 (2008), which is incorporated herein by reference). Binding of the toxins to cell surface receptors is mediated by a C-terminal region, including a series of repetitive oligopeptides. After uptake of the toxins, a central region mediates translocation from endosomes into the cytosol. The glucosyltransferase domain is located at the N-terminal end of the toxins. The glucosyltransferase domain is released into the cytosol by auto-catalytic cleavage induced by an adjacent cysteine protease domain (CPD). (See FIG. 1 of Egerer et al., *J. Biol. Chem.* 282(35):25314-21 (2007), which is incorporated herein by reference in its entirety particularly).

[041] The CPD domain is a member of the peptidase C80 family and is represented in the National Center for Biotechnology Information (NCBI) Conserved Domain database under accession pfam11713 or accession number PF11713 of the Pfam database of the Sanger Institute, the information associate with both of these database accession numbers are incorporated herein by reference as of the filing date of the current application. Amino acids 576 to 756 of SEQ ID NO:1 provide an example of a CPD, other similar domains can be identified by using various search algorithms.

[042] Clostridial Glucosylating Toxins (CGT) include, but are not limited to *Clostridium sordellii* lethal toxin, *C. novyi* α -toxin, *C. difficile* toxin A, and *C. difficile* toxin B. This group of toxins are major virulence factors. *C. difficile* toxins A and B cause antibiotic-associated diarrhea and pseudomembranous colitis, *C. sordellii* lethal toxin is implicated in toxic-shock syndrome, and *C. novyi* α -toxin plays a pathogenetic role in gas gangrene syndrome.

[043] CPD domains are also found in the Multifunctional Autoprocessing RTX-like (MARTX) polypeptides, such as those found in *Vibrio cholerae*, *Vibrio vulnificus*, and *Vibrio anguillarum*.

[044] Polypeptides classified as CPDadh polypeptides also contain CPD domains (See Pei et al. February 10, 2009 at proteinscience.org, which is incorporated herein by reference in its entirety). CPDadh polypeptides are homologous to CPD_{MARTX} polypeptides. CPDadh polypeptides are found in bacteria and eukaryotes. In certain aspects, CPDadh can be limited to bacterial CPDadh. In a further aspect, CPDadh polypeptides can be limited to pathogenic bacterial CPDadh polypeptides. The pathogenic CPDadh polypeptides can include those

polypeptides that are allosterically regulated by inositol phosphate or analogs thereof. Examples of CPDadh polypeptides include GenBank accession numbers gi89075996 and gi90578280, each of which is incorporated herein by reference.

[045] Members of the gingipain polypeptide family contain CPD domains. Gingipain is a protease secreted by *Porphyromonas gingivalis*. *Porphyromonas gingivalis* belongs to the phylum *Bacteroidetes* and is a non-motile, Gram-negative, rod-shaped, anaerobic pathogenic bacterium. *P. gingivalis* is found in the oral cavity, where it is implicated in certain forms of periodontal disease, as well as the upper gastrointestinal tract, respiratory tract, and in the colon. Additionally *P. gingivalis* has been linked to rheumatoid arthritis.

II. S-Nitrosylating agents

[046] S-nitrosylating agents include agents that generate or increase GSNO *in situ*. In one embodiment, the composition comprises nitrite. The nitrite is either an organic nitrite or an inorganic nitrite. Organic nitrite does not require acidic conditions to form GSNO and is much more potent; but inorganic nitrite can be used if conditions are acidic and amounts are larger (e.g., up to 100 μ M - 50 mM). The organic nitrite may be, for example, ethyl nitrite or amyl nitrite. In one embodiment, the composition contains a nitrite and glutathione. In another embodiment, the composition contains a nitrite and N-acetylcysteine. In another embodiment, the composition contains a nitrite and a phytocheletin or hydrogen sulfide. In one aspect, the S-nitrosylating agents (either individually or combined) are present in the composition/administered in an amount of about 1 μ M to about 1 mM.

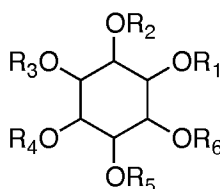
[047] In any of the embodiments described herein, the composition may further comprise one or more inositol phosphates or analogs thereof. The combination of inositol phosphates (e.g., IP6 and IP7 and there analogs) with S-nitrosylating agents (such as GSNO and GSNO-generating agents) demonstrate synergistic effects (therapeutic allostery).

III. Inositol Phosphates and Analogs Thereof

[048] In certain aspects a composition or method includes one or more inositol phosphates or analogs thereof. Representative inositol phosphates include but are not limited to inositol hexakisphosphate, inositol pyrophosphate, or an inositol phosphate analog. In one

aspect, the inositol phosphates and analogs thereof (either individually or combined) are present in the composition/administered in an amount of about 1 μ M to about 1 mM.

[049] Certain aspects are directed to inositol hexakisphosphate analogs (*See*, U.S. Provisional Patent Application serial number 61/516,639 filed April 6, 2011 and U.S. Patent Application 13/441,017 filed April 6, 2012, both of which are incorporated herein by reference in their entirety. In certain aspects, an inositol hexakisphosphate analog will be an allosteric enhancer of *C. difficile* exotoxin, or similar proteins, that results in a conformational change in the protein, which in turn provides greater access for S-nitrosylation of an active site cysteine. In further aspects, the analog will be a degradation resistant (e.g., phytase resistant) allosteric enhancer of *C. difficile* exotoxin or similar proteins. In certain embodiments the derivative or analog compound has a chemical structure of Formula I:



Formula I

where R_1 - R_6 independently are $-\text{PO}(\text{OH})_2$, $-\text{PS}(\text{OH})_2$, $-\text{PSe}(\text{OH})_2$, $-\text{AsO}_3$, or $(\text{NO})_{1-2}$, or NO associated with $-\text{PO}(\text{OH})_2$ (i.e., $-\text{PO}(\text{OH})_2\text{NO}$). In certain aspects, at least one of R_1 - R_6 is $-\text{PS}(\text{OH})_2$, $-\text{PSe}(\text{OH})_2$, or NO associated with $-\text{PO}(\text{OH})_2$ (i.e., $\text{PO}(\text{OH})_2\text{NO}$). In certain aspects R_1 and R_3 are not both $-\text{PSe}(\text{OH})_2$ or $-\text{PS}(\text{OH})_2$ when R_2 , R_4 , R_5 , R_6 are $-\text{PO}(\text{OH})_2$. In certain aspects, R_1 is not $-\text{PS}(\text{OH})_2$ or $-\text{PSe}(\text{OH})_2$ if R_2 - R_6 are $-\text{PO}(\text{OH})_2$. In further aspects, the analog can be a pharmacologically effective salt of the compounds described herein. In other aspects, the analog can be a derivative, such as the pyrophosphates IP7 and IP8.

[050] In certain embodiments, the inositol analog is a myo-inositol analog. In further aspects, the inositol analog is a neo-inositol analog. In still further aspects, the inositol analog is a D-chiro-inositol analog. In further aspects, the inositol analog is a L-chiro-inositol analog. In certain aspects, the inositol analog is a muco-inositol analog. In still further aspects, the inositol analog is an allo-inositol analog. In still further aspects, the

inositol analog is a scyllo-inositol analog. In yet further aspects, the inositol analog is an epi-inositol analog. In certain aspects, the inositol analog is a cis-inositol analog.

[051] As used herein, “analog” refers to a chemical compound that is structurally similar to a parent compound, but differs in composition (e.g., differs by appended functional groups or substitutions). The analog may or may not have different chemical or physical properties than the original compound and may or may not have improved biological and/or chemical activity. For example, the analog may be more hydrophilic or it may have altered reactivity as compared to the parent compound. The analog may mimic the chemical and/or biologically activity of the parent compound (i.e., it may have similar or identical activity), or, in some cases, may have increased or decreased activity.

[052] In one aspect, the derivative or analog compound has a chemical structure of Formula I wherein R_1 is $-PSe(OH)_2$ and (i) R_2 - R_6 (i.e., R_2 , R_3 , R_4 , R_5 , and R_6) are $-PO(OH)_2$ or (ii) R_2 - R_6 are independently $-PO(OH)_2$, $-PS(OH)_2$, $-PSe(OH)_2$, $-AsO_3$, or $PO(OH)_2NO$, but not all are $-PO(OH)_2$. In certain aspects, R_2 is $-PSe(OH)_2$, and R_1 and R_3 - R_6 are $-PO(OH)_2$. In further aspects, R_4 is $-PSe(OH)_2$, and R_1 - R_3 and R_5 - R_6 are $-PO(OH)_2$. In still further aspects, R_5 is $-PSe(OH)_2$, and R_1 - R_4 and R_6 are $-PO(OH)_2$. In certain aspects, R_1 - R_4 are $-PSe(OH)_2$ and R_5 - R_6 are $-PO(OH)_2$. In certain aspects, one or more of the $-PO(OH)_2$ groups is further modified to a $-PO(OH)_2NO$. The NO group can be covalently or non-covalently bound to the analog. In a further aspect, the compound is a pharmacologically effective salt or derivative of these compounds.

[053] In certain aspects, the derivative or analog compound has a chemical structure of Formula I where R_1 is $-PS(OH)_2$ and R_2 - R_6 are $-PO(OH)_2$. In further aspects, R_2 is $-PS(OH)_2$ and R_1 and R_3 - R_6 are $-PO(OH)_2$. In still further aspects, R_4 is $-PS(OH)_2$ and R_1 - R_3 and R_5 - R_6 are $-PO(OH)_2$. In certain aspects, R_1 , R_5 , and R_3 are $-PS(OH)_2$, and R_2 and R_4 - R_6 are $-PO(OH)_2$. In further aspects, R_5 is $-PS(OH)_2$ and R_1 - R_4 and R_6 are $-PO(OH)_2$. In still further aspects, R_1 - R_4 are $-PS(OH)_2$ and R_5 - R_6 are $-PO(OH)_2$. In certain aspects the analog is an inhibitor of exotoxin cleavage. In certain aspects, the compounds are pharmacologically effective salt or derivative of these compounds.

[054] In a further aspect, the derivative or analog compound has a chemical structure of Formula I where R_1 - R_6 independently are $-PO(OH)_2$ or $-PO(OH)_2NO$ (NO associated

covalently or ionically with $-\text{PO}(\text{OH})_2$), whereby at least one of $\text{R}_1\text{-R}_6$ is NO associated with $-\text{PO}(\text{OH})_2$, or a pharmacologically effective salt or derivative thereof.

[055] In certain embodiments an inositol analog has a chemical structure of Formula I where R_1 , R_2 , R_3 , R_4 , R_5 , and R_6 are independently $-\text{PS}(\text{OH})_2$, $-\text{PSe}(\text{OH})_2$, $-\text{AsO}_3$, or $-\text{PO}(\text{OH})\text{NO}$. In certain aspects, R_1 , R_2 , R_3 , R_4 , R_5 , and R_6 are $-\text{PS}(\text{OH})_2$. In certain aspects, R_1 , R_2 , R_3 , R_4 , R_5 , and R_6 are $-\text{PSe}(\text{OH})_2$. In certain aspects, R_1 , R_2 , R_3 , R_4 , R_5 , and R_6 are $-\text{AsO}_3$. In certain aspects, R_1 , R_2 , R_3 , R_4 , R_5 , and R_6 are $-\text{PO}(\text{OH})_2\text{NO}$. In certain aspects, R_1 , R_2 , and 1, 2, 3, or 4 of R_3 , R_4 , R_5 , and R_6 are $-\text{PS}(\text{OH})_2$, $-\text{PSe}(\text{OH})_2$, $-\text{AsO}_3$, or $-\text{PO}(\text{OH})_2\text{NO}$. In further aspects R_1 , R_4 , and 1, 2, 3, or 4 of R_2 , R_3 , R_5 , and R_6 are $-\text{PS}(\text{OH})_2$, $-\text{PSe}(\text{OH})_2$, $-\text{AsO}_3$, or $-\text{PO}(\text{OH})\text{NO}$. In still further aspects, R_1 , R_5 , and 1, 2, 3, or 4 of R_2 , R_3 , R_4 , and R_6 are $-\text{PS}(\text{OH})_2$, $-\text{PSe}(\text{OH})_2$, $-\text{AsO}_3$, or $-\text{PO}(\text{OH})\text{NO}$. In certain aspects, R_1 , R_6 , and 1, 2, 3, or 4 of R_2 , R_3 , R_4 , and R_5 are $-\text{PS}(\text{OH})_2$, $-\text{PSe}(\text{OH})_2$, $-\text{AsO}_3$, or $-\text{PO}(\text{OH})\text{NO}$. In certain embodiments the inositol analog is a myo-inositol analog.

IV. Pharmaceutical Preparations

[056] Pharmaceutical compositions of the present invention comprise an effective amount of an S-nitrosylating agent and/or an inositol phosphate or analog thereof dissolved or dispersed in a pharmaceutically acceptable carrier. The phrases "pharmaceutical or pharmacologically acceptable" refers to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to an animal, such as, for example, a human. The preparation of a pharmaceutical composition that contains at least one S-nitrosylating agent and/or an inositol phosphate or analog thereof. Moreover, for animal (e.g., human) administration, it will be understood that preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biological Standards.

[057] As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, surfactants, antioxidants, preservatives (e.g., antibacterial agents, antifungal agents), isotonic agents, absorption delaying agents, salts, preservatives, drugs, drug stabilizers, gels, binders, excipients, disintegration agents, lubricants, sweetening agents, flavoring agents, dyes, such like materials and combinations thereof, as would be known to one of ordinary skill in the art (see, for example, Remington's Pharmaceutical

Sciences, 18th Ed. Mack Printing Company, 1990, pp. 1289-1329). Except insofar as any conventional carrier is incompatible with the active ingredient, its use in the therapeutic or pharmaceutical compositions is contemplated.

[058] The pharmaceutical compositions of the invention may comprise different types of carriers depending on whether it is to be administered in solid, liquid, or aerosol form, and whether it needs to be sterile for such routes of administration, e.g., injection. The present invention can be administered orally or rectally (e.g., to facilitate delivery to the gastrointestinal tract), but may also be administered by any other local or systemic route (e.g., intratracheally, intranasally, subcutaneously, mucosally, inhalation (e.g., aerosol inhalation), injection, infusion, continuous infusion, localized perfusion bathing target cells directly, via a catheter, via a lavage, or by other method or any combination of the foregoing as would be known to one of ordinary skill in the art) (see, e.g., Remington's Pharmaceutical Sciences, 18th Ed. Mack Printing Company, 1990).

[059] The actual dosage amount of a composition administered to a patient can be determined by physical and physiological factors such as body weight, severity of condition, the type of disease being treated, previous or concurrent therapeutic interventions, idiopathy of the patient and on the route of administration. The practitioner responsible for administration will, in any event, determine the concentration of active ingredient(s) in a composition and appropriate dose(s) for the individual subject. In any case, the composition may comprise various antioxidants to retard oxidation of one or more component. Additionally, the prevention of the action of microorganisms can be brought about by preservatives such as various antibacterial and antifungal agents, including but not limited to parabens (e.g., methylparabens, propylparabens), chlorobutanol, phenol, sorbic acid, thimerosal or combinations thereof.

[060] In embodiments where the composition is in a liquid form, a carrier can be a solvent or dispersion medium comprising but not limited to, water, ethanol, polyol (e.g., glycerol, propylene glycol, liquid polyethylene glycol, etc.), lipids (e.g., triglycerides, vegetable oils, liposomes) and combinations thereof. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin; by the maintenance of the required particle size by dispersion in carriers such as, for example liquid polyol or lipids; by the use

of surfactants such as, for example hydroxypropylcellulose; or combinations thereof such methods. In many cases, it will be preferable to include isotonic agents, such as, for example, sugars, sodium chloride or combinations thereof. Liposomal delivery is useful when the agents are gaseous, volative or need stabilization.

[061] In certain embodiments, the compositions of the present invention are prepared for administration by such routes as oral ingestion. In these embodiments, the solid composition may comprise, for example, solutions, suspensions, emulsions, tablets, pills, capsules (e.g., hard or soft shelled gelatin capsules), delayed release capsules, sustained release formulations, buccal compositions, troches, elixirs, suspensions, syrups, wafers, or combinations thereof. Oral compositions may be incorporated directly with food. Preferred carriers for oral administration comprise inert diluents, assimilable edible carriers or combinations thereof. In other aspects of the invention, the oral composition may be prepared as a syrup or elixir. A syrup or elixir, and may comprise, for example, at least one active agent, a sweetening agent, a preservative, a flavoring agent, a dye, a preservative, or combinations thereof.

[062] In certain embodiments, an oral composition may comprise one or more binders, excipients, disintegration agents, lubricants, flavoring agents, and combinations thereof. In certain embodiments, a composition may comprise one or more of the following: a binder, such as, for example, gum tragacanth, acacia, cornstarch, gelatin or combinations thereof; an excipient, such as, for example, dicalcium phosphate, mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate or combinations thereof; a disintegrating agent, such as, for example, corn starch, potato starch, alginic acid or combinations thereof; a lubricant, such as, for example, magnesium stearate; a sweetening agent, such as, for example, sucrose, lactose, saccharin or combinations thereof, a flavoring agent, such as, for example peppermint, oil of wintergreen, cherry flavoring, orange flavoring, etc.; or combinations thereof the foregoing. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, carriers such as a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both.

[063] Additional formulations suitable for other modes of administration include suppositories. Suppositories are solid dosage forms of various weights and shapes, usually medicated, for insertion into the rectum. After insertion, suppositories soften, melt or dissolve in the cavity. In general, for suppositories, traditional carriers may include, for example, polyalkylene glycols, triglycerides or combinations thereof. In certain embodiments, suppositories may be formed from mixtures containing, for example, the active ingredient in the range of about 0.5% to about 10%, and preferably about 1% to about 2%.

[064] Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and/or the other ingredients. In the case of sterile powders for the preparation of sterile injectable solutions, suspensions or emulsion, the preferred methods of preparation are vacuum-drying or freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered liquid medium thereof. The liquid medium should be suitably buffered if necessary and the liquid diluent first rendered isotonic prior to injection with sufficient saline or glucose. The preparation of highly concentrated compositions for direct injection is also contemplated, where the use of DMSO as solvent is envisioned to result in extremely rapid penetration, delivering high concentrations of the active agents to a small area.

[065] The composition should be stable under the conditions of manufacture and storage, and preserved against the contaminating action of microorganisms, such as bacteria and fungi. It will be appreciated that endotoxin contamination should be kept minimally at a safe level.

[066] The compositions of the present invention may be specially formulated for release after administration. More particularly, the compositions may be designed for release or synthesis in the stomach, the small intestine, the ileum, the jejunum, the duodenum, the large intestine, the cecum, the ascending colon, the transverse colon, the descending colon, the sigmoid colon or the rectum.

V. Examples

[067] The following examples as well as the figures are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples or figures represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1

A. Materials and Methods

[068] *Materials.* Materials were obtained from Sigma-Aldrich Chemical Company (St Louis, MO) or Invitrogen (Carlsbad, CA) unless otherwise stated and r-InsP₇ ((α)-1(3)-PP-(2,3A,5,6,)-*nsP_s*) and m-InsP₇ (D-myo-5-PP-(1,2,3,4,6)InsP₅ was obtained from Dr. Glenn Prestwich (University of Utah, UT)).

[069] *Generation, treatment and testing of native and recombinant C. difficile toxins.* Purification of TcdA and TcdB from toxigenic *C. difficile* strain 10463 was performed and tested for cytotoxicity as described (Savidge et al., *Gastroenterology* 125:413-20 (2003)). Toxin purity was assessed by SDS-PAGE, confirming molecular masses for TcdA and TcdB as 308 kDa and 270 kDa, respectively. The EZ-Link® Sulfo-NHS-LC-Biotinylation Kit (Pierce) was used to biotinylate toxin on primary amide-groups without altering its biological activity or interfering with cysteine-thiol groups required for activity or S-nitrosylation. N-Hydroxysuccinimide (NHS) ester-activated biotins react efficiently with primary amino groups (-NH₂) at pH 7.4 to form stable amide bonds. One mg of TcdA or TcdB was dissolved in 0.85 ml of phosphate-buffered saline (PBS). The Sulfo-NHS-LC-Biotin solution (150 μ l) was then added to the protein solution and incubated on ice for two hours. Remaining biotin reagent was then removed by using a desalting column (Zeba™ Desalt Spin Column). Biotinylated toxin concentrations were measured by the Bradford method (Bio-Rad, Hercules, CA) and purity assessed by gel electrophoresis, confirming the expected molecular mass of 308 kDa for TcdA and 270 kDa for TcdB. For S-nitrosylation of *C.*

difficile toxins, 100 µg of purified TcdA or TcdB in 20 mM Hepes, 5mM EDTA, pH 7.2 was S-nitrosylated with 100 µM GSNO for 10-30 min and then separated to remove excess GSNO using Vivaspin 500 (100,000 mw cut-off filters; Sartorius biotech) or acetone precipitation. S-nitrosylation reactions were also run in the presence of increasing concentrations of InsP₆ or r-InsP₇ ((rac1)-1(3)-PP-(2,3,4,5,6)InsP5). *C. difficile* toxins with an inactivated cysteine protease domain were prepared by incubation with 100 µM N-ethylmaleimide and InsP₆ for 30 min before removing excess inhibitor using Vivaspin 500 filters.

[070] Full-length, bioactive recombinant TcdA and TcdB have been successfully cloned and expressed in a *B. megaterium* system, achieving a toxin expression level of 10 mg/L (Yang et al. *BMC Microbiol* 8:192-98 (2008); Sun et al. *Microb Pathog* 46:298-305 (2009)). To test S-nitrosylation effects on intracellular autocleavage efficiency in cells, several recombinant TcdB proteins were generated, including the point mutations Cys698Ser (catalytic cysteine), His653Ala (catalytic histidine), Glu743Ala (glutamic acid forming part of the S-nitrosylation motif), and glucosyltransferase (GT)-domain substrate binding residues (Trp102Ala and Asp288Asn) that essentially abolish TcdB toxicity (but do not alter affect cellular internalization or InsP₆ induced self-cleavage)(Sun et al. *Microb Pathog* 46:298-305 (2009)). The GT-mutant recombinant toxin was named aTcdB and contains a C-terminus His-tag to facilitate toxin recovery as described using an anti-His tag monoclonal antibody or HisPur™ cobalt resin (Thermo Scientific) (Yang et al. *BMC Microbiol* 8:192-98 (2008); Sun et al. *Microb Pathog* 46:298-305 (2009)).

[071] *In vitro* and BioCore toxin cleavage assays. Autocleavage of 1 µg TcdA and TcdB holotoxins, and recombinant native and mutant TcdB was performed in 25 µl 20 mM Tris-HCl, 150 mM NaCl (pH 7.4) with and without InsP₆, GSNO, GSH, or DTT for 10 min to overnight at 37°C as described (Egerer et al., *J Biol Chem* 282: 25314-21 (2007)). For nitroso-thiocyanobenzoic acid (NTCB)-induced toxin cleavage reactions, 1 µg of toxin was incubated with a 5-fold molar excess of NTCB in 1M Glycine-Tris buffer (pH 9.0) for 2 hrs to overnight at 37°C as previously described (Tang et al., *Analyt Biochem* 334:48-61 (2004)). Cleavage reactions were stopped with SDS-PAGE loading buffer and boiling at 96°C for 5 min. Samples were then run under reducing conditions on 4-20% gradient gels and cleavage

products were stained with GelcodeBlue™ (Pierce) for 1 hr and cleared in water overnight. Cleavage fragments were then identified by mass spectrometry. AC_{50} and IC_{50} concentrations were calculated by measuring the relative absorbance of cleavage fragments relative to intact toxin using a LiCor Odyssey infrared scanner ($\lambda = 680$ nm). Cleavage was plotted against ligand concentration using four-parameter logistic curve fitting on SigmaPlot 11.0 software. Real time BiaCore-X toxin cleavage assays were performed essentially as described (Lupardus et al., *Science* 322: 265-68 (2008)), with the following modifications: unconjugated or biotinylated toxin was bound to a captured anti-toxin (A1H3) or anti-biotin (BN34) antibodies on CM5 sensor chips as recommended by the manufacturer. Flow rates for toxin cleavage assays were run at 5-10 $\mu\text{l min}^{-1}$ in degassed HBS-EP buffer (BIAcore). Analysis was performed using BIAsimulations software (BIAcore).

[072] *[³H] inositol hexakisphosphate binding assays.* (³H-InsP₆, 10-30 Ci/mmol) NET-1023 was from Perkin Elmer (Waltham, MA). The binding buffer (BB) in the assays was 25 mM Hepes (pH 7.2), containing 100 mM KCl and 1 mM EDTA. The ³H-InsP₆ stock (in BB) was cleared at 18,000 x g for 3 min at 4 C° before use in assays. The binding reactions in protein LoBind tubes (Eppendorf, Westbury, NY) included 2 μg of purified TcdA and various concentrations of ³H InsP₆. The binding reactions were incubated for 10 min with gentle shake at 4 C° and then cold 30% PEG8000 (in BB) was added to final concentration of 20 % and the incubation was continued without shaking for 10 min. TcdA was precipitated at 18,000 x g for 20 min at 4 C°. The supernatant was thoroughly removed, and the pellet was resuspended with 200 μl of 2 % SDS into 5 ml of ScintiVerse II (Fisher Scientific, Pittsburg, PA) and counted in a LS6500 scintillation counter (Beckman, Fullerton CA). Nonspecific background at 1 mM of cold InsP₆ was subtracted and B-Max and K_d values were analyzed by nonlinear and Scatchard analyses with GraphPad Prism 5 software. The effect of GSNO on InsP₆ binding was assayed by preincubating TcdA in the dark at RT for 30 minutes with and without 100 μM GSNO. UV treatment (302 nm) was for 10 min at 9000 $\mu\text{W/cm}^2$.

[073] *Circular Dichroism (CD) Spectroscopy.* CD spectra were recorded on an Aviv 62 spectropolarimeter in the wavelength range of 190-260 nm, with a bandwidth of 1.0 nm and scan step of 0.5 nm using a 0.1-cm path length in a 1-cm quartz cell at 22°C. The protein

concentration was in the range of 50-200 µg/ml. In each case, at least five spectra was accumulated, smoothed, averaged, and corrected for the contribution of solutes.

[074] *Cell culture.* Human-derived colonic adenocarcinoma Caco-2 cells were obtained from the American Tissue Culture Collection. Cells were cultured at 37°C in a 5% CO₂ atmosphere in Dulbecco's Modified Eagle Media (DMEM) with 10% fetal calf serum, 50 U/ml penicillin and 50 µg/ml streptomycin.

[075] *Toxin-exposure of cell lines.* Caco-2 cells transiently-transfected with pCMV6-eNOS (seq identical to NM_000603, which is incorporated herein by reference) or control vector pCMV6 were seeded in 6 well or 96 well tissue culture plates at a concentration of 5×10^4 cells per well, in 100 µl of media. Amaxa-Nucleofector based electroporations were performed according to the manufacturer's instructions (Lonza Walkersville Inc. Walkersville, MD) and typically resulted in 50% transfection rates. After 48 hrs, cells were treated to facilitate calcium influx and 12 hours later intoxicated with TcdA or TcdB (dose-range of 0.4-to-40 nM) for 3-15 min and cells were then washed 3 x with fresh media. For short term experiments testing Rac1 glucosylation levels, cells were intoxicated for 10-120 min and cell lysates were probed with Rac23A8 (total Rac1) and Rac102 (non-glucosylated) antibodies. For acute cellular autocleavage assays, transfected cells were intoxicated with recombinant aTcdB (20 nM) for 3 min before harvesting cell lysates. For MTT assays, cells were cultured for a minimum of 48hrs after toxin exposure. Cells were washed with PBS and 90 µl of fresh media without phenol red and 10 µl of MTT (5 mg/ml) added for 3 hours at 37°C. The insoluble formazan salt is dissolved by adding 100 µl of 20% (w/v) SDS in 10 mM HCl, followed by overnight incubation at 37°C. Absorbance of the converted dye is measured at 550 nm. In test cultures where toxin was retrieved for SNO analysis and mass spectrometry, transfected cells were seeded in 75 cm² tissue culture flasks for 48 hours and were intoxicated with 1-10 µg of toxin or biotinylated toxin for 10 min. Cells were then processed for precipitation of toxin using streptavin-beads or immunoprecipitation with antibody (rabbit anti-nitrocysteine (SNO) specific antibody (Sigma; N5411)). Recombinant His-tagged toxins were enriched as described (Yang et al. *BMC Microbiol* 8:192-98 (2008)). Alternatively, TcdB intoxicated cells were processed for SNO-immunoblotting or biotin-switch assay. Additional control experiments included 1-10 mM membrane-permeable GSH-

ethyl ester (GSH-EHH) to elevate intracellular GSH in eNOS-transfected cells prior to intoxication. Intracellular GSH is generated by the action of cytoplasmic esterases. Inhibiting eNOS activity in Caco-2 cells was achieved using N^G-nitro-L-arginine methyl ester (L-NAME; 100 μ M). iNOS-deficient mice (B6.128S2-Nos2^{tm1Mrl} N12) were obtained from Taconic and intraperitoneal macrophages were harvested as described (Ng et al., *Gastroenterology* April 13 (2010); Sun et al. *Microb Pathog* 46:298-305 (2009)).

[076] *Cell free Rac1 glucosylation assay.* Glucosyltransferase activity of TcdA and TcdA-SNO was measured by their ability to glucosylate Rho GTPase Rac1 in a cell-free assay. Caco-2 cell pellets were resuspended in glucosylation buffer (50 mM HEPES, pH 7.5, 100 mM KCl, 1 mM MnCl₂ and 2 mM MgCl₂) and lysed with a syringe (25G, 40 passes through the needle). After centrifugation (167,000 x g, 3 min), the supernatant was used as a post-nuclear cell lysate. To perform the glucosylation assay, the cell lysates were incubated with TcdA or TcdA-SNO (final concentration of the toxins was 5 μ g/ml) at 37°C for 30 min. The reaction was terminated by heating at 100°C for 5 min in SDS-sample buffer. To measure Rac1 glucosylation levels, lysates were separated on a 4-20% gradient SDS-PAGE gel and transferred onto a nitrocellulose membrane. An antibody that specifically recognizes the non-glucosylated form of Rac1 (clone 102, BD Bioscience) was used for detection.

[077] *TcdA-exposure of mouse ileal loops.* Cd-1 mice (10 week old males; Charles River Labs) were fasted overnight. Ileal loops were ligated in anesthetized animals via laparotomy and injected with 0.15 ml of purified TcdA, TcdA-SNO or TcdA-NEM (10 μ g) or buffer as described (Savidge et al., *Gastroenterology* 125:413-20 (2003); Oiu et al., *Gastroenterology* 111: 409-18 (1996)). Test animals were additionally pretreated with GSNO, InsP₆ or GSNO + InsP₆ (0.1 ml; 10 mg/kg + InsP₆ 1 mM) for 15 min prior to inoculation of TcdA. Four hrs after TcdA administration, mice were euthanized and fluid secretion was determined as the loop weight (100 mg)-to-length (cm) ratio. Full thickness loops were frozen in liquid nitrogen or fixed in 10% formalin in PBS (pH 7.2) for 24 hours and embedded in paraffin using routine procedures. Histological severity of enteritis was evaluated by assessing (1) epithelial cell necrosis, (2) hemorrhagic congestion and edema of the mucosa, and (3) neutrophil margination and tissue infiltration (Savidge et al., *Gastroenterology* 125:413-20 (2003); Oiu et al., *Gastroenterology* 111: 409-18 (1996)).

[078] *Murine C. difficile infection model.* A conventional mouse model of antibiotic-induced *C. difficile* infection that closely resembles the spectrum of disease manifest in humans was used as described (Chen et al., *Gastroenterology* 135:1984-92 (2008)). Disease severity varies from fulminant with typical histopathologic features of *C. difficile* infection, to minimal diarrhea depending on challenge dose. 12 week-old C57BL/6 mice were pre-treated for three days with a mixture of antibiotics shown to disrupt the intestinal microflora (Chen et al., *Gastroenterology* 135:1984-92 (2008)). After two days, mice were injected with clindamycin and were challenged intragastrically with 10^6 *C. difficile* VPI 10463 vegetative cells the following day. Therapeutic efficacy of GSNO and InsP₆ alone or in combination were performed by oral gavage of GSNO (10 mg/kg/day) + InsP₆ (0.25 mg/Kg/day). Oral administration of vancomycin (50 mg/kg/day) was used as a positive control. In addition, GSNO combinations were administered intracecally via catheter using 7 day ALZET mini-osmotic pumps (Durect Corp, Cupertino, CA) surgically implanted 3 days before *C. difficile* challenge as described (Savidge et al., *Gastroenterology* 132:1344-58 (2007)).

[079] *Myeloperoxidase Assay.* Neutrophil myeloperoxidase (MPO) activity is an indicator of tissue inflammation. Intestinal segments (100-250 mg) were homogenized in 1 ml HTAB buffer and centrifuged at 20,000 x g for 10 min at 4°C. Pellets were resuspended in 1 ml HTAB buffer containing 1% hexadecyltrimethylammonium to negate pseudoperoxidase activity. MPO activity was measured in supernatants following 3 cycles of sonication, freezing and thawing. After centrifugation at 40,000 x g for 15 min at 4°C, supernatants (10 µl) were mixed with 90 µl of potassium phosphate buffer containing 0.167 mg/ml O-dianiside dihydrochloride and 0.0005% hydrogen peroxide. Activity was measured every 2 minutes for 20 minutes at 450 nm.

[080] *Quantitative cytokine mRNA measurements.* Total RNA was extracted from cells or frozen tissues, treated with 1 U DNase I and reversed transcribed (Gene Amp RNA-PCR Kit). Real-time rtPCR reactions were run with SYBR Green PCR Master-Mix for 40 cycles on a Chromo4 detector (Bio-Rad Ltd) (94°C for 2 min; 94°C for 1 min; 60°C for 1 min; 72°C for 1min; repeat step 2-to-4 for 40 cycles; 72°C for 10 min). Primer sets were for human IL-8: (Forward 5'-GCCGTGGCTCTCTTGGC-3' SEQ ID NO:2; Reverse 5'-GCACTCCTTGGCAAACTGC-3' SEQ ID NO:3); murine TNFα (Forward 5'-ATGAGCACAGAAAGCATGATC-3' SEQ ID NO:4, Reverse 5'-TACAGGCTTG

TCACTCGAATT-3' SEQ ID NO:5); murine IL-1 γ (Forward 5'-TTGACGGACCCCAAA AGATG-3' SEQ ID NO:6, Reverse 5'-AGAAGGTGCTCATGTCCTCA-3' SEQ ID NO:7). Samples were normalized against commercial GAPDH or 18S rRNA primers and probes (Applied Biosystems) and relative expression levels were calculated as previously described (Savidge et al., *Gastroenterology* 132:1344-58 (2007)).

[081] *Hg-coupled photolysis/chemiluminescence determination of tissue GSNO concentrations.* Briefly, tissue samples were homogenized in PBS (pH 7.4), 0.1 mM EDTA, 0.1 mM DTPA and processed for tissue GSNO as described (Hausladen et al., *Proc Natl Acad Sci USA*. 104: 2157-62 (2007)). Samples were introduced into a photolysis unit (a borosilicate glass coil illuminated with a 200 W mercury vapor lamp for photolytic cleavage of bound NO) via an HPLC capillary pump. NO is then carried in a helium gas stream to a chemiluminescence analyzer (Thermo Electron Corp. TEA 610).

[082] *Biotin-switch assay.* The biotin-switch assay was performed away from direct sunlight essentially as described (Jaffrey et al., *Sci STKE* 12:86 (2001)) with the following modifications. Cell lysates were incubated at 0.8 mg of protein per ml in HENS buffer (200 mM Hepes, 1 mM EDTA, 0.1 mM neocuproine, 2.5 % SDS, pH 7.7) containing 20 mM S-methylmethane thiosulfonate (MMTS) for blocking of free thiols at 50°C for 40 min. Excess MMTS was then removed by precipitating the proteins with four volumes of cold 100 % acetone for one hour at -20°C. After centrifugation at 4000 x g for 30 min, the pellet was washed 3 times with 70% acetone and re-suspended in 0.5 ml of 50 mM Hepes, 1 % SDS, 1 μ M CuCl. Reduction and biotinylation of the SNO's was done by adding 0.5 ml of Reducing and Labeling reagent (S-Nitrosylated Protein Detection Assay Kit, Cayman Chemical Co., Ann Arbor, MI) and incubating at room temperature for one hour. The proteins were then precipitated with 100 % acetone and resuspended in 0.5 ml of 25 mM Hepes, 1 mM EDTA, 1% SDS. For Streptavidin pull-down, 250 μ l of the suspension was diluted with 750 μ l of neutralization buffer (25 mM Hepes, 100 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, pH 7.7). This solution was tumbled overnight at 4 °C with 30 pi of streptavidin agarose beads, which had been pre-washed with neutralization buffer. The beads were pelleted at 200 x g for 30 s, washed with neutralization buffer containing 600 mM NaCl, pelleted and resuspended in 5x SDS-PAGE sample loading buffer. Supernatants of boiled samples were separated on 10% SDS-PAGE and immunoblotted for TcdB.

[083] *Cysteine-saturation fluorescence assay for 2-D gel separation and analyses of the S-nitrosoproteome.* After processing tissue samples to the ascorbate step as described above, the biotin-switch label was substituted with use of a BODIPY-FI-maleimide fluorescent conjugate (Life Technologies, Inc., Carlsbad, CA). Amino acid analysis was first used to determine the cysteine content of the protein sample. Protein (400 µg) was then labeled with BODIPY® FL N-(2-aminoethyl)maleimide at a 1:75 ratio cysteine:BodipyFL maleimide. The reaction buffer was 7M urea, 2M thiourea, 2% CHAPS, 50mM Tris pH 7.5. Incubation time for labeling was 2 hrs at RT. To quench the reaction, 10x molar excess β-mercaptoethanol (BodipyFL: βME) was added and incubated for 30 min at room temperature. The final reaction volume (400 µl) used for isoelectric focusing contained 200 µg labeled protein + 0.5% IPG buffer pH3-10 (GE Healthcare), and was loaded onto a 11cm pH3-10 IPG strip (GE Healthcare) in duplicate and proteins were focused using the following protocol: (1) 50V x 11 hrs (hydration of strip); (2) 250V x 1hr; (3) 500V x 1hr; (4) 1.000V x 1hr; (5) 8,000V x 2 hr (steps 2-5 are gradient increases in voltage); and (6) 8,000V x 48,000 V/hr. Prior to running the second dimension, IPG strips were equilibrated in 6M Urea, 2% SDS, 50mM Tris, pH 8.8, 20% glycerol x 30 min RT and applied to wells of 8-16% Tris-glycine-SDS gels. Gels were run at 150V x 2.25 hr at 4°C, fixed for 1 hr in 10% methanol, 7% acetic acid and washed overnight in 10% ethanol. Finally, gels were imaged on a ProXpress 2D Proteomic Imaging System (Perkin Elmer; excitation λ =480/40nm & emission λ =535/50nm). It was demonstrated that this covalent derivatization method using an uncharged thio-reactive dye: (i) exhibits good specificity for reduced cysteine residues, (ii) has minimal effects on the pi of proteins allowing separation by isoelectric focusing, and (iii) can provide precise quantification due to saturation labeling (Pretzer, et al., *Analyt. Biochem.* 374:250-62 (2008)). Furthermore, this sensitive thiol-labeling method is amenable for in-gel digestion for identification by peptide mass fingering.

[084] *S-nitrosocysteine (SNO) in gel and western blotting.* Purified toxin-SNO samples were run on 4-20% gradient SDS polyacrylamide gels under non-reducing conditions. Gels were then incubated in 50% isopropanol 5% acetic acid for 15 min with gentle shaking, washed in ultrapure water (1 mM EDTA, 0.1 mM neocuproine) for 15 min, before incubating with anti-nitrosocysteine (SNO) antibody (1:200; Sigma) overnight. Gels were then washed 3 x with PBS+0.1% tween-20. Alternatively, toxin was transferred onto nitrocellulose

membrane. SNO-toxin was visualized following incubation with an anti-rabbit IR800 antibody (1:5000) using infrared imaging on an Odyssey imager (Li-Cor Biosciences).

[085] *SNO-immunofluorescence.* Frozen tissue sections of murine ileal loops or colonic mucosa from control and ulcerative colitis patients (n=4/group), were fixed in FACS™ lysing solution (BD Biosciences) for 20 minutes and blocked with rat serum (1:20 in PBS) for 15 min at room temperature. Samples were then incubated with AF®647 conjugated anti-S-Nitroso-Cysteine (SNO) polyclonal rabbit Abs (1:200 in PBS) at room temperature for one hour. Rabbit conjugated IgG was included as a control. Each staining step was followed by six washes with PBS. Samples were then mounted in SlowFade® Gold antifade reagent with DAPI. Confocal microscopy was performed with a Zeiss LSM510 META laser scanning confocal microscope (Carl Zeiss, Thornwood, NY). DAPI stain was visualized with an excitation of 351-364 nm and emission at 385-470 nm (UV laser). The AF®647 staining was detected with an excitation wavelength of 633 nm and an emission wavelength longer than 650 nm (red helium/neon laser). Specificity was demonstrated by a loss of immunofluorescence following pretreatment of sections with 0.1% mercuric chloride as previously described (Gow et al., *J Biol Chem* 277: 9637-40 (2002)).

[086] *C. difficile patients and stool cytotoxicity (ICT) assay.* Coded stool samples from patients with antibiotic-associated diarrhea were investigated for *C. difficile* infection. Eight unformed stool specimens tested positive for TcdA by western blot (A1H3) and for stool cytotoxicity by ICT assay (He et al., *J Microbiol Methods* 78:97-100 (2009)). For the ICT cytotoxicity assay, stool samples were diluted in PBS (50-fold) and filtered through a 0.2 µm membrane. Samples were mixed with A1H3 (2 µg/ml final conc.) and mRG1 cells (2x10⁴/well) before adding to E-plates as described (He et al., *J Microbiol Methods* 78:97-100 (2009)). A control set of wells included neutralizing antibody (1:1000 of goat anti-TcdA from TechLab, Inc) to specifically block TcdA activity. Cell indexes (CIs) of wells were monitored and a positive sample was defined as a CI value (in the absence of anti-sera) equal or less than 50% of that in the presence of anti-sera. TcdA S-nitrosylation was investigated by anti-nitrosocysteine (SNO) antibody immunoprecipitation and detection with A1H3 antibody as described.

[087] *Mass spectrometry.* Two complementary strategies were used to conduct more detailed mapping of the site(s) of S-nitrosylation in TcdA and TcdB. In the first, toxins were subjected to limited proteinase K digestion, followed by the biotin-switch assay resulting in cleavage of full length toxin into smaller biotinylated fragments. Mass spectrometry was then used to identify which fragments were mostly associated with S-nitrosylation following streptavidin pulldown. Alternatively, gel samples of SNO-labeled or autocleaved toxin fragments were cut into 1mm size pieces or smaller and placed into separate 0.5 mL polypropylene tubes. 100µl of 50 mM ammonium bicarbonate buffer was added to each tube and the samples were then incubated at 37°C for 30 min. After incubation, the buffer was removed and 100 µl of water was added to each tube. The samples were then incubated again at 37°C for 30 min. After incubation, the water was removed and 100 µl of acetonitrile was added to each tube to dehydrate the gel pieces. The samples were vortexed, and after 5 min the acetonitrile was removed. 100 µl of acetonitrile was again added to each of the sample tubes, vortexed, and acetonitrile removed after 5 minutes. The samples were then placed in a speedvac for 45 minutes to remove any excess solvent. A 25 mM ammonium bicarbonate solution was prepared at pH 8.0. To a 20 µg vial of lyophilized trypsin (Promega Corp.) was added 2 mL of 25 mM ammonium bicarbonate. The trypsin solution was then vortexed. Trypsin solution was added to each sample tube in an amount (approximately 10 µL) to just cover the dried gel. The samples were then incubated at 37°C for 6 hrs. After digestion, 1 µL of sample solution was spotted directly onto a MALDI target plate and allowed to dry. 1 µL of alpha-cyano-4-hydroxycinnamic acid (Aldrich Chemical Co.) matrix solution (50:50 acetonitrile/water at 5 mg/mL) was then applied on the sample spot and allowed to dry. The dried MALDI spot was blown with compressed air (Decon Laboratories, Inc.) before inserting into the mass spectrometer.

[088] Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI TOF-MS) was used to analyze the samples and determine protein identification. Data were acquired with an Applied Biosystems 4800 MALDI TOF/TOF Proteomics Analyzer. Applied Biosystems software package included 4000 Series Explorer (v. 3.6 RC1) with Oracle Database Schema Version (v. 3.19.0), Data Version (3.80.0) to acquire both MS and MS/MS spectral data. The instrument was operated in positive ion reflectron mode, mass range was 850 - 3000 Da, and the focus mass was set at 1700 Da. For MS data, 2000-4000

laser shots were acquired and averaged from each sample spot. Automatic external calibration was performed using a peptide mixture with reference masses 904.468, 1296.685, 1570.677, and 2465.199. Following MALDI MS analysis, MALDI MS/MS was performed on several (5-10) abundant ions from each sample spot. A 1kV positive ion MS/MS method was used to acquire data under post-source decay (PSD) conditions. The instrument precursor selection window was ± 3 Da. For MS/MS data, 2000 laser shots were acquired and averaged from each sample spot. Automatic external calibration was performed using reference fragment masses 175.120, 480.257, 684.347, 1056.475, and 1441.635 (from precursor mass 1570.700). Applied Biosystems GPS Explorer™ (v. 3.6) software was used in conjunction with MASCOT to search the respective protein database using both MS and MS/MS spectral data for protein identification. Protein match probabilities were determined using expectation values and/or MASCOT protein scores. MS peak filtering included the following parameters: mass range 800 Da to 4000 Da, minimum S/N filter =10, mass exclusion list tolerance = 0.5 Da, and mass exclusion list (for some trypsin and keratin-containing compounds) included masses 842.51, 870.45, 1045.56, 1179.60, 1277.71, 1475.79, and 2211.1. For MS/MS peak filtering, the minimum S/N filter =10.

[089] For protein identification, eukaryotic and bacterial taxonomy was searched in the NCBI database. Other parameters included the following: selecting the enzyme as trypsin; maximum missed cleavages = 1; fixed modifications included BODIPY FI-maleimide for 2-D gel analyses only; variable modifications included oxidation (M); precursor tolerance was set at 0.2 Da; MS/MS fragment tolerance was set at 0.3 Da; mass = monoisotopic; and peptide charges were only considered as +1. The significance of a protein match, based on both the peptide mass fingerprint (PMF) in the first MS and the MS/MS data from several precursor ions, is based on expectation values; each protein match is accompanied by an expectation value. The expectation value is the number of matches with equal or better scores that are expected to occur by chance alone. The default significance threshold is $p < 0.05$, so an expectation value of 0.05 is considered to be on this threshold. A more stringent threshold of 10^{-3} was used for protein identification; the lower the expectation value, the more significant the score.

[090] *Ligand docking studies to the toxin cysteine protease domain.* There are four toxin cysteine protease crystal structures available (3EEB and 3FZY of RTXvc, 3H06 of

TcdA and 3PA8 of TcdB). These structures show that the catalytic residues, the β -flap and larger helices are conserved. The RTXvc 3FZY structure includes the uncut N-terminus substrate. The crystal structures of RTXvc (3FZY), TcdB (3PA8) and the TcdB glucosyltransferase domain (2BVL) were combined to generate a combined model of the glucosyltransferase and cysteine protease domain in TcdB. An N-terminus elongated TcdA model was also generated from the uncut RTXvc 3FZY structure and imposed on the 3H06 TcdA structure using methods described (Navaratnarajah et al. *Nat Struct Mol Biol.* 18:128-34 (2011)). For ligand docking studies to the toxin crystal structures, the properties and structures of the inositolphosphate family members were evaluated and the most favorable AutoDock scores were measured and the estimated binding energy was compared to that of InsP₆ as described (Chen et al. *Bioorg Med Chem* 16:7225-33 (2008)).

[091] *Statistical analysis.* Results are presented as mean values + S.E.M. Statistical significance was determined using t-test, Mann-Whitney U test on Ranks and ANOVA on ranks on SigmaStat software, n = 3 unless otherwise stated; P < 0.05 was considered statistically significant.

B. Results

[092] *C. difficile* toxins are molecular targets of S-nitrosylation. Using a well characterized *C. difficile* toxigenic disease model (Oiu et al., *Gastroenterology* 111: 409-18 (1996); Ng et al., *Gastroenterology* 139(2):542-52 (2010)), there was significant pathophysiology when purified TcdA was injected into ileal-loops in Cd-1 mice. TcdA induced significant epithelial cell damage, neutrophil infiltration and edema of the intestinal mucosa, fluid secretion into the intestinal lumen and accumulation of proinflammatory gene transcripts for iNOS, TNF- α and IL-1 γ (FIG. 1A-1B).

[093] To identify S-nitrosylation signals that may regulate disease severity to *C. difficile* toxins, tissue concentrations of S-nitrosoglutathione (GSNO), a small endogenous S-nitrosothiol that constitutes the main source of NO bioactivity in the respiratory (Que et al., *Science* 308:1618-21 (2005)) and gastrointestinal tract (Savidge et al., *Gastroenterology* 132:1344-58 (2007)) where it protects against inflammatory disease were measured. Using HgCl₂-coupled photolysis-chemiluminescence (Que et al., *Science* 308:1618-21 (2005); Hausladen et al., *Proc Natl Acad Sci USA.* 104: 2157-62 (2007)), a 12.1-fold increase in

tissue GSNO concentrations was demonstrated following TcdA-intoxication (84 ± 29.1 versus 1020 ± 475 nM/mg protein, respectively; $p < 0.05$; $\pm$ SD), which correlated with elevated iNOS expression levels (FIG. 1D). The effects of GSNO are mediated primarily by SNO-proteins (Benharefa, *Nat Rev Mol Cell Bio.* 10:721-31 (2009)). Immunofluorescence labeling of SNO-proteins in tissue sections using an anti-nitrosocysteine antibody demonstrated large increases within TcdA-exposed intestinal mucosa, and in particular, apical brush border epithelial staining of intoxicated, but not normal, samples. A similar SNO-protein tissue distribution was evident in biopsies from patients with active colitis, which suggests that epithelial SNO accumulation is also of pathophysiological relevance in the human colon during inflammatory conditions (FIGs. 1E-1F).

[094] The toxin-induced S-nitrosoproteome was characterized using a cysteine saturation fluorescence assay (Pretzer et al., *Analyt. Biochem.* 374:250-62 (2008)). Cysteine saturation labeling and mass fingering of up regulated SNO-proteins identified several species that are known targets of S-nitrosylation, including hemoglobin, cytoskeletal, heat-shock and various cell signaling proteins (FIG. 1G). *C. difficile* infection is therefore associated with increases in tissue S-nitrosylation that reflect toxin-induced pro-inflammatory responses. Cysteine saturation labeling also consistently identified TcdA as a molecular target of S-nitrosylation in inflamed tissues (FIG. 1G), which was confirmed by a biotin-switch assay (Jaffrey et al., *Sci STKE* 12:86 (2001)) that specifically labels Cys-NO adducts (FIG. 1H).

[095] *S-nitrosylation inhibits C. difficile toxin virulence.* Because there is no precedent for *in situ* S-nitrosylation of foreign proteins in host tissues, an *in vitro* model was established to examine the potential significance of toxin S-nitrosylation. To recapitulate the epithelial protein-SNO accumulation observed during *C. difficile* infection, human Caco-2 colonocytes were transfected with a calcium-inducible endothelial nitric oxide synthase construct (pCMV6-eNOS) to transiently raise cellular SNO levels prior to intoxication (Gow et al., *J Biol Chem* 277:9637-40 (2002)) (FIGs. 2A-2C).

[096] Biotin-switch assay and anti-SNO immunoblotting confirmed the *in vivo* findings that toxin recovered from cellular lysates was preferentially S-nitrosylated in pCMV6-eNOS transfected Caco-2 cells, and S-nitrosylation was abolished by addition of the membrane-permeable denitrosylating agent, GSH-ethyl ester (Gow et al., *J Biol Chem* 277:9637-40

(2002)) (FIG. 2D). Toxin-induced cell rounding and viability assays demonstrated that elevated SNO in pCMV6-eNOS transfected Caco-2 cells conferred significant protection against intoxication (FIGs. 2E-2G). Moreover, toxin UDP-glucosylation of RhoGTPases (Popoff et al., *Biochim Biophys Acta*. 88:797-812 (2009)) was significantly reduced in eNOS-transfected cells, indicating an early SNO-based protective mechanism (FIG. 2H). This protective effect was significantly attenuated by GSH-ethyl ester and by inhibition of eNOS enzymatic activity using N (G)-nitro-L-arginine methyl ester (L-NAME) (FIG. 2G). In support of the premise that toxin S-nitrosylation is a physiological inhibitory mechanism of the *C. difficile* toxins, murine iNOS-deficient peritoneal macrophages were also more susceptible to intoxication when compared with wild type cells which express abundant SNO (Kim et al., *Science* 2005 310:1966-70 (2005)) (FIG. 2I).

[097] To confirm that these cytoprotective effects were directly linked to S-nitrosylation of the exotoxin, the S-nitrosylated toxin was assessed in pure form. Purified TcdA was treated with GSNO and formation of SNO was confirmed by biotin-switch assay (FIG. 3A). *In vitro* studies showed that the S-nitrosylated toxin (toxin-SNO) was significantly less cytotoxic when compared with unmodified toxin (FIG. 3A), and the protective effects of S-nitrosylation were abolished by glutathione (GSH) or dithiothreitol (DTT), which denitrosylated the toxin (FIG. 2D). TcdA-SNO was also significantly less cytotoxic *in vivo* in the murine ileal loop model (FIG. 1C). Pathophysiological relevance of these data was further suggested by finding S-nitrosylated toxin in stool samples from patients with *C. difficile* infection, and by the inverse relationship between levels of toxin-SNO and stool cytotoxicity (He et al., *J Microbiol Methods* 78:97-100 (2009)) (FIG. 3B-3D). Thus, toxin S-nitrosylation is both physiologically and functionally significant.

[098] *Allosteric inositolphosphate potentiates S-nitrosylation of the toxin cysteine protease active site.* The S-nitrosylated residues responsible for inactivation of *C. difficile* toxins was investigated. Cysteine residues targeted by S-nitrosylation often conform to an exposed acid-base consensus motif (Marino et al., *J Mol Biol* 395: 844-59 (2010)). There are seven cysteine residues in TcdA and nine in TcdB, with four being conserved between the two toxins. Because cysteine proteases may be regulated by S-nitrosylation of the cysteine-histidine catalytic dyad¹⁷, whether the toxin cysteine protease domain is a preferred target for posttranslational modification by GSNO was examined. High resolution crystal structures of

the TcdA (Pruitt et al., *J Biol Chem* 284:21934-40 (2009)) and TcdB (Puri et al., *Chem Biol.* 17:1201-11 (2010)) cysteine protease domains, and the closely aligned *Vibrio cholerae* RTX toxin (Lupardus et al., *Science* 322:265-68 (2008); Prochazkova et al., *J Biol Chem* 284:26557-68 (2009)), show a well-defined catalytic cleft that is separated from a positively charged InsP₆ binding pocket abutting a β -hairpin fold (β -flap) (FIG. 4A). Data confirmed that InsP₆ binds to *C. difficile* TcdA holotoxin, yielding an equilibrium binding affinity constant (K_d) of 62.6 $\pm$ 7.0 nM, which is in close agreement with the AC₅₀ concentration for the TcdA cysteine protease domain (Pruitt et al., *J Biol Chem* 284: 21934-40 (2009)) (FIG. 4B). *In silico* determination of the binding energies of the various inositolphosphate family members to the allosteric binding pocket in TcdA and TcdB demonstrated good docking scores for InsP₆, and even better values for InsP₇, a member of the higher inositolpyrophosphates (Chakraborty et al., *Cell* 143:897-910 (2010)) (FIG. 4C). The prevailing binding affinity of InsP₇ was confirmed experimentally by demonstrating significantly greater toxin autocleavage activity for InsP₇ than for InsP₆ (FIG. 4D). Moreover, the enhanced cleavage activity of InsP₇ was not due to toxin autophosphorylation as a result of the high energy pyrophosphate bond. Thus, the phylogenically ancient inositolpyrophosphates may play an important role in regulating *C. difficile* toxin virulence, perhaps via privileged access to the toxin at the plasma membrane (Chakraborty et al., *Cell* 143:897-910 (2010)).

[099] Because of the heterotropic nature of the inositolphosphate interaction with the toxin through specific binding to the cysteine protease domain (Prochazkova et al., *J Biol Chem* 283:23656-64 (2008); Popoff et al., *Biochim Biophys Acta.* 88:797-812 (2009); Pruitt et al., *J Biol Chem* 284:21934-40 (2009)), the enzymatically active inositolphosphate bound form appears to be the preferential target for S-nitrosylation. Multiple lines of experimental evidence supported this view: (i) InsP₆ and InsP₇ potentiated S-nitrosylation of TcdB by GSNO (FIG. 4E), and this effect was abolished by site-directed mutation of Cys698 to alanine in TcdB (FIG. 4F); (ii) Standard *in vitro* (FIGs. 4G-4H) and realtime toxin cleavage assays demonstrated that GSNO rapidly inhibits InsP₆ induced toxin self-cleavage with an IC₅₀ of 12.9 $\pm$ 4.2 μ M for TcdB (analogous to inhibition by N-ethylmaleimide and novel peptide analogues that inhibit TcdB virulence via covalent modification of the catalytic cysteine(Puri et al., *Chem Biol.* 17:1201-11(2010)), only GSNO is an endogenous species),

(iii) cysteine-specific cyanylation of *C. difficile* toxins using nitro-thiocyanobenzoic acid (NTCB)-based cleavage assays (Tang et al., *Analyt Biochem* 334: 48-61 (2004)) identified TcdA Cys700 and TcdB Cys698 as preferential cleavage fragments following InsP₆ treatment. GSNO inhibited this cleavage reaction demonstrating that these cysteine residues are modified by S-nitrosylation and are, as such, not amenable to cyanylation; (iv) S-nitrosylated cysteine protease domain peptide fragments were preferentially identified by mass spectrometry after InsP₆ and GSNO co-treatment; (v) Significant SNO inhibition of TcdB-autocleavage is evident in eNOS-Caco-2 cells, demonstrating that S-nitrosylation prevents the intracellular release of the toxin N-terminus effector domain, which is dependent on inositolphosphate binding; (vi) GSNO treatment of toxin significantly inhibited InsP₆ binding to the cysteine protease domain, an effect that was reversed by UV-cleavage of the SNO bond (FIG. 4B). This 'linkage' indicates that S-nitrosylation of the active site cysteine residue regulates communication with the InsP₆ binding pocket, possibly by disordering the β -flap (Lupardus et al., *Science* 322: 265-268 (2008); Pruitt et al., *J Biol Chem* 284: 21934-40 (2009); Prochazkova et al., *J Biol Chem* 284:26557-68 (2009); Kreimeyer et al., *Naunyn Schmiedebergs Arch Pharmacol.* 2010 Nov 3). (vii) QSNO did not inhibit toxin binding to Caco-2 cells or alter glucosyltransferase activity in cell lysates. Taken together, these data indicate that inositol phosphate cofactors enable GSNO to specifically inhibit *C. difficile* toxin via S-nitrosylation of the cysteine protease catalytic residue, and that S-nitrosylation of the active site Cys has the added effect of displacing the allosteric activator. Thus, GSNO inhibits the toxin by a novel dual orthosteric and allosteric mechanism of action. Notably, several ions (Ca^{2+} , Mg^{2+} , H^{+}) and O₂/redox have been shown to allosterically regulate protein S-nitrosylation (Hess, et al., *Nature Rev* 6: 150-166 (2005)), and NO binding to Cys93 in hemoglobin, which provides vasodilatory activity upon deoxygenation (Hess, et al., *Nature Rev* 6: 150-166 (2005); MW et al., *Trends Mol Med* 15:391-404 (2009)), is a prototypic example of allosteric regulation, with predicted consequences for InsP₆ binding (McMahon et al., *J Biol Chem* 275:16738-45 (2000)). Taken together, these data raise the idea that crosstalk between NO signaling and the inositolphosphate family may be more widespread.

[0100] *A novel toxin S- nitrosylation-catalytic motif.* The premise that *C. difficile* toxins are regulated by S-nitrosylation is strengthened further by *in silico* docking studies of GSNO to the toxin cysteine protease crystal structure, which predicts an excellent alignment of the

S-NO bond with the active site cysteine which forms part of an exposed acid-base S-nitrosylation consensus motif (FIG. 5A). This dual S-nitrosylation-catalytic motif is structurally conserved amongst microbial cysteine proteases, with glutamic acid (Glu) and histidine (His) residues juxtaposing the catalytic Cys700 in TcdA, Cys698 in TcdB, Cys3568 in *V. cholera* RTX toxin (Prochazkova et al., *J Biol Chem* 284:26557-68 (2009)) and Cys244 in gingipain R (Eichinger, et al., *EMBO J* 18:5453-62 (1999)) (FIGs. 5A-5B). Genetic disruption of this S-nitrosylation motif in TcdB by site-directed mutagenesis of Glu743 to alanine diminished the ability of GSNO to transnitrosylate the toxin (FIG. 5Q). However, site-directed mutagenesis of the catalytic His653 to alanine in TcdB played a relatively minor role in this transnitrosylation reaction (FIG. 5C). This observation is consistent with the unusually large distances ($>6 \text{ \AA}$) between the catalytic cysteine and histidine in all of the toxin cysteine protease crystal structures (Pruitt et al., *J Biol Chem* 284: 21934-40 (2009)). It is more likely that this histidine residue plays a role in substrate orientation within the active site rather than conferring nucleophilicity to the cysteine thiolate, with the catalytic aspartic acid stabilizing the histidine imidazolium ring (FIG. 5D). Site-directed mutagenesis of His to alanine in TcdB confirmed its role in this catalytic activity (FIG. 5E). Moreover, a potential regulatory role for Glu743 in the cysteine protease active site is predicted via hydrogen bonding and modulation of catalytic cysteine thiolate reactivity (FIG. 5D). Mutagenesis of Glu743 to alanine in TcdB confirmed its role in actively regulating toxin self-cleavage in the presence of inositol phosphate cofactor (FIGs. 5E-5G). Thus, this highly conserved regulatory residue appears to facilitate an allosteric-switch mechanism (Cui et al., *Protein Science* 17:1295-1307 (2008)), possibly to restrict toxin self-cleavage in response to situational exposure to InsP_6 cofactor which can reach micromolar concentrations in the extracellular gut environment from dietary sources (Bohn et al., *J Zhejiang Univ Sci B* 9:165-191 (2008); Letcher et al., *Biochem J* 416:261-270 (2008)).

[0101] *Therapeutic allostery of the C. difficile toxins.* Metronidazole and vancomycin can effectively treat *C. difficile* infection, but the association of these drugs with high relapse rates represents a major health problem. These considerations necessitate the development of alternative non-antibiotic therapeutic strategies that can inactivate the exotoxin activity. The observation that S-nitrosylation inhibits toxin self-cleavage and that GSNO likely serves as

the endogenous S-nitrosylating agent prompted the testing of whether GSNO can be exploited therapeutically to confer protection against *C. difficile* infection.

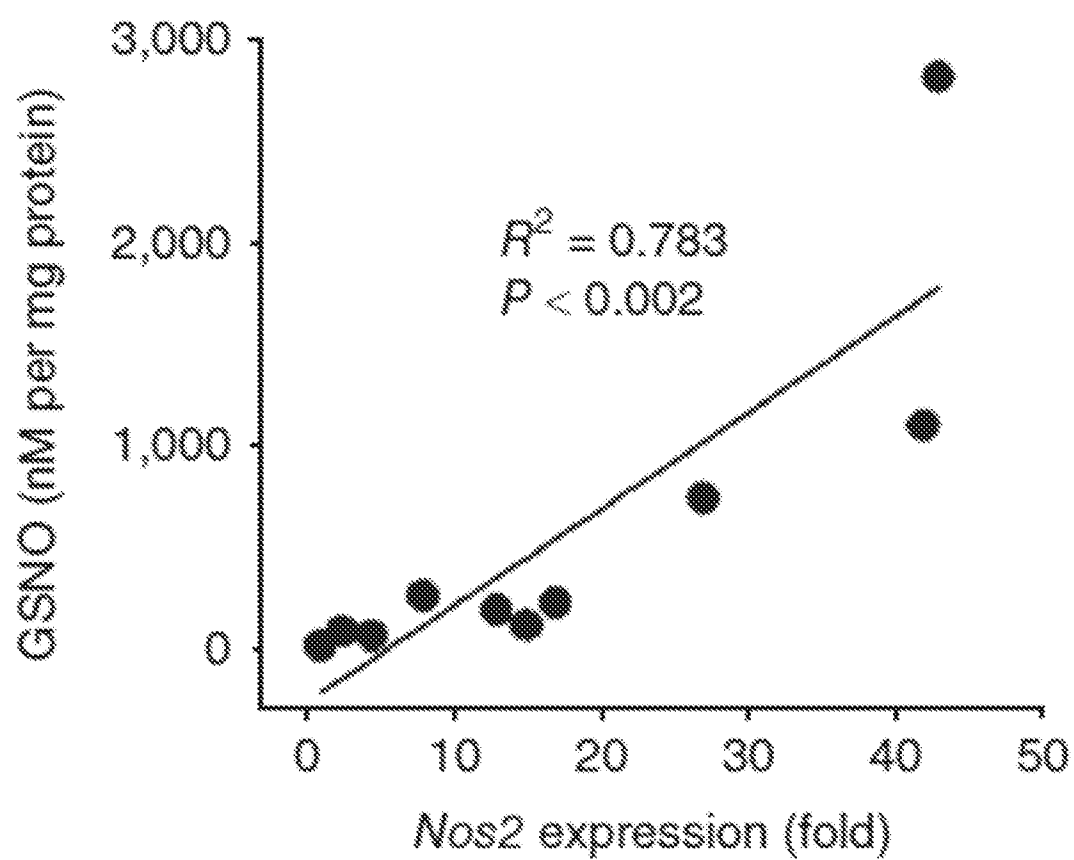
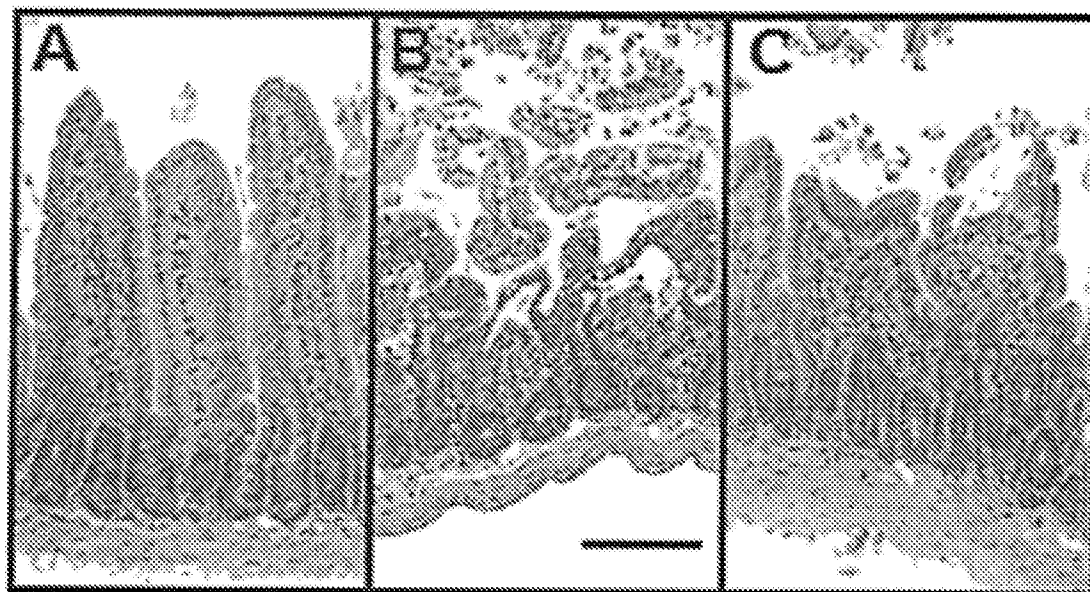
[0102] *In vitro* studies demonstrated that exogenous GSNO dose-dependently S-nitrosylated epithelial cell proteins (FIG. 6A), and prevented cytotoxicity in Caco-2 cells with a half-maximum inhibition concentration (IC₅₀) of 57.9±13.7 and 46.3±7.1 μM for TcdA and TcdB, respectively (FIG. 6B). Inhibition of toxin activity was reversed by GSH- or dithiothreitol-mediated protein denitrosylation, and cytoprotection was greatly enhanced by the addition of inositolphosphate cofactor, which reduced the IC₅₀ of GSNO into the low micromolar range (FIG. 6B). Using the Cd-1 murine loop model, exogenous GSNO markedly reduced toxin-induced disease activity, including a significant inhibition of histological damage, inflammation and intestinal secretion when co-injected with TcdA into ileal loops for 4 hrs (FIGs. 6C-6D). In addition, as was evident in the *in vitro* studies, InsP₆ enhanced the therapeutic actions of exogenous S-nitrosothiol *in vivo* (FIGs. 6C-6D).

[0103] The therapeutic efficacy of GSNO was also tested in a murine infectious disease model that closely mimics the human disease (Chen et al., *Gastroenterology* 135:1984-92 (2008)). Kaplan-Merier survival plots of infected mice demonstrated a survival benefit of GSNO that was potentiated by InsP₆ (FIG. 6E). Thus, the most significant protection was evident in animals that received both oral GSNO and InsP₆, and this was potentiated further by direct therapeutic delivery into the cecum (FIGs. 6E-6F). Synergistic benefits of GSNO and InsP₆ identify the toxin as a primary locus of GSNO action.

CLAIMS

1. A method for ameliorating the pathophysiology of a microbial cysteine protease exotoxin in a subject comprising, administering to the subject an effective dose of an S-nitrosylating agent and an inositol phosphate or analog thereof.
2. The method of claim 1, wherein the S-nitrosylating agent is a nitrosylator, nitrosylation potentiator, or a nitric oxide generator.
3. The method of claim 1, wherein the nitrosylator is S-nitrosoglutathione.
4. The method of claim 2, wherein the potentiator is N acetyl cysteine.
5. The method of claim 2, wherein the nitric oxide generator comprises (a) a nitrate and (b) glutathione and/or N-acetylcysteine.
7. The method of claim 5, wherein the nitrate is an organic nitrate.
8. The method of claim 7, wherein the organic nitrate is ethyl nitrite or amyl nitrite.
9. The method of claims 1 to 8, wherein the S-nitrosylating agent is administered in an amount of about 1 μ M to about 1 mM.
10. The method of claim 1 to 9, wherein the exotoxin is a *Clostridium* exotoxin.
11. The method of claim 10, wherein the exotoxin is a *Clostridium difficile*, *Clostridium sordellii*, *Clostridium novyi*, *Clostridium botulinum*, *Clostridium perfringens*, or *Clostridium tetani* exotoxin.
12. The method of claim 1, wherein the exotoxin is *Vibrio cholera* RTX, gingipains, CPD_{MARTX}, or CDP_{adh} exotoxin.
13. The method of claim 1 to 12, wherein the inositol phosphate is a hexakisphosphate, or inositol pyrophosphate.
14. The method of claim 1 to 13, wherein the inositol phosphate or analog thereof is administered in an amount of about 1 μ M to about 10 mM.

15. The method of claim 1 to 14, wherein the S-nitrosylating agent and the inositol phosphate or analog thereof are comprised in the same pharmaceutical composition.
16. The method of claim 15, wherein the composition is formulated for delivery to the gastrointestinal tract.
17. A method of treating a *Clostridium* infection in a subject comprising administering to the subject an effective dose of an S-nitrosylating agent and an inositol phosphate or analog thereof.
18. The method of claim 17, wherein the *Clostridium* is *Clostridium difficile*, *Clostridium sordellii*, *Clostridium novyi*, *Clostridium botulinum*, *Clostridium perfringens*, or *Clostridium tetani*.
19. A pharmaceutical composition comprising an S-nitrosylating agent and an inositol phosphate or analog thereof.
20. The pharmaceutical composition of claim 19, wherein the inositol phosphate is a hexakisphosphate, or inositol pyrophosphate.
21. The pharmaceutical composition of claim 19, wherein the S-nitrosylating agent is S-nitrosoglutathione, nitrite, glutathione, N-acetylcysteine, a GSNO reductase inhibitor, N-acetylcysteine, and/or phytocheletins.
22. The pharmaceutical composition of claim 19, wherein the S-nitrosylating agent comprises (a) a nitrate and (b) glutathione and/or N-acetylcysteine.
23. The pharmaceutical composition of claim 22, wherein the nitrate is an organic nitrate.
24. The pharmaceutical composition of claim 23, wherein the organic nitrate is ethyl nitrite or amyl nitrite.

**FIG. 1D**

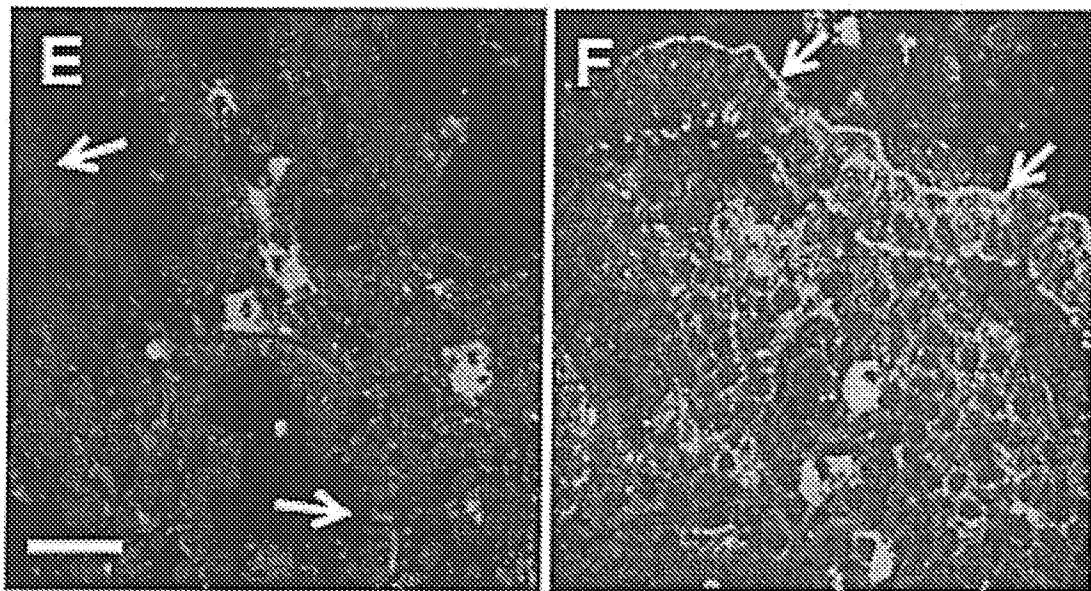


FIG. 1E

FIG. 1F

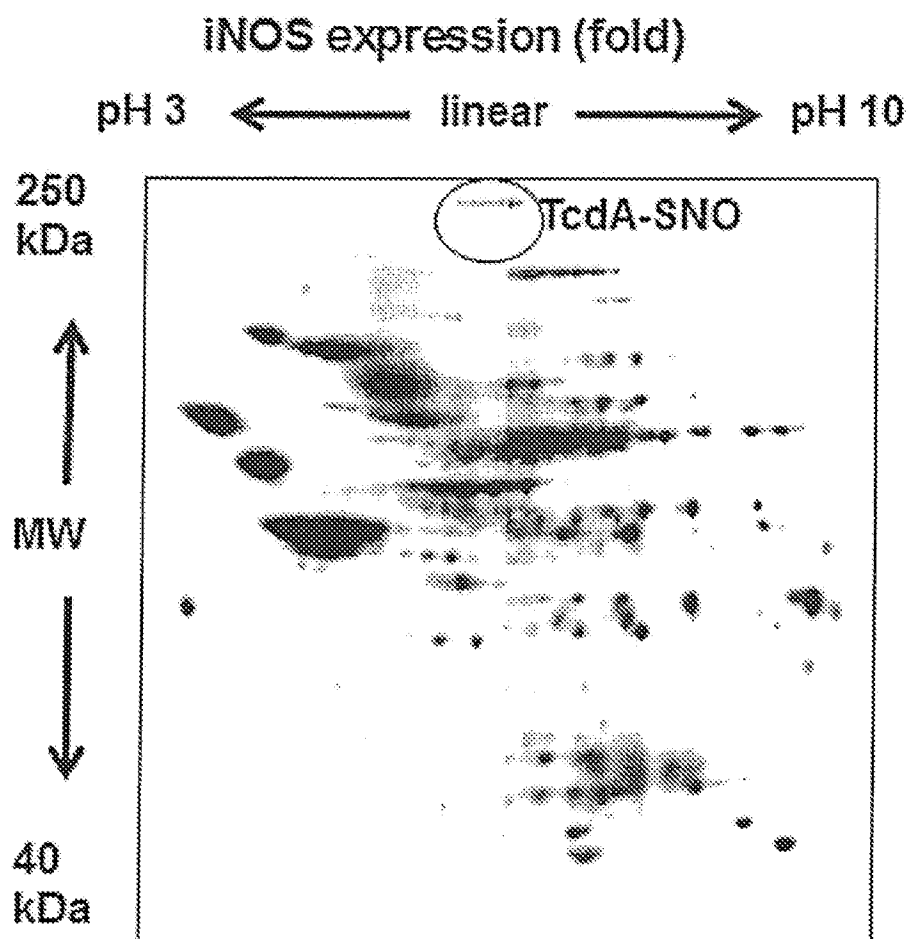


FIG. 1G

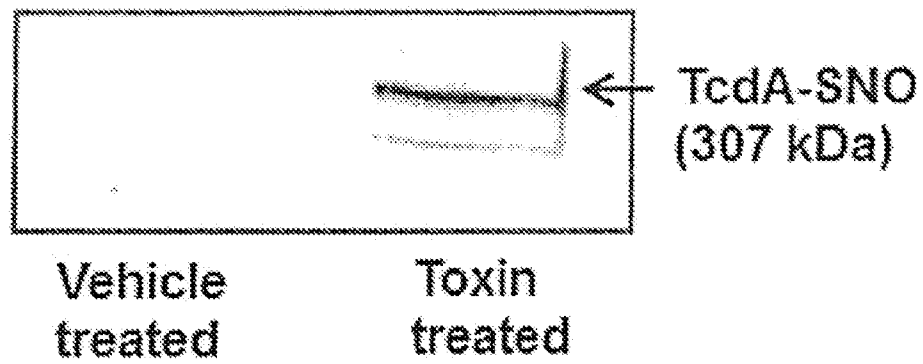


FIG. 1H

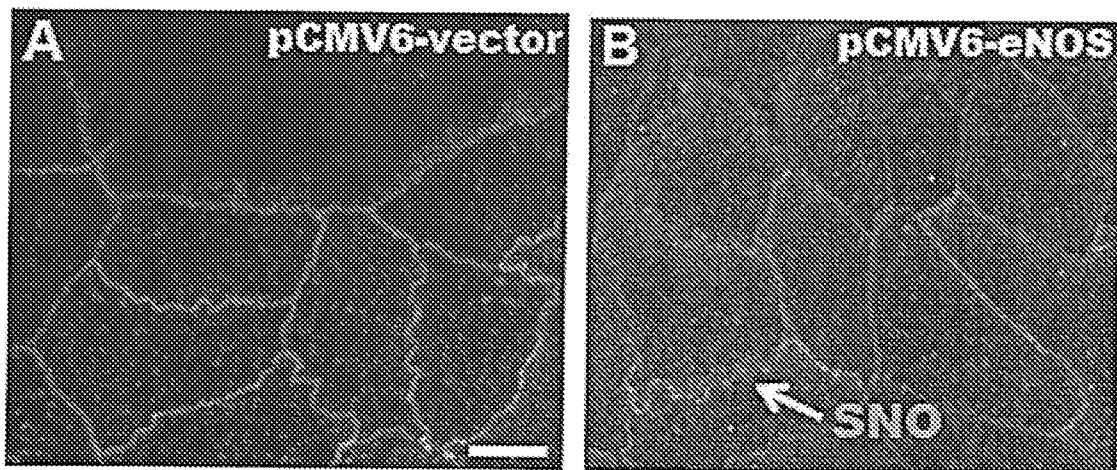


FIG. 2A

FIG. 2B

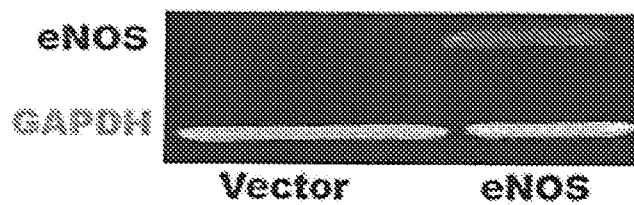


FIG. 2C

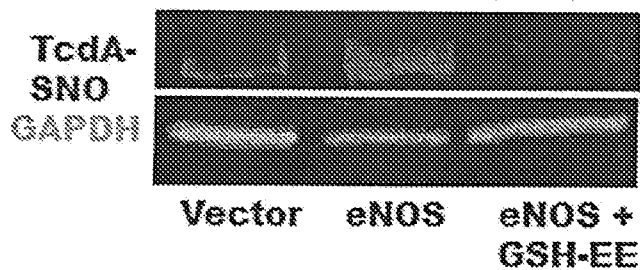


FIG. 2D

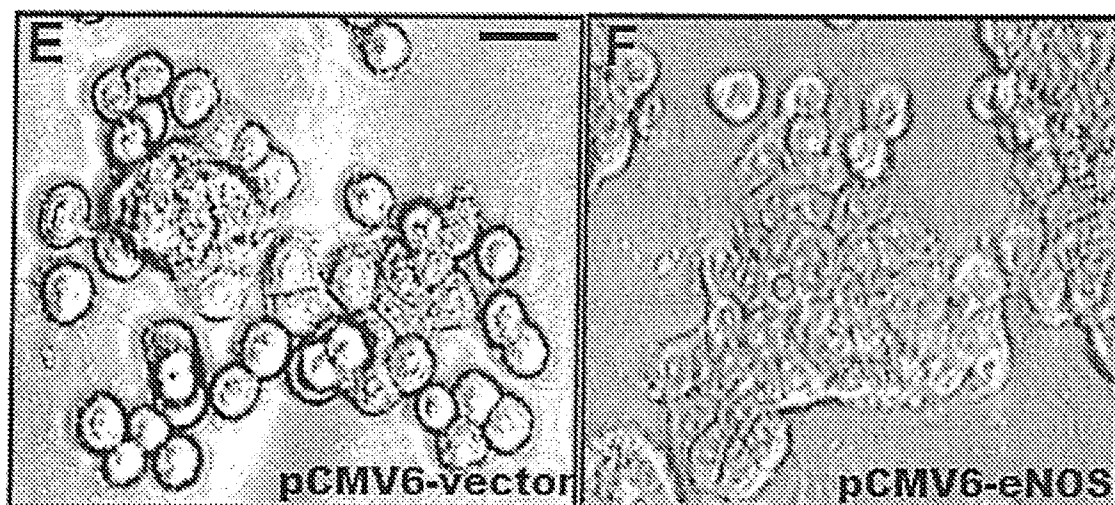


FIG. 2E

FIG. 2F

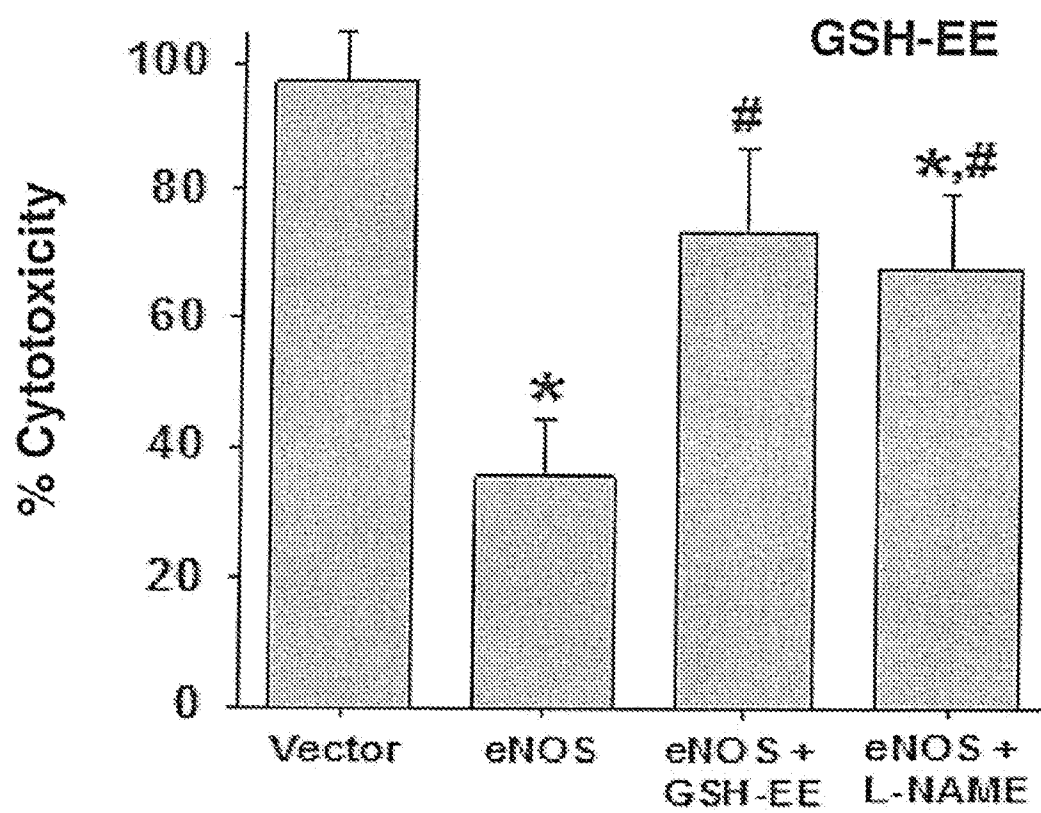


FIG. 2G

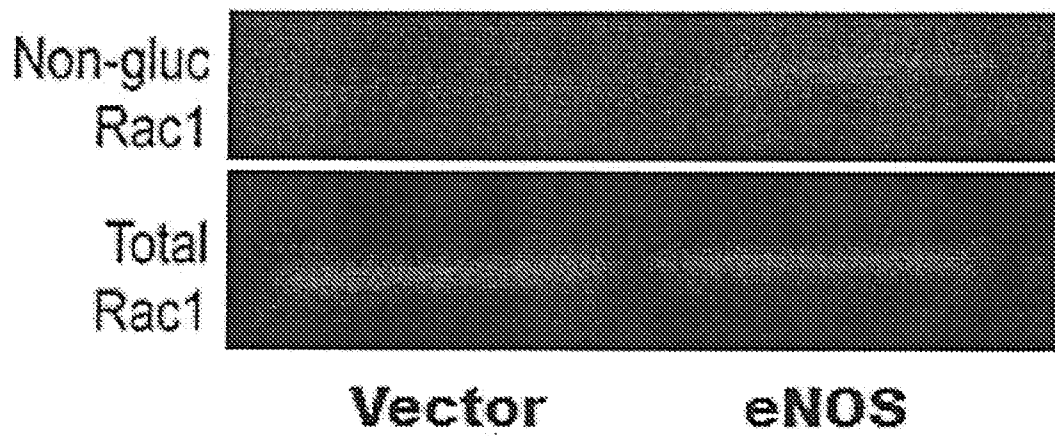


FIG. 2H

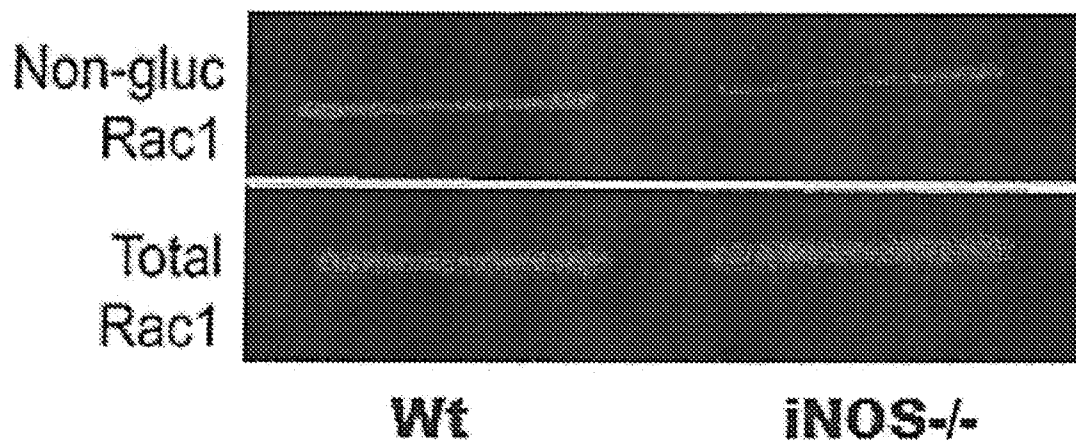


FIG. 2I

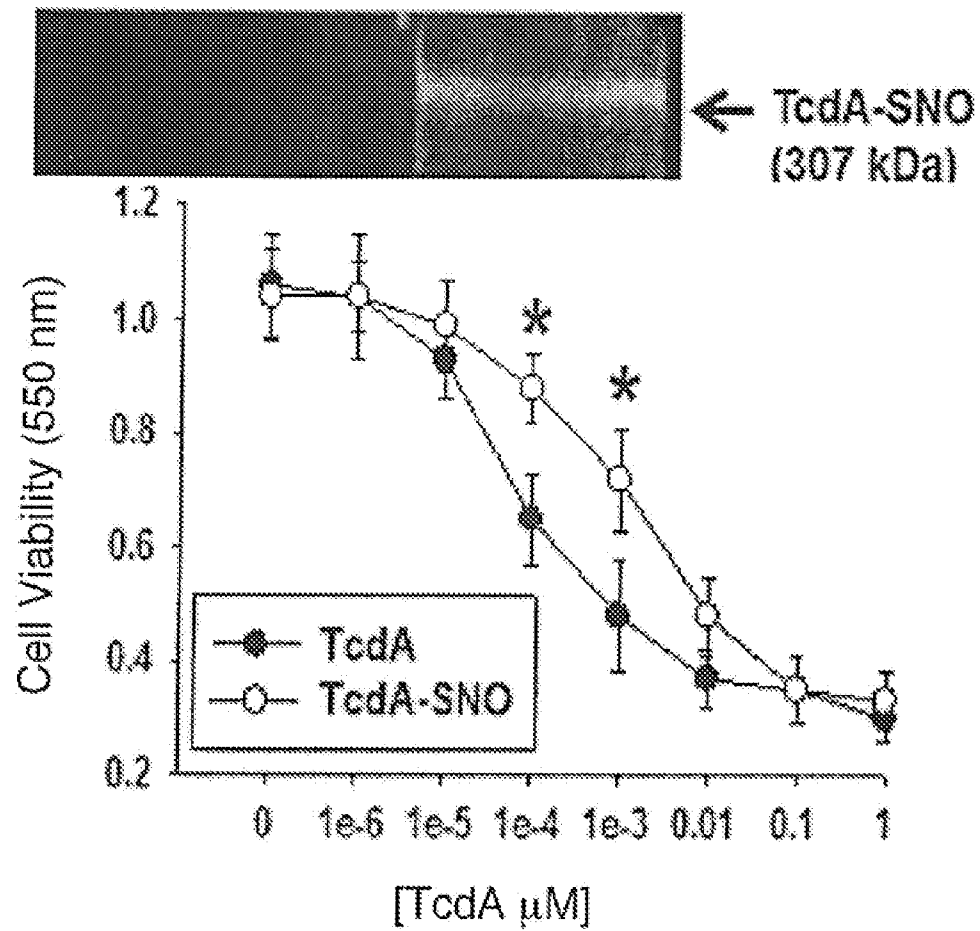


FIG. 3A

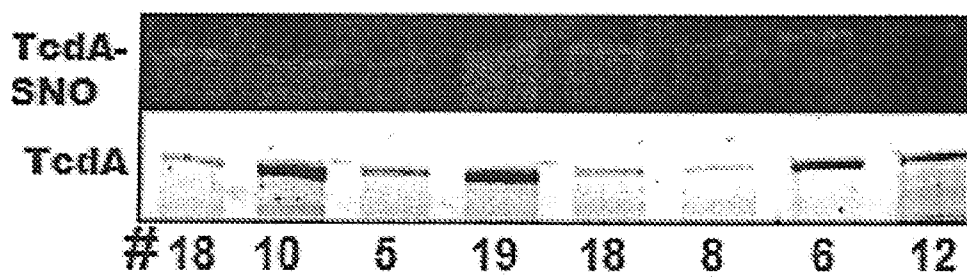


FIG. 3B

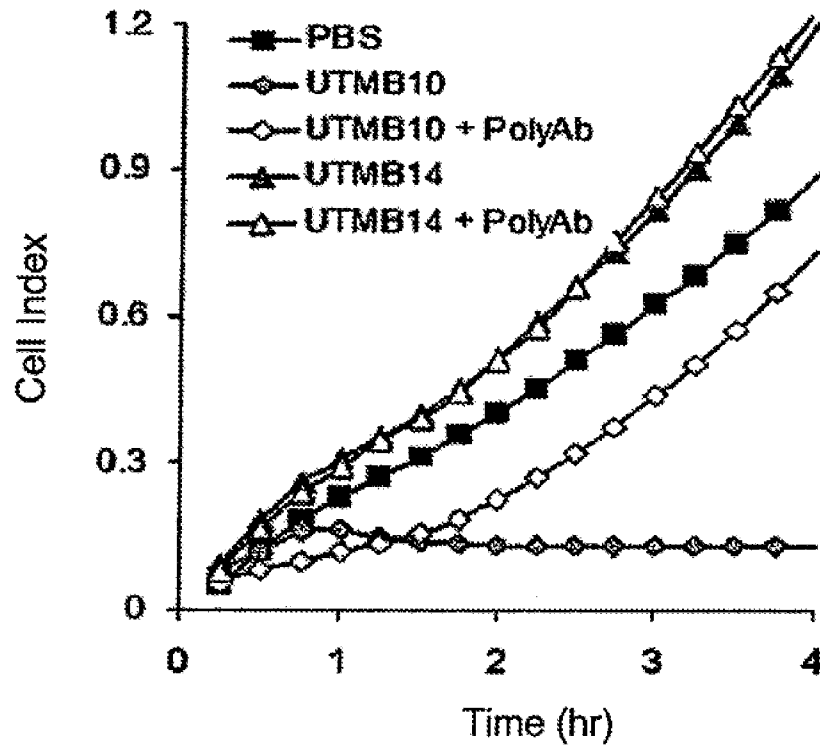


FIG. 3C

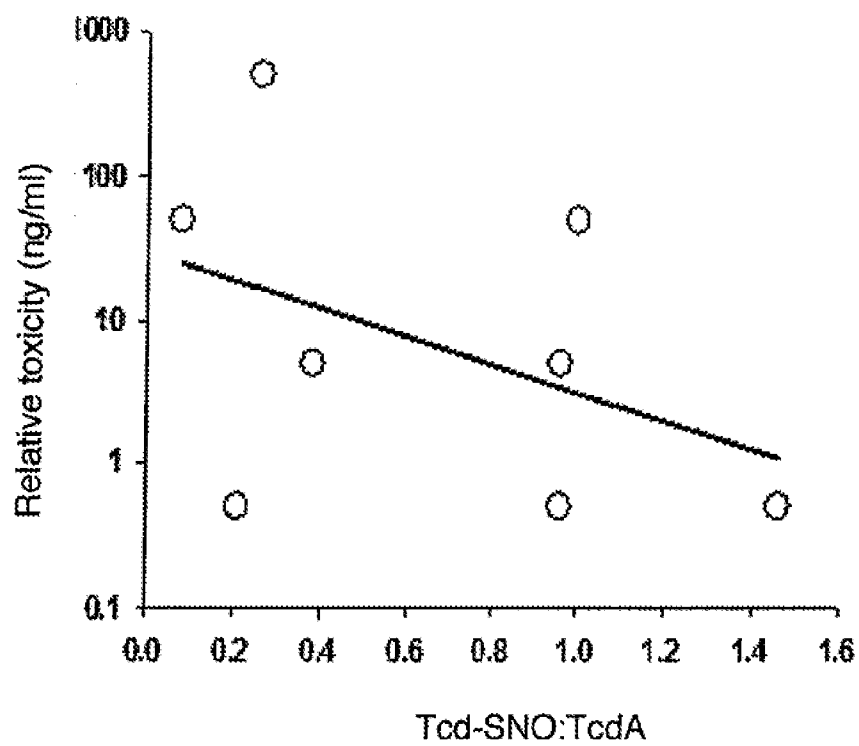


FIG. 3D

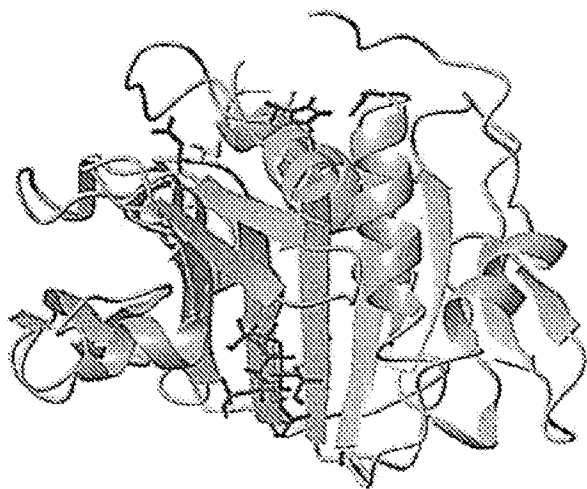


FIG. 4A

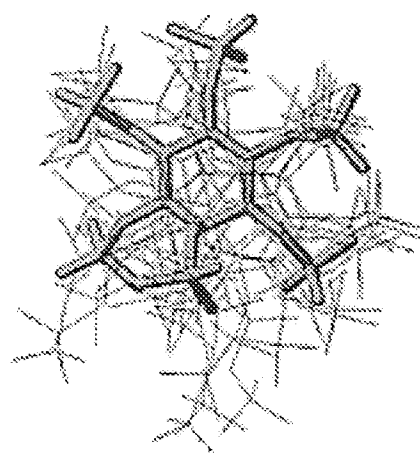


FIG. 4C

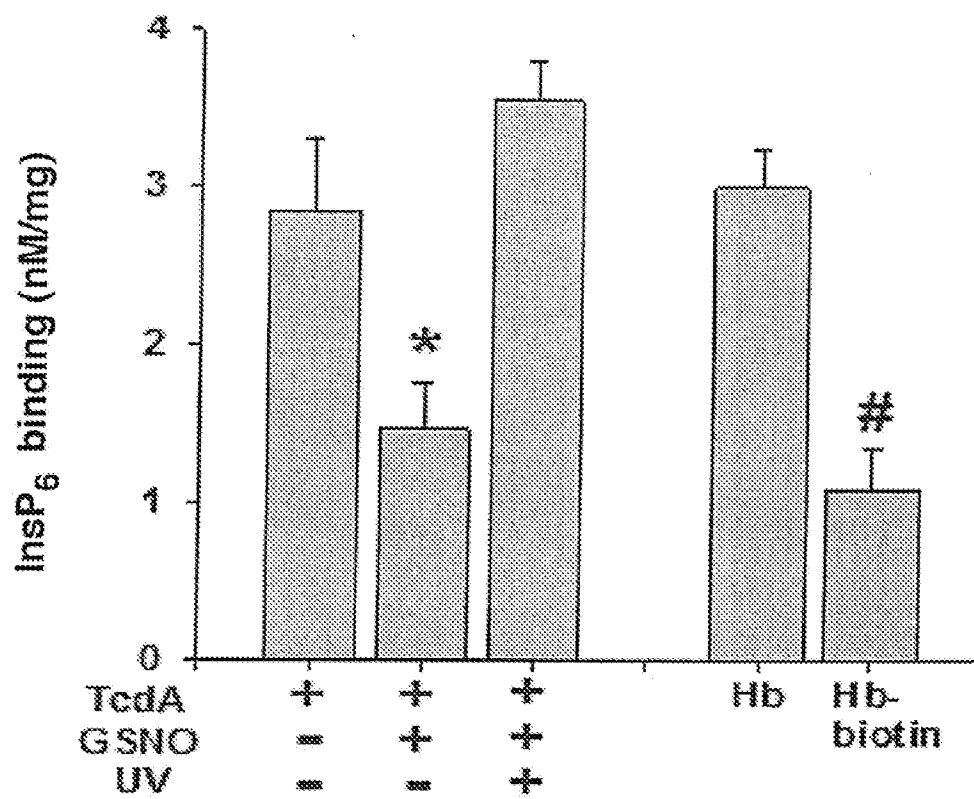


FIG. 4B

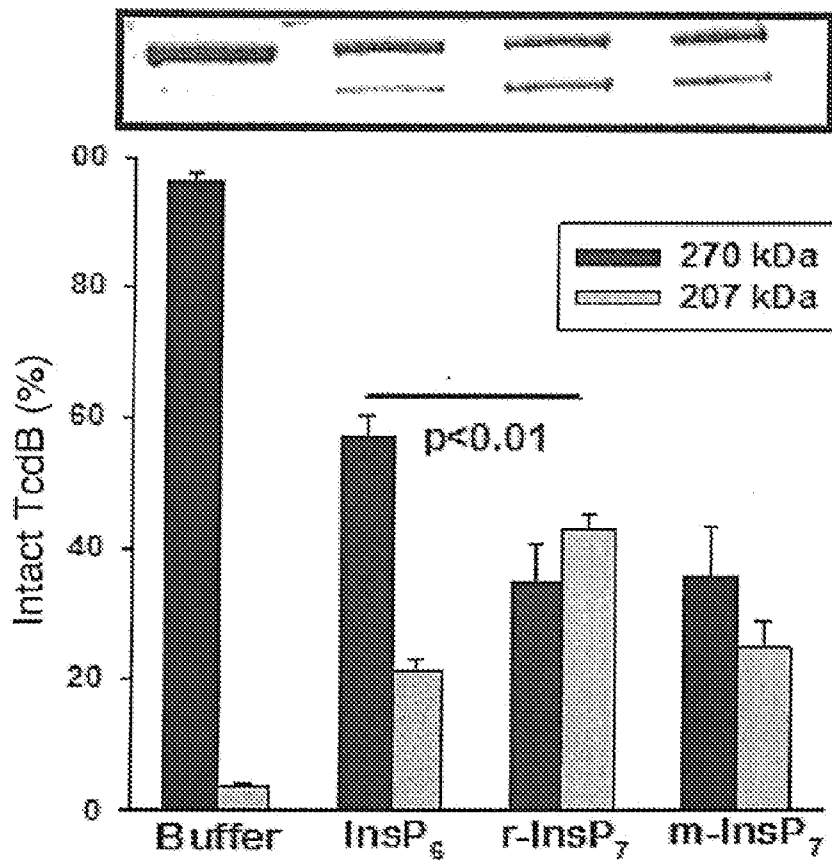


FIG. 4D

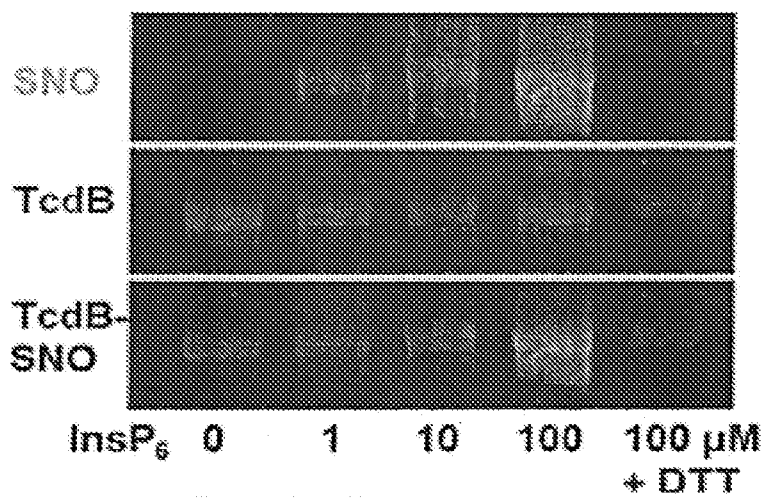


FIG. 4E

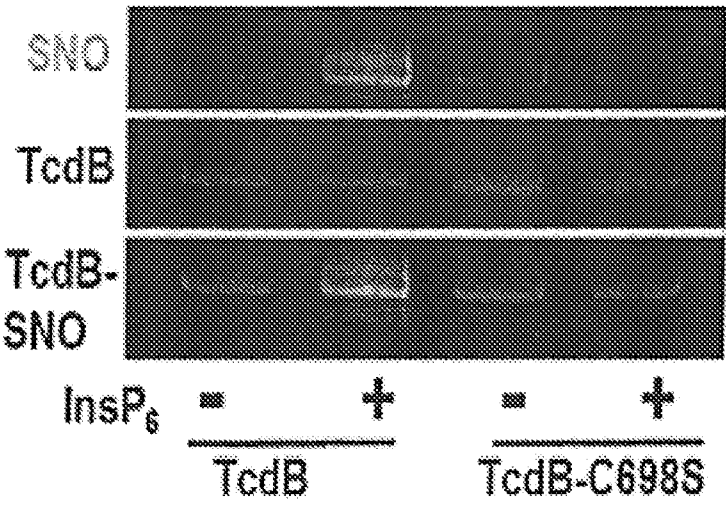


FIG. 4F

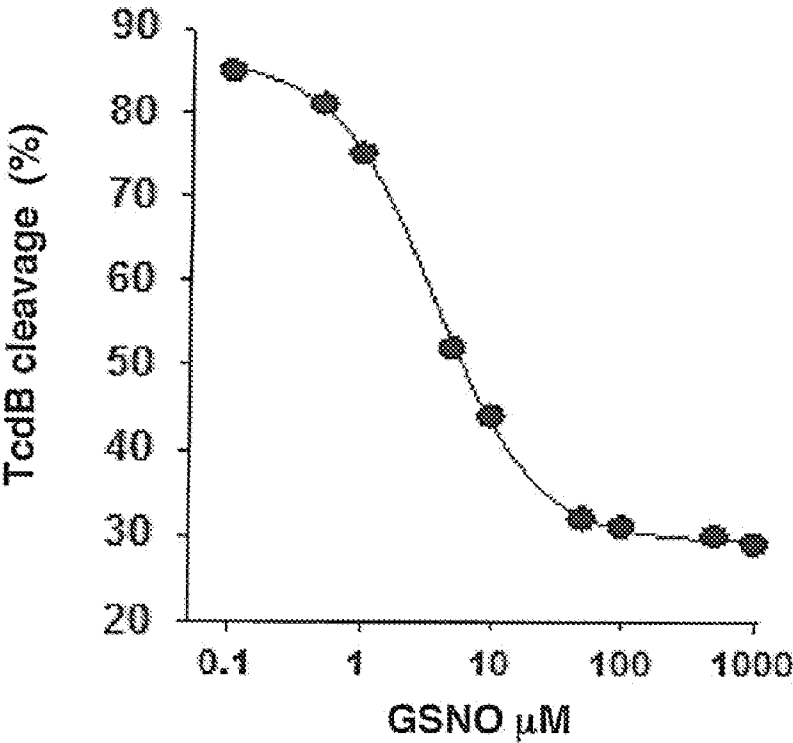


FIG. 4G

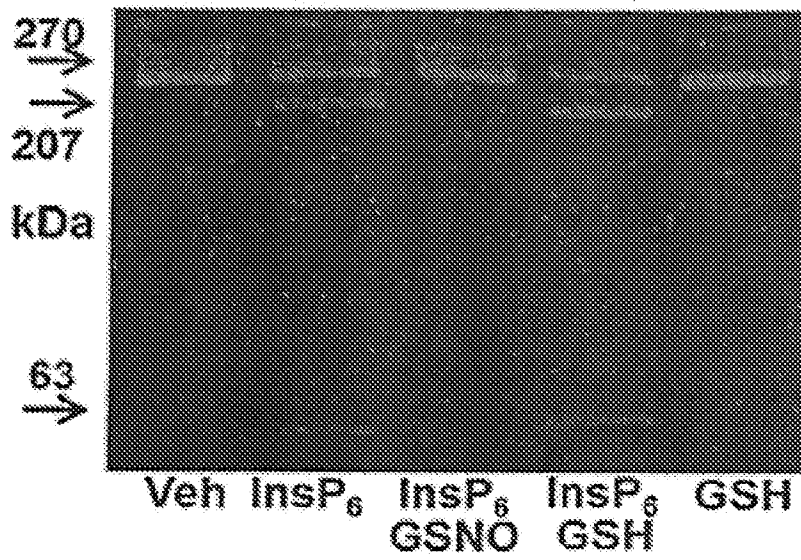


FIG. 4H

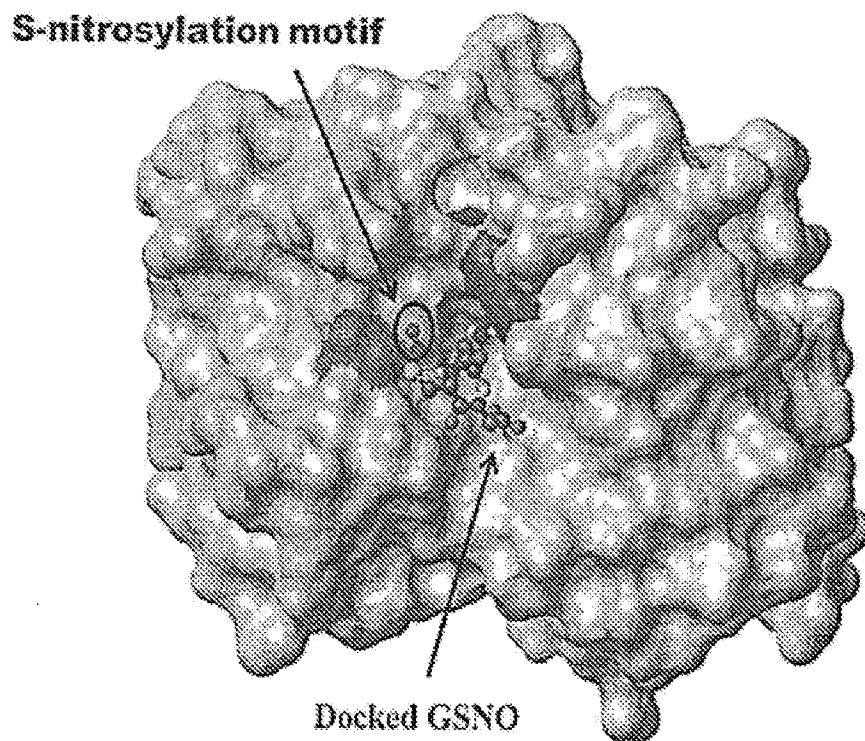


FIG. 5A

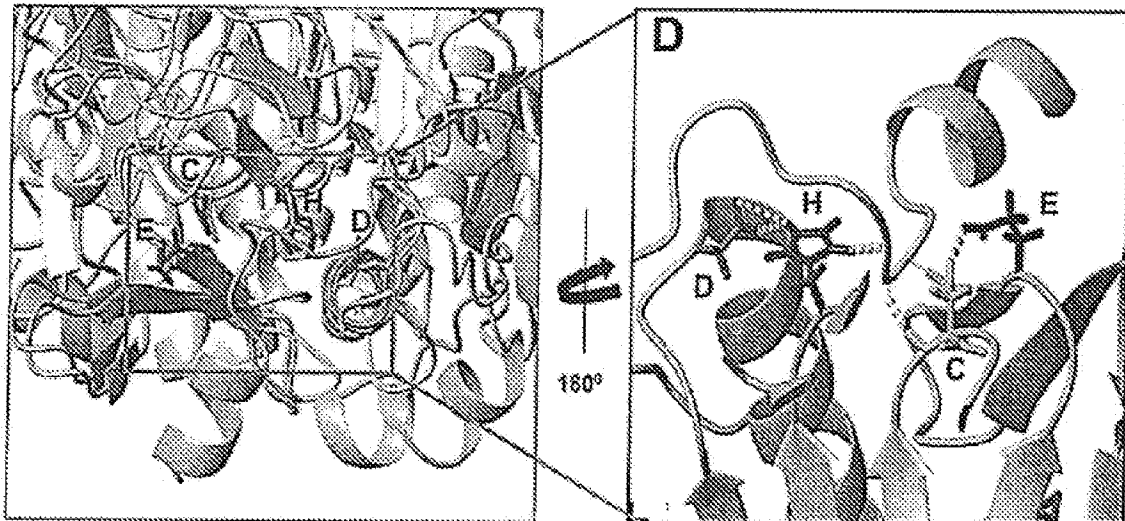


FIG. 5B

FIG. 5D

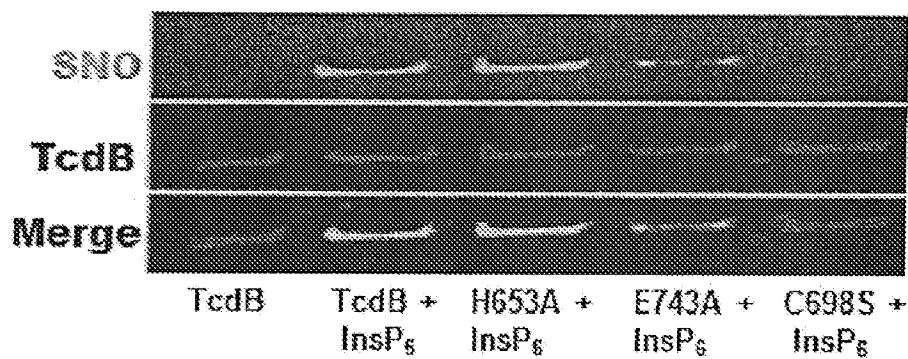


FIG. 5C

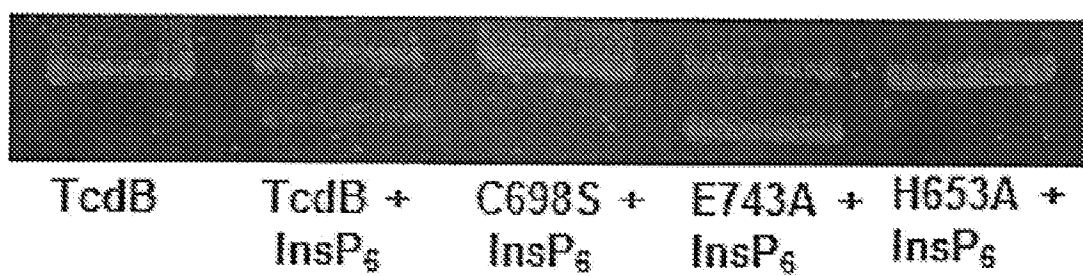


FIG. 5E

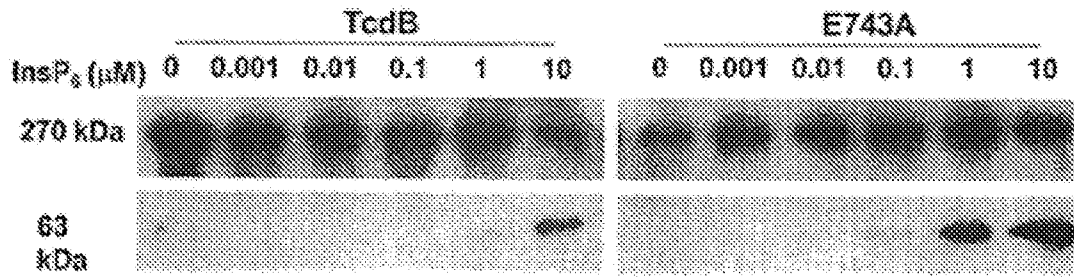


FIG. 5F

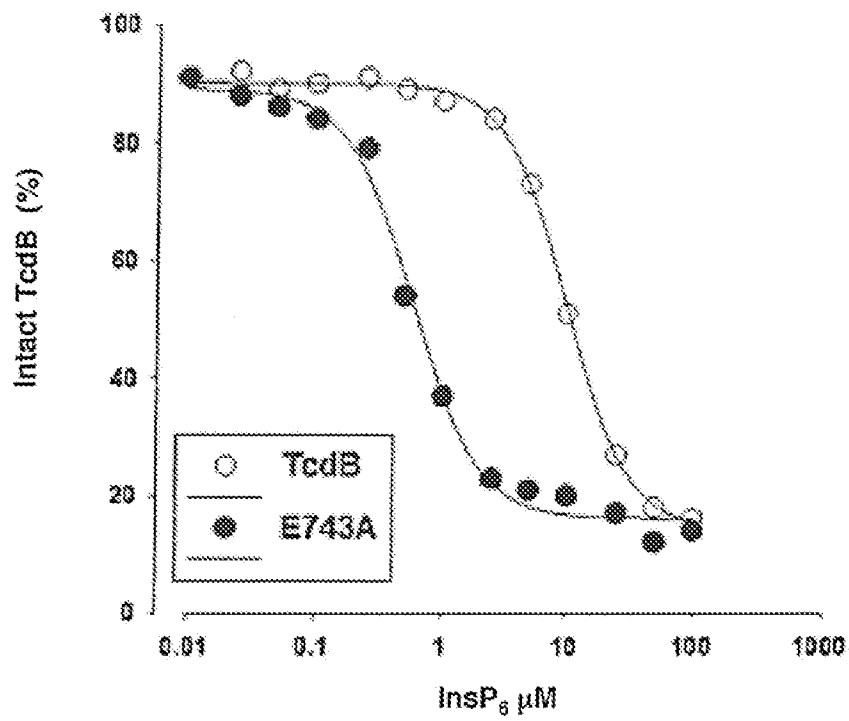


FIG. 5G

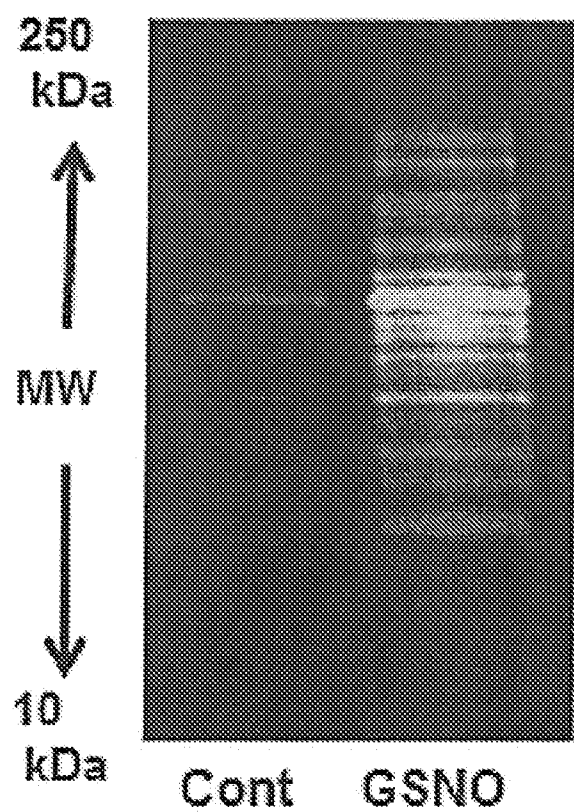


FIG. 6A

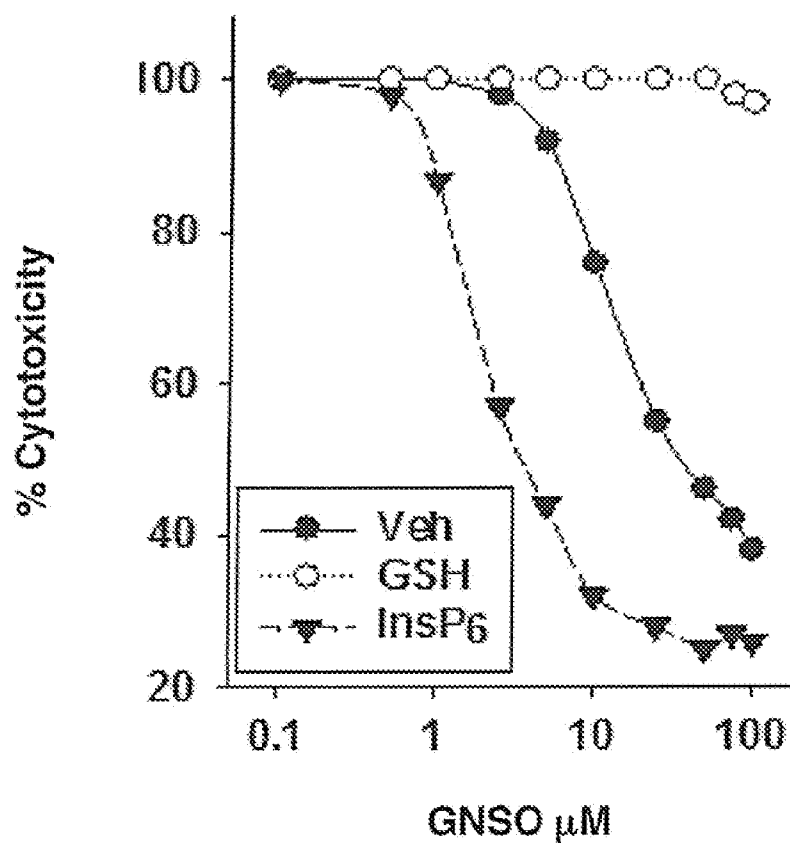


FIG. 6B

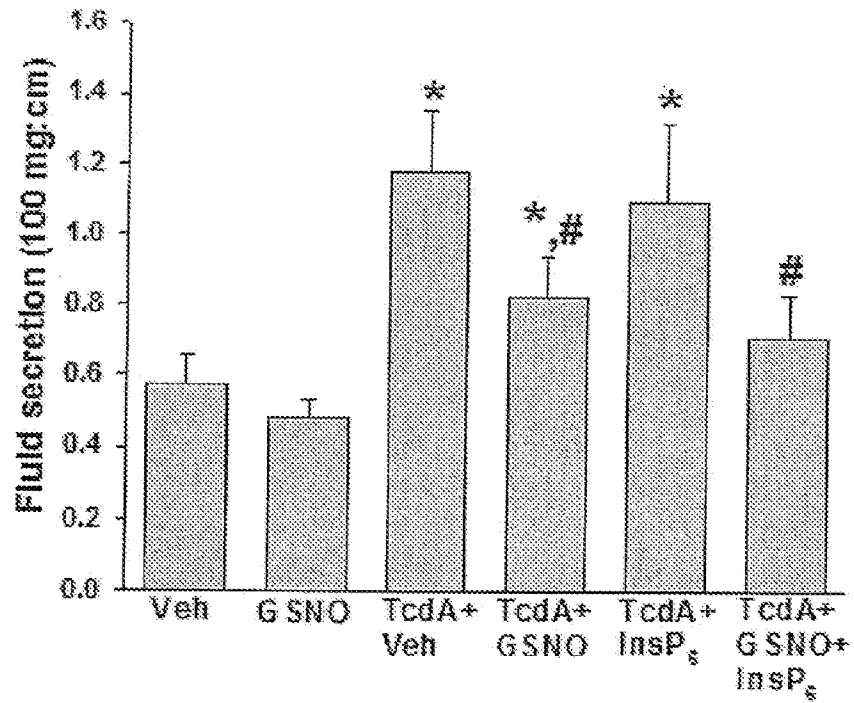


FIG. 6C

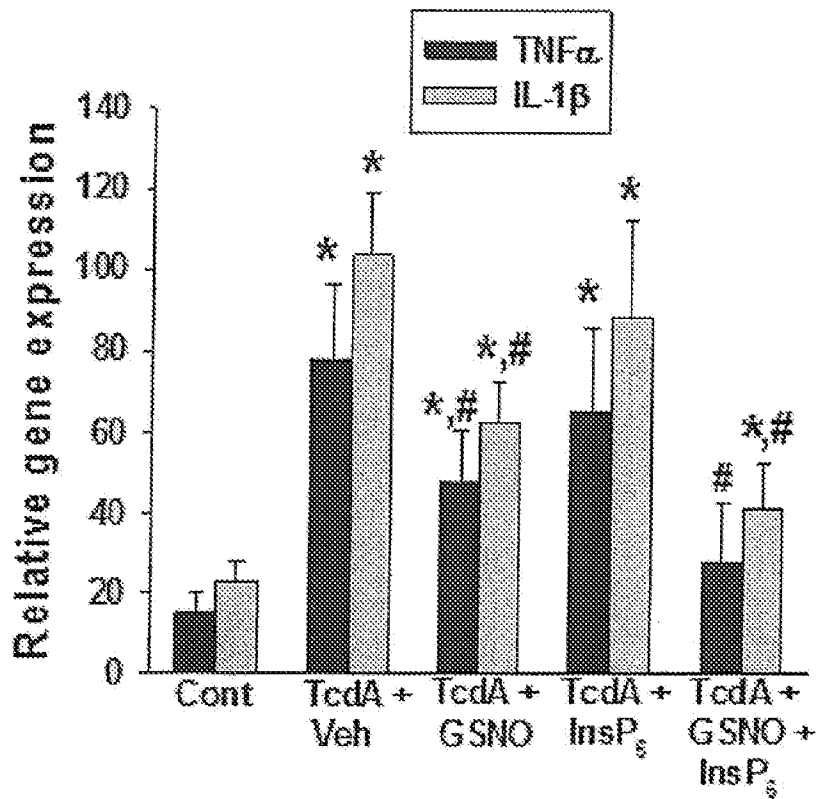


FIG. 6D

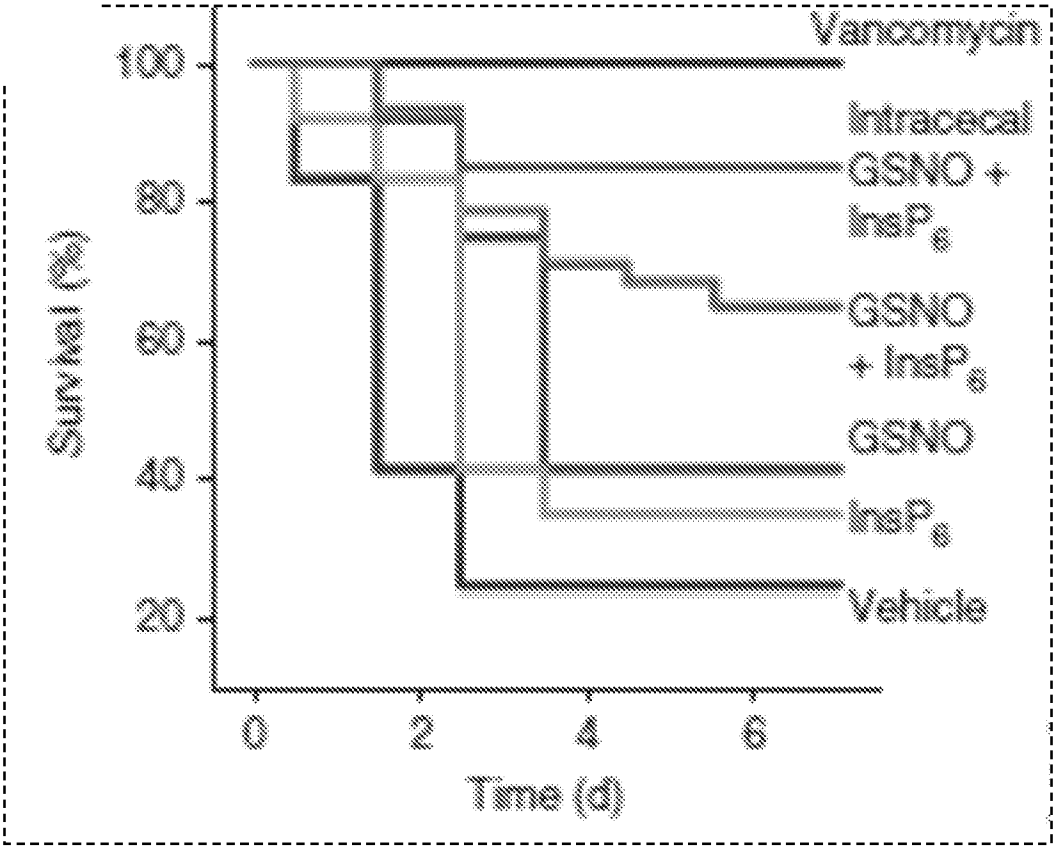


FIG. 6E

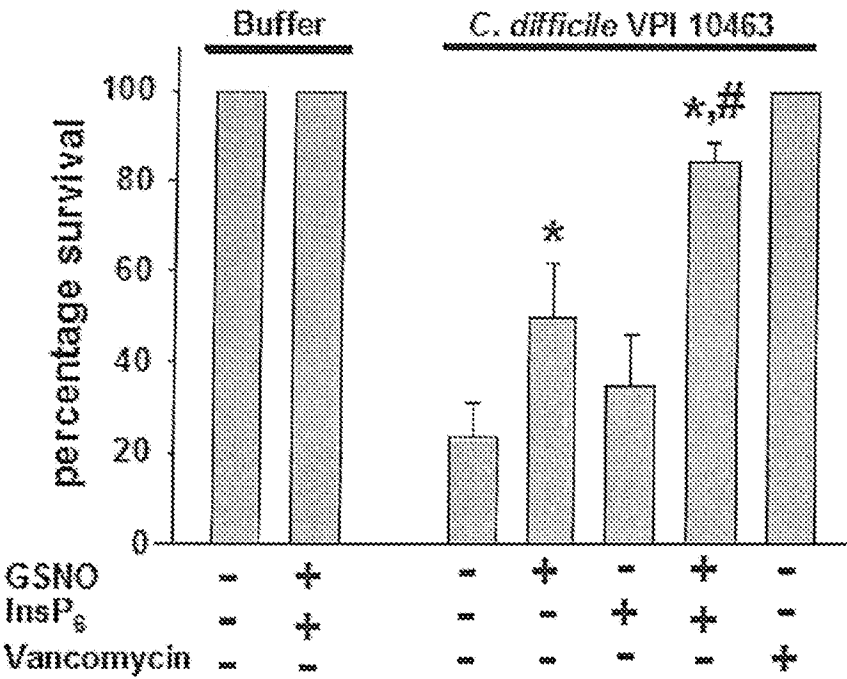


FIG. 6F

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 12/40315

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 39/08; A01N 37/06; A61K 31/225 (2012.01)

USPC - 424/239.1; 514/547

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

USPC: 424/239.1; 514/547

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

USPC: 424/239.1, 236.1, 234.1; 514/547, 740

(keyword limited; terms below)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PubWEST (PGPB,USPT,USOC,EPAB,JPAB); Google; PubMed

Search terms: s-nitrosylating, s-nitrosylation, agent, inositol, phosphate, hexakisphosphate, clostridium, vibrio cholerae, toxin, exotoxin, allosteric, cysteine protease, administer

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SHEN et al. Defining an allosteric circuit in the cysteine protease domain of Clostridium difficile toxins. Nat. Struct. Mol. Biol. March 2001, Vol. 18, No. 3, pages 364-371; abstract; pg 364, para 4; pg 365, para 3-5; pg 369, para 4; Figs. 2, 4	1-8, 12, 17-24
Y	HESS et al. Protein S-nitrosylation: purview and parameters. Nat. Rev. Mol. Cell. Biol. February 2005, Vol. 6, No. 2, pages 150-166; pg 155, para 2 - pg 156, para 2	1-8, 12, 17-24
Y	US 2004/0224899 A1 (GASTON et al.) 11 November 2004 (11.11.2004) abstract	4-8, 22-24
A	EGERER et al. Auto-catalytic cleavage of Clostridium difficile toxins A and B depends on cysteine protease activity. J. Biol. Chem. 31 August 2007 (31.08.2007), Vol. 282, No. 35, pages 25314-25321	1-8, 12, 17-24
A	EGERER et al. Inositol hexakisphosphate-induced autoprocessing of large bacterial protein toxins. PLoS Pathog. 8 July 2010 (08.07.2010), Vol. 6, No. 7, Article e1000942, pages 1-8;	1-8, 12, 17-24
A	CARIELLO et al. In vitro amoebicidal activity of S-nitrosoglutathione and S-nitroso-N-acetylcysteine against trophozoites of Acanthamoeba castellanii. J. Antimicrob. Chemother. March 2010, Vol. 65, No. 3, pages 588-591;	1-8, 12, 17-24
A	ZAMAN et al. S-nitrosylating agents: a novel class of compounds that increase cystic fibrosis transmembrane conductance regulator expression and maturation in epithelial cells. Mol. Pharmacol. October 2006, Vol. 70, No. 4, pages 1435-1442	1-8, 12, 17-24



Further documents are listed in the continuation of Box C.



* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

16 August 2012 (16.08.2012)

Date of mailing of the international search report

07 SEP 2012

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450

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PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 12/40315

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DE SOUZA et al. Leishmanicidal activity of primary S-nitrosothiols against <i>Leishmania major</i> and <i>Leishmania amazonensis</i> : implications for the treatment of cutaneous leishmaniasis. Nitric Oxide. November 2006 , vol 15, 209-216.	1-8, 12, 17-24
X,P	SAVIDGE et al. Host S-nitrosylation inhibits clostridial small molecule-activated glucosylating toxins. Nat. Med. 21 August 2011 (21.08.2011), Vol. 17, No. 9, pages 1136-1141. (author's manuscript uploaded for reference)	1-8, 12, 17-24

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 12/40315

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 9-11 and 13-16
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.