INOSITOL HEXAKISPHOSPHATE ANALOGS AND USES THEREOF

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ABSTRACT

Provided herein are analog and derivative compounds of inositol hexakisphosphate effective to treat a Clostridium difficile infection and to neutralize the bacterial toxins produced by the same. In addition, methods of treating the C. difficile infection and for neutralizing its toxins with the compounds are provided.
FIG. 1

FIGS. 2A-2B
FIG. 4A

UV absorption spectra of IP6 and IP6-NO

FIG. 4B

Retention Time (min)
INOSITOL HEXAKISPHOSPHATE ANALOGS AND USES THEREOF

PRIORITY CLAIM

This application claims priority to U.S. Provisional Patent Application Ser. No. 61/516,639 filed Apr. 6, 2011, which is incorporated herein by reference in its entirety.

STATEMENT REGARDING FEDERALLY FUNDED RESEARCH

This invention was made with government support under 1UL1RC029876-01 and DK078032-01 awarded by the John S. Dunn Gulf Coast Consortium for Chemical Genomics Robert A. Welch Collaborative Grant Program and NIDDK, respectively. The government has certain rights in the invention.

BACKGROUND

I. Field of the Invention

The present invention relates generally to microbiology, pharmaceutical chemistry and antibiotic formulations. More specifically, the present invention relates to inositol hexakisphosphate analogs.

II. Description of the Related Art

Clostridium difficile is a Gram-positive, spore-forming anaerobic bacillus that is a common cause of nosocomial antibiotic-associated diarrhea and is the etiologic agent of pseudomembranous colitis. The disease ranges from mild diarrhea to life threatening fulminating colitis. Antibiotic use in patients results in a reduction of the commensal gut microflora. C. difficile is resistant to most antibiotics, which gives it a competitive advantage over normal bacterial flora resulting in its proliferation and toxin production.

C. difficile enterotoxins (TcdA and TcdB) are the major cause of the disease since toxin-deficient strains are avirulent. Standard therapy depends on treatment with vancomycin or metronidazole, neither of which is fully effective. Moreover, up to 35% of patients infected with C. difficile relapse following treatment. The primary treatment option for recurrent C. difficile infection (CDI) is still metronidazole or vancomycin. C. difficile infection accounts for approximately 25% of cases of antibiotic-associated diarrhea and the incidence of infection is rising steadily in North America, with yearly costs in the U.S. estimated at $3.2 billion. Several recent hospital outbreaks of C. difficile infection associated with high morbidity and mortality rates have been attributed to the widespread use of broad-spectrum antibiotics. The emergence of new and more virulent C. difficile strains also contributes to the increased incidence and severity of the disease. Because of the steadily rising incidence and severity, C. difficile infection is an important emerging drug-resistance associated disease.

The incidence of C. difficile carriage in healthy adults is around 3.5%. By contrast, in hospitalized adults taking antibiotics, the rate of colonization increases substantially to 20-40%, and is associated with a high disease burden. According to the U.S. Agency of Healthcare Research and Quality (AHRQ), the prevalence of hospital patients infected with C. difficile jumped 200% from 2000 to 2005, which follows a 74% increase from 1993 to 2000. This rapid increase in C. difficile infection cases is attributed to the use of broad-spectrum antibiotics and/or the emergence of new hypervirulent C. difficile strains, such as BI/NAP1/027.

[0009] C. difficile infection is associated with a wide spectrum of clinical outcomes ranging from asymptomatic carriage to fulminant and fatal colitis. Severe C. difficile infection may also be associated with systemic manifestations including marked leukocytosis, hypotension, renal failure, respiratory failure, coagulopathy, and lactic acidosis. Refractory cases, not responding to vancomycin and/or metronidazole treatment is not uncommon. A recent study found that 22.1% of hospital in-patients with C. difficile infection had severe disease. The incidence of in-hospital deaths in the cohort of patients with C. difficile infection was 12.1%, and mortality caused primarily by C. difficile infection was 4.0%. Surgical intervention in the form of subtotal colectomy can be lifesaving in severe, fulminant, or refractory C. difficile infection. However, patients with severe C. difficile infection are typically elderly, critically ill, and are at high risk for surgical and anesthetic complications.

[0010] Several reports have described clinical improvement following use of passive antitoxin immunotherapy with normal pooled intravenous immunoglobulin to avoid surgery and prevent death. More recently, passive immunotherapy using human IgG monoclonal antitoxins was reported to be effective in preventing recurrent C. difficile infection. However, it did not confer protection against toxin activity, and the length of hospitalization was not significantly reduced. Other options, such as probiotics and anion-exchange resins, have limited efficacy and are potentially harmful. Complementary therapy is therefore urgently warranted to neutralize toxin activity. Experimental therapy currently under clinical development includes toxin-absorbing polymers and new antibiotics.

[0011] There is a recognized need in the art for alternative therapies for Clostridium difficile infections. The present invention fulfills this long-standing need and desire in the art by providing inositol hexakisphosphate-based compounds effective to treat C. difficile infections and to neutralize its toxins.

SUMMARY

The present invention is directed to an inositol hexakisphosphate analog compound. In certain aspects, an inositol hexakisphosphate analog will be an allosteric activator or inhibitor of C. difficile exotoxin cleavage. In further aspects, the analog will be a degradation resistant (e.g., phytase resistant) allosteric activator of C. difficile exotoxin cleavage. In certain embodiments the derivative or analog compound has a chemical structure of Formula I

\[
\text{Formula I}
\]

where R₁, R₆ independently are —PO(OH)₂, —PS(OH)₂, —PSe(OH)₂, —AsO₃, or NO associated with —PO(OH)₂ (i.e., —PO(OH)₂NO), whereby at least one of R₁, R₆ is —PS(OH)₂, —PSe(OH)₂, or NO associated with —PO(OH)₂ (i.e., PO(OH)₂NO). In certain aspects R₁ and R₆ are not both
—PSe(OH)₂ or —PS(OH)₂ when R₂, R₄, R₆, R₈ are —PO(OH). In certain aspects, R₈ is not —PS(OH)₂ or —PSe(OH)₂ if R₁-R₄ are —PO(OH). In further aspects, the analog can be a pharmaceutically effective salt of the compounds described herein. In other aspects, the analog can be a derivative, such as the pyrophosphates IP7 and IP8.

[0013] Certain embodiments are directed to inositol analogs having the chemical formula of Formula I. 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 an epi-inositol analog. In certain aspects, the inositol analog is a cis-inositol analog.

[0014] 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.

[0015] The present invention is directed to an inositol hexakisphosphate analog compound. The analog compound has a chemical structure of Formula I wherein R₈ is —PSe(OH)₂, and (i) R₁-R₄ (i.e., R₂, R₃, R₅, R₆, R₇) are —PO(OH), or (ii) R₁-R₄ are independently —PO(OH), —PS(OH)₂, —PSe(OH)₂, —AsO₃, or PO(OH)NO, but not all are —PO(OH). In certain aspects, R₈ is —PSe(OH)₂, and R₁ and R₄-R₇ are —PO(OH). In further aspects, R₁ is —PSe(OH)₂, and R₄-R₇ are —PO(OH). In certain aspects, R₁-R₇ are —PSe(OH)₂ and R₄-R₇ are —PO(OH). In certain aspects, one or more of the —PO(OH) groups is further modified to a —PO(OH)NO. The NO group is covalently bound to the analog. In a further aspect, the compound is a pharmaceutically effective salt or derivative of these compounds.

[0016] In certain aspects, the derivative or analog compound has a chemical structure of Formula I wherein R₈ is —PS(OH)₂, R₁-R₄ are —PO(OH). In further aspects, R₁-R₄ are —PS(OH)₂, and R₁-R₄ are —PO(OH). In still further aspects, R₁-R₄ are —PS(OH)₂, R₁-R₄ are —PO(OH). In certain aspects, R₁-R₄ are —PS(OH)₂, and R₁-R₄ are —PO(OH). In still further aspects, R₁-R₄ are —PS(OH)₂, and R₅-R₆ are —PO(OH). In certain aspects the analog is an inhibitor of exotoxin cleavage. In certain aspects, the compounds are pharmaceutically effective salt or derivative of these compounds.

[0017] In a further aspect, the derivative or analog compound has a chemical structure of Formula I wherein R₈ are —PO(OH), independently R₁-R₄ are —PO(OH), or —PO(OH)NO (NO associated covalently or ionically with —PO(OH)₂), whereby at least one of R₁-R₄ is NO associated with —PO(OH)₂, or a pharmaceutically effective salt or derivative thereof.

[0018] In certain embodiments an inositol analog has a chemical structure of Formula I wherein R₁, R₂, R₃, R₅, R₆, R₇, and R₈ are independently —PO(OH)₂, —PSe(OH)₂, —AsO₃, or —PO(OH)NO. In certain aspects, R₁, R₂, R₃, R₅, R₆, R₇, and R₈ are —PO(OH)₂. In certain aspects, R₁, R₂, R₃, R₅, R₆, R₇, and R₈ are —PSe(OH)₂. In certain aspects, R₁, R₂, R₃, R₅, R₆, R₇, and R₈ are —AsO₃. In certain aspects, R₁, R₂, R₃, R₅, R₆, R₇, and R₈ are —PO(OH)NO. In certain aspects, R₁, R₂, R₃, and 1, 2, 3, or 4 of R₅, R₆, R₇, and R₈ are —PO(OH)₂, —PSe(OH)₂, —AsO₃, or —PO(OH)NO. In further aspects R₁-R₄, R₅, and 1, 2, 3, or 4 of R₅, R₆, R₇, and R₈ are —PO(OH)₂, —PSe(OH)₂, —AsO₃, or —PO(OH)NO. In further aspects R₁, R₂, R₃, and 1, 2, 3, or 4 of R₅, R₆, R₇, and R₈ are —PS(OH)₂, —PSe(OH)₂, —AsO₃, or —PO(OH)NO. In certain embodiments the inositol analog is a myo-inositol analog.

[0019] The present invention is also directed to a method for neutralizing a pathogenic Clostridium difficile bacteria toxin. The method comprises contacting the C. difficile bacteria or toxin with the compounds described herein, where the compound potentiates or inhibits cysteine-dependent toxin self-cleavage, thereby neutralizing the same.

[0020] The present invention is directed further to a method of treating a pathogenic Clostridium difficile infection in a subject. The method comprises administering to the subject a pharmaceutically effective dose of the compounds described herein.

[0021] As used herein, the term, “a” or “an” may mean one or more. As used herein in the claim(s), when used in conjunction with the word “comprising”, the words “a” or “an” may mean one or more than one. As used herein “another” or “other” may mean at least a second or more of the same or different claim element or components thereof.

[0022] As used herein, the term “or” in the claims refers to “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”.

[0023] As used herein, the term “contacting” refers to any suitable method of bringing the analog and derivative compounds of inositol hexakisphosphate into contact with a Clostridium difficile toxin or the bacterial cell comprising the same. In vitro or ex vivo this is achieved by exposing the compound to the toxin and/or C. difficile bacteria in a suitable medium. For in vivo applications, any known method of administration is suitable as described herein.

[0024] As used herein, the term “subject” refers to any recipient of the novel compounds or a pharmaceutical composition thereof provided herein that are effective as therapeutics or inhibitors against a Clostridium difficile infection.

[0025] Other and further aspects, features, and advantages of the present invention will be apparent from the following description of the presently preferred embodiments of the invention. These embodiments are given for the purpose of disclosure.

DESCRIPTION OF THE DRAWINGS

[0026] So that the matter in which the above-referred features, advantages and objects of the invention, as well as others which will become clear, are attained and can be understood in detail, more particular descriptions and certain embodiments of the invention briefly summarized above are illustrated in the appended drawings. These drawings form a
part of the specification. It is to be noted, however, that the appended drawings illustrate preferred embodiments of the invention and therefore are not to be considered limiting in their scope.

[0027] FIG. 1 shows CPD domain boundaries in TcdB.

[0028] FIGS. 2A-2B shows the Toxin InsP₃ sensor. FIG. 2A shows InsP₃-induced toxin cleavage and the allosteric sensor activity of the glutamic acid (E743) residue. Enhanced InsP₃-induced self-cleavage is evident in the toxin E743A mutant, whereas no cleavage is evident in the inactive catalytic cysteine (C698S) mutant. FIG. 2B shows that, using an N-terminus specific anti-TcdB antibody, it is demonstrated that toxin cleavage is approximately 2-orders of magnitude more sensitive following genetic disruption of the toxin allosteric-sensor mechanism (E743A).

[0029] FIGS. 3A-3D show therapeutic allostery and examples of the assays to test designed compounds. FIG. 3A shows that InsP₃₆-autocleaves (left) and inhibits toxin-induced cytotoxicity (right) in CaCo-2 colonocytes (AC₅₀ and IC₅₀ = 10⁻⁸ M). Toxic (TcdB) cleavage fragments are shown in FIG. 3B, and are absent in the presence of a CPD inhibitor. (FIGS. 3C-3D) Kaplan-Meier survival plots of infected mice. C57BL/6 mice were inoculated with 10⁶ C. difficile. Oral InsP₃ (but not inositol) therapy dose dependently protects mice from CDI (0.25 and 2.5 mg/kg/day delivered intragastrically; n=12/group; survival at day 6). The protective effect is statistically significant in the 2.5 mg/kg group, which is within the daily recommended dose for humans.

[0030] FIGS. 4A-4B show Nitroso-InsP₃ experimental UV spectra (FIG. 4A) and LC-MS for nitroso-InsP₃ and InsP₃ (FIG. 4B).

[0031] FIGS. 5A-5B shows that Nitroso-InsP₃ shows greater amelioration in experimental CDI. FIG. 5A shows antimicrobial activity of InsP₃ vs. InsP₃-NO against C. difficile. FIG. 5B demonstrates how mini-osmotic pumps (7 day pumps; n=10/group) were surgically implanted to deliver inositol derivatives at 12.5 mg/kg/day, starting 1 day prior to C. difficile inoculation (10⁶ bacteria). In this system of experiments, vehicle treatment was associated with 100% mortality, vs. 40% and 10% for InsP₃ and nitroso-InsP₃ groups, respectively.

[0032] FIG. 6 shows TcdB autocleavage in the presence of 10⁻⁸ M InsP₃ derivatives.

[0033] FIG. 7 shows an increased phytase resistance of myo-inositol hexakisphosphorothioate (InsP₆(S)).

DESCRIPTION

[0034] C. difficile infection is a toxin-mediated disease. Two exotoxins, toxin A (TcdA) and toxin B (TcdB), are the major virulence factors. C. difficile strains that lack both toxin genes are non-pathogenic. TcdA and TcdB are structurally similar to each other. Both toxins consist of at least three functional domains that are now well defined. The C-terminus receptor binding domain (RBD) has a α-solenoid structure and is involved in receptor binding. The middle part is involved in translocation of the toxins into the target cells, and the N-terminus is a catalytic glucosyltransferase (GT) domain. Interactions between the C-terminal receptor binding domain and host cell receptors initiate receptor-mediated endocytosis. Although the precise intracellular mode of action remains unclear, the toxins undergo a conformational change at the low pH of the endosomal compartment, leading to a membrane insertion and channel formation. An essential host-derived virulence cofactor inositol hexakisphosphate (InsP₆) is then required to trigger an allosteric structural change that activates a cysteine protease domain to induce toxin self-cleavage, resulting in the release of the GT-effector domain into the cytosol. Once in the cytosol, the catalytic GT-domain mono-O-glucosylates small GTases of the Rho family, including RhoA, Rac1, and Cdc42. Glucosylation of Rho proteins inhibits their "molecular switch" function, thus blocking Rho GTase-dependent signaling in intestinal epithelial cells, leading to alterations in the actin cytoskeleton, massive fluid secretion, acute inflammation and necrosis of the colonic mucosa.

[0035] Cysteine-dependent cleavage is a crucial activation mechanism for TcdA and TcdB because it facilitates toxin entry into cells. Specific inhibition of this cleavage reaction significantly attenuates the toxin. This virulence mechanism is dependent on cellular InsP₃ that activates the cysteine protease domain (CPD) to facilitate toxin self-cleavage. Cysteine protease domain crystal structures for TcdA and the closely aligned Vibrio cholerae RTX toxin demonstrate a well defined catalytic cleft separated from a positively charged InsP₃-binding pocket abutting a flexible β-hairpin fold (β-hairpin).

[0036] In one embodiment of the present invention there is provided an inositol hexakisphosphate analog or derivative compound having a chemical structure:

![Chemical Structure](attachment://image.png)

where R₁-R₆ independently are —PO(OH)₂, —PS(OH)₂, —PSe(OH)₂, —As(OH)₂, or NO associated with —PO(OH), whereby at least one of R₁-R₆ is —PS(OH)₂, —PSe(OH)₂, or NO associated with —PO(OH)₂, and R₁ and R₃ are not both —PSe(OH)₂ or a pharmacologically effective salt or derivative thereof. In certain aspects, R₁ is —PO(OH)₂ or —PSe (OH)₂ when R₂-R₆ are not —PO(OH)₂. In all embodiments and aspects, the inositol hexakisphosphate analog or derivative compound may comprise a pharmaceutical composition with a pharmaceutically acceptable carrier.

[0037] In one aspect R₁ may be —PS(OH)₂ and R₂-R₆ may be —PO(OH)₂. In one aspect R₃ may be —PS(OH)₂ and one or more of R₄-R₆, but not all of R₃-R₆ may be —PO(OH)₂. In another aspect R₃ may be —PS(OH)₂ and R₄-R₆ may be —PO(OH)₂. In yet another aspect R₃ may be —PS(OH)₂ and R₄-R₆ may be —PO(OH)₂. In yet another aspect R₃ may be —PS(OH)₂ and R₄-R₆ may be —PO(OH)₂. In yet another aspect R₃ may be —PS(OH)₂ and R₄-R₆ may be —PO(OH)₂. In yet another aspect R₃ may be —PS(OH)₂ and R₄-R₆ may be —PO(OH)₂. In yet another aspect R₃ may be —PS(OH)₂ and R₄-R₆ may be —PO(OH)₂. In yet another aspect R₃ may be —PS(OH)₂ and R₄-R₆ may be —PO(OH)₂. In yet another aspect R₃ may be —PS(OH)₂ and R₄-R₆ may be —PO(OH)₂. In yet another aspect R₃ may be —PS(OH)₂ and R₄-R₆ may be —PO(OH)₂. In yet another aspect R₃ may be —PS(OH)₂ and R₄-R₆ may be —PO(OH)₂. In yet another aspect R₃ may be —PS(OH)₂ and R₄-R₆ may be —PO(OH)₂.
R₁-R₆ may be —PSe(OH)₂ and R₂-R₆ may be —PO(OH)₂. In yet another aspect of this embodiment the NO substituent may be associated covalently or ionically with —PO(OH)₂.

In a related embodiment, there is provided an inositol hexakisphosphate analog or derivative compound having a chemical structure as described supra where R₁ is —PSe(OH)₂ and R₂-R₆ are —PO(OH)₂; R₃ is —PSe(OH)₂ and R₁ and R₃-R₆ are —PO(OH)₂; R₃ is —PSe(OH)₂ and R₁-R₆ are —PO(OH)₂; R₃ is —PO(OH)₂; R₁ and R₃-R₆ are —PSe(OH)₂; and R₁ and R₃-R₆ are —PO(OH)₂; or R₁-R₆ are —PSe(OH)₂ and R₂-R₆ are —PO(OH)₂; a pharmaceutically effective salt or derivative thereof.

In another related embodiment there is provided an inositol hexakisphosphate analog or derivative compound having a chemical structure as described supra where R₁ is —PSe(OH)₂ and R₂-R₆ are —PO(OH)₂; R₃ is —PS(OH)₂ and R₁ and R₃-R₆ are —PO(OH)₂; R₃ is —PS(OH)₂ and R₁-R₆ are —PO(OH)₂; R₃ is —PSe(OH)₂ and R₁-R₆ are —PO(OH)₂; R₃ is —PO(OH)₂; R₁ and R₃-R₆ are —PS(OH)₂; and R₁ and R₃-R₆ are —PO(OH)₂; or R₁-R₆ are —PS(OH)₂ and R₂-R₆ are —PO(OH)₂; a pharmaceutically effective salt or derivative thereof.

In another embodiment of the present invention there is provided a method for neutralizing a toxin in a pathogenic *Clostridium difficile* bacteria, comprising contacting the *C. difficile* bacteria with a compound as described supra, wherein said compound inhibits cysteine-dependent toxin self-cleavage, thereby neutralizing the same. In this embodiment the toxin may be one or both of TcdA or TcdB.

In yet another embodiment of the present invention, there is provided a method for treating a pathogenic *Clostridium difficile* infection in a subject, comprising administering to the subject a pharmaceutically effective dose of the compound as described supra. In certain embodiment the compound may be comprised an oral formulation and/or administered orally.

In yet another embodiment of the present invention there is provided a method for identifying an inositol hexakisphosphate analog or derivative compound effective to inhibit self-cleavage of a pathogenic *Clostridium difficile* toxin, comprising designing a 3D-pharmacophore, at least in part in silica, based on a crystal structure of inositol hexakisphosphate bound to the toxin; selecting a potential inhibitor compound; and analyzing a structure activity relationship of the potential inhibitor with the toxin 3D-pharmacophore to determine inhibitory activity of toxin self-cleavage, thereby identifying an inhibitory inositol hexakisphosphate analog or derivative compound. Further to this embodiment the method may comprise optimizing the structure of the inhibitory inositol hexakisphosphate analog or derivative compound.

In a related embodiment, there is provided a 3D-pharmacophore based on the crystal structure of inositol hexakisphosphate bound to the *C. difficile* toxin as described supra.
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.

**EXAMPLES**

[0051]  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**

**Allosteric Activation of TcdA and TcdB Autocleavage**

[0052]  In order to better understand how InsP₆ allosterically activates TcdA and TcdB autocleavage, cysteine protease domain homology models were generated that included the uncut N-terminus substrate cleavage fragment within the P1-substrate residue catalytic cleft (FIG. 1). These studies have identified a reaction mechanism in response to conformational-coupling by InsP₆. Analysis of these N-terminus extended cysteine protease domain models demonstrated an extensive network of interconnecting hydrogen bonds within the catalytic active site. Because of the unusually large distances (>6 Å) between the catalytic cysteine and histidine in all of the microbial cysteine protease domain crystal structures, the histidine residue appears to play a role in substrate orientation within the P1 pocket rather than conferring nucleophile to the catalytic cysteine thiolate as conventionally happens in cysteine proteases. The aspartic acid may stabilize the histidine imidazolium ring, and hydrogen bonding between a novel glutamic acid residue and the catalytic cysteine modulates thiolate reactivity. Thus, this tetrad active site motif appears to have developed an allosteric sensor mechanism (achieved via hydrogen bonding between a highly conserved glutamic acid (Glu) and the catalytic cysteine) in order to restrict toxin self-cleavage to situational exposure to InsP₆ cofactor. Applicants note that their analysis is not to be construed as a limitation on the claimed subject matter unless expressly included as a limitation.

[0053]  This catalytic tetrad function is demonstrated experimentally by site-directed mutagenesis in TcdB, where there was generated (i) a catalytically dead Cys698Ser mutation; (ii) a highly attenuated toxin His655Ala mutation (10⁶ fold inhibition), and (iii) an enhancing Glu743Ala mutation, which sensitizes toxin self-cleavage into the nM InsP₆ range (a concentration that is readily achieved in the gut lumen by dietary InsP₆) (FIGS. 2A-2B). Further, this regulatory glutamic acid is located on the flexible cysteine protease domain 13-flap, a structure likely to be regulated by InsP₆. However, because of the instability of the cysteine protease domain in the absence of InsP₆ cofactor, it has not been possible to generate crystal structures of the native unbound configuration to elucidate this β-flap mechanism. Therefore, in order to better understand the structural basis for the InsP₆ allostery, molecular dynamics (MD) structural simulations of InsP₆ binding to the cysteine protease domain of TcdA, TcdB, and *V. cholerae* RTX toxin were performed.

[0054]  InsP₆ binds to a highly conserved positively charged binding pocket that conforms to the edge of the flexible allosteric 13-flap. The molecular dynamics-simulation models consistently showed that allosteric InsP₆ binding facilitates substrate access to the active site cysteine in the catalytic groove by inducing conformational changes that leverage the flexible β-flap away from catalytic clef. Moreover, InsP₆ re-orientates the glutamic acid side chain relative to the catalytic cysteine. Thus, InsP₆ allostery appears to facilitate toxin self-cleavage by promoting accessibility and reactivity of the active site cysteine thiolate for the cleavage substrate.

[0055]  Extracellular InsP₆ concentrations in blood and plasma are generally too low (<1 nM) to facilitate autocleavage of the *C. difficile* toxins. However, in the gut lumen InsP₆ can reach much higher concentrations from dietary sources, although it seems unlikely that sufficiently high levels are achieved to neutralize the toxins because gut-associated enzymes rapidly degrade InsP₆ to inactive myo-inositol ((1R, 2R, 3S, 4S, 5R, 6S)-cyclohexane-1,2,3,4,5,6-hexol)) and inorganic phosphate. Phytic acid (InsP₆) is the principle storage form of phosphorous in many plant tissues, especially in the fiber of bran and seeds. Because InsP₆ is currently regarded as the main chemoprotective agent in dietary fiber due to its potent anti-oxidant and metal ion chelator properties, it is available to the public as a nutritional supplement. The enzyme meso-inositol hexaphosphate phosphohydrolase (phytase) actively degrades InsP₆ into lower inositol phosphate derivatives, rapidly reducing its bioavailability in the colon.

[0056]  Three classes of phytase enzymes exist which initiate dephosphorylation of InsP₆ at different positions on the inositol ring, and provide different isomers of lower inositol phosphates. These phytase families have pronounced stereospecificity targeting the P3, P5, and/or P4/6 positions, and have a strong preference for equatorial over axial phosphate groups. Because the lower inositol phosphate products are poor allosteric activators of the toxin cysteine protease, the present invention generates non-hydrolysable phytase-resistant InsP₆ analogs that remain potent toxin inhibitors in the colon. Echelon Biosciences has generated stable racemic P1/3 phosphoroselenuim and phosphorothiolate InsP₆ derivatives that are resistant to the P3 family of microbial phytases, which represents the major enzyme class in the human colon. Computational 3D-pharmacophores and structure activity relationship analysis of the toxin allosteric binding site will aid in the optimization of these phytase-resistant InsP₆ derivatives for therapy.

[0057]  *C. difficile*, newly emerged in its present drug resistant hyper-virulent form, causes serious and potentially fatal inflammation of the colon. Currently, there is an urgent need to find alternative therapy for CDI as *C. difficile* is rapidly developing resistance to antibiotic treatment. Although an antitoxin vaccine program is in clinical trials, the efficacy of this approach remains highly uncertain and problematic since
patients with severe CDI typically tend to be the elderly and the critically ill. Passive systemic antitoxin immunotherapy has been reported to be effective in preventing disease recurrence in CDI patients, but failed to confer significant clinical benefits or reduce the length of hospitalization. Oral adaptation of passive immunotherapy is not feasible or economical. Probiotics and anion-exchange resins remain unproven treatment options for CDI with limited efficacy. Thus, in CDI, there is an urgent need to develop therapeutics not subject to antimicrobial resistance. A goal of the present invention is to address these critical issues by developing antimicrobial inositol phosphate-based therapy that also neutralizes toxin activity in the colon.

Cysteine proteases degrade polyopeptides via a common catalytic mechanism that normally involves a nucleophilic cysteine thiol in a catalytic triad. This important enzyme class regulates many cellular activities in eukaryotic cells and in infectious pathogens, including C. difficile. Thus, various strategies are being explored to combat infectious disease by specific inhibition of microbial cysteine proteases. Proof-of-concept for such an approach has been provided by demonstrating that wide-spectrum cysteine protease inhibitors suppress both viral and parasitic disease. Vinyl sulfone-based peptides are efficient inhibitors of microbial cysteine proteases, such as cruzain and falcipains, by forming irreversible covalent bonds with the thiolate of the catalytic cysteine.

Although such irreversible inhibitors are quite potent, with IC_{50} values in the nanomolar range, the poor selectivity for parasitic over human cysteine proteases remains a significant concern. Also, it is desirable to design non-peptide based reversible inhibitors to minimize the potential toxicity that can be observed with irreversible inhibitors.

With the recent discovery that C. difficile toxins require cysteine protease activity for virulence, specific targeting of this enzyme class represents an important therapeutic strategy in combating CDI. The present invention designs small molecule therapeutics to combat CDI that mimics the cytosolic allosteric cofactor, inositol hexakisphosphate (InsP_6) of these toxins. As the exotoxin cysteine protease active site self-processes the toxin to an active state, is normally inaccessible to inhibitors. In certain embodiments the InsP_6 analog is designed to trigger the accessible and highly conserved allosteric site, inducing autolytic cleavage and preventing the toxins from entering mammalian cells. This approach has been validated by showing that high levels of dietary InsP_6 can mitigate CDI in an animal model. However, oral InsP_6 therapy may be limited due to enzymatic degradation by gut phytases. Thus, the present invention develops new degradation resistant or phytase-resistant inositol analogs that bind to the allosteric site and rapidly trigger the autolysis while the toxin is still in the extracellular milieu. Computational modeling of the toxin-InsP_6 allosteric mechanism and structure-based design of the allosteric binding site assist in the design and selection of inhibitors. Data for structure-activity relationship analysis is generated for phosphoseleno-, phosphorothiolate-, and antimicrobial nitroso-derivatives of InsP_6. Thus, the present invention exploits the reliance of the C. difficile toxins on InsP_6, as a virulence factor to develop inositol phosphate-based-therapy for CDI.

Optimization of phytase-resistant InsP_6 analogues for CDI therapy. Aspects of the present invention provide compositions and methods of neutralizing C. difficile exotoxins in the extracellular gut environment. C. difficile toxins are autocleaved by InsP_6 in the μM range (IC_{50}=10 μM; FIGS. 3A-3B). When this autocleavage occurs outside of the cell (by means of InsP_6 supplementation), this renders the toxin inactive as the effector domain does not enter the cytosol. The present invention demonstrates that oral InsP_6 supplementation is protective in a murine CDI model that closely resembles the human disease. C57BL/6 mice were administered antibiotics and then orally inoculated with 10^5- to 10^6 C. difficile (strain VPI 10463). Disease outcome measures included evidence of inflammation and fluid secretion in the caecum and colon of severely afflicted animals, and of characteristic pseudomembranous histopathological lesions in the colonic mucosa. Surviving mice developed diarrhea for 5 days post infection and weight loss lasted for 3 to 4 days. Mice continued to shed C. difficile bacteria in the stool for up to 13 days post challenge, at which time the experiments were terminated. The highest bacterial dose (10^6 CFU) was used to test for InsP_6 efficacy given intra-gastrically (0.25-2.5 mg/kg/day; range is within the recommended oral daily dose for humans (1,020 mg/day)). These studies demonstrated dose-dependent protective effects of InsP_6 that were not evident with inactive myo-inositol (FIGS. 3C-3D).

Phytases are gut-associated acid phosphatases that degrade dietary InsP_6 into myo-inositol and inorganic phosphorus. Toxin models indicate that the highly conserved InsP_6 binding pocket is readily accessible to inhibitors. The present invention provides an analysis of the allosteric site and tests new small molecule derivatives for toxin cleavage activity, and designs—pharmacophores for use in developing novel phytase-resistant InsP_6 derivatives that will not degrade in the intestine. The present invention discloses structural predictions of chemical modifications that optimize toxin cleavage activity for the synthesis of therapeutic InsP_6 analogs. This involves targeted synthesis of P1/P3 modifications, followed by targeted polymodifications, and finally combinatorial mono-modifications.

Modeling of the toxin allosteric binding site. As described above, the present invention used homology modeling and MD simulations to determine the likely structure of the CDI in the absence of allosteric InsP_6. The flexible β-flap must move to open the InsP_6 binding site and block access to the 1α protase active site in the InsP_6 free toxin. Thus, a series of conformations are chosen from the molecular dynamics simulations that reflect different degrees of opening and solvent exposure of the β-flap. A series of InsP_6-derivatives are then docked to these conformations, and select the conformation that is most consistent with the experimentally derived activities of these compounds. This conformation is then used for designing 3D-pharmacophores.

To demonstrate feasibility, InsP_6 (12 rotatable bonds) was docked to a conformer of TcdA. Of 224 Autodock poses for InsP_6 binding to the open β-flap (inactive) TcdA conformation, 139 clustered together at the lowest binding energy. Their position was confirmed as the InsP_6 binding site in the TcdA crystal structure. Probably due to the symmetry of the molecule, the InsP_6 in the docking positions was rotated by 60° clockwise about the ring axis and tilted about 50°.

Designing 3D-pharmacophores specific for the C. difficile toxins. Design begins with analysis of the toxin homology models and the bound conformation of InsP_6 and the analogs of known activity. A 3D-pharmacophore based on the crystal structure of the bound InsP_6 is designed that can be used to optimize the design of phytase-stable InsP_6 derivatives. In addition, structure activity relationship analysis of
InsP₃ derivatives are used with activities determined in toxin cleavage and binding assays. Alternative conformations of the modeled InsP₃ binding site, taken from the molecular dynamics simulations, may be used in the structure activity relationship to refine the binding model. Structurally related compounds from the ZINC database in toxin autocleavage assays are examined. Preliminary studies have demonstrated that the inositolhexakisulfate (Ins₆) analog is an active compound, as are InsP₃ derivatives. Of interest, InsP₃ derivatives demonstrate enhanced toxin cleavage activity vs. InsP₃, indicating that autocleavage efficacy can be achieved in the IC₅₀ nM range by optimizing the design for synthesis of the phytase-resistant inositol phosphate derivatives.

[0066] Development of antimicrobial nitroso InsP₃ derivatives for CDI therapy. As described above, stable phytase-resistant InsP₃ derivatives that inactive the C. difficile toxins are synthesized. As described below, combinatorial antimicrobial activity can be imparted to these derivatives. Because C. difficile is highly susceptible to nitric oxide (NO) signals and this has been suggested as an antimicrobial strategy for CDI, NO-derivatives of InsP₃ that exert both antimicrobial and anticynotoxin activity were generated. A 5-molar excess of ethylnitrite was added to InsP₃ and incubated at room temperature in the dark for three days. The reaction mixture was dried to completion under nitrogen gas. Dried nitroso-InsP₃ was dissolved in water and a UV spectrum analysis was performed. Equilibrium geometries at global minimum energy were calculated using Spartan 08 for windows (See URL wavefun.com on the world wide web), density functional theory (DFT, 6-31G*) level in vacuum phase. The experimental UV spectrum for nitroso-InsP₃ is in close agreement with the predicted spectrum for mono-nitrosylated InsP₃ with a λ-max at 300 nm (Fig. 4A). Further, calculated energies of InsP₃ and InsP₃-NO (18.4 kcal/mole) suggested similar stability. Aqueous nitroso-InsP₃ was stable at ambient temperature over a period of two weeks.

[0067] LC-MS was used to separate InsP₃ and nitroso-InsP₃ derivatives. The mass spectrum showed four peaks. The first peak matched the InsP₃ standard, and later peaks showed a mass spectrum that corresponded to a mixture of nitroso-InsP₃(NO)₃, derivatives, with approximate yields ranging from 2-22% (Fig. 4B). Further, the nitroso-InsP₃ derivatives demonstrated significant antimicrobial activity towards C. difficile and showed enhanced disease amelioration in experimental CDI (Fig. 5A-B), without altering toxin cleavage efficiency (Fig. 6).

[0068] Characterization and preparation of optimal nitroso InsP₃ derivatives. The LC conditions are refined to separate out the different NO-derivatives in sufficient quantities of pure material so as to test them individually in antimicrobial and toxin assays, and phytase-enzymatic assays. MS is used to determine the size of each adduct, and as an approximate indicator of purity. With a reasonably pure, active molecule, the structure can be determined in more detail, using a combination of ³¹P- and Proton NMR. Initially, these studies determine whether the NO is covalently linked or remains as a free radical adduct. In the latter case, the distinctive proton resonances for the hydrogens should allow one to discriminate which position on the InsP₃ has been modified. If the bond is indeed covalent, the synthesis is repeated, incorporating ¹⁵NO. In an effort to isolate and quantify nitroso-InsP₃, a Q-Trap 2000 (Hybrid MS) is used, combination of trap [identification of unknown InsP₃(NO) by mass spectral fragmentation] and triplequad mass spectrometry. Finally, synthesis conditions (variations in time, temperature, molar excess of ethylnitrite and other NO donors) are optimized to increase the yield of the various active nitroso-InsP₃ derivatives.

[0069] In vitro testing of nitroso-InsP₃ derivatives. In vitro testing and measurement of binding affinities of compounds are performed using toxin cleavage assays, and by radioligand or BIACORE-toxin binding studies, respectively. For medium throughput toxin cleavage assays, autocleavage of 1 μg TecA and TecB holotoxins is performed in 25 μL 20 mM Tris-HCl, 150 mM NaCl (pH 7.4) with and without InsP₃ for 0-60 min at 37° C. (Fig. 6). Cleavage reactions are then stopped with SDS-PAGE loading buffer and boiling at 96° C for 5 min. Samples are then run under reducing conditions on 4-20% gradient gels and cleavage products stained with GelcodeBlue™ for 1 hr and cleared in water overnight. AC₅₀ and IC₅₀ concentrations are calculated by measuring the relative absorbance of cleavage fragments relative to intact toxin using a LiCor Odyssey infrared scanner (λ=860 nm). Cleavage is plotted against ligand concentration using four-parameter log-logistic curve fitting on SigmaPlot 11.0 software. If the toxin cleavage efficiency remains unaltered in purified nitroso-InsP₃ fractions, then toxin Rac1 glycosylation and cell rounding assays are initiated to evaluate cytoprotection in cell culture systems. Antimicrobial activity is then recorded as shown in Fig. 5A-5B. Finally, if a covalent modification is formed with NO, it is contemplated that this shows phytase-resistance. Thus, enzymatic digestions of nitroso-InsP₃ derivatives are carried out with EC 3.1.3.8 (type 3 phytase); EC 3.1.3.72 (type 5 phytase), and EC 3.1.3.26 (type 4/6 phytase) to test whether the NO-modifications are nonhydrolysable (e.g., Fig. 7).

### TABLE 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>AC₅₀ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>myo-inositol-(1,3,4,5-tetrahydrophosphate)</td>
<td>Myo-InP₄(2,6) &gt;1000</td>
</tr>
<tr>
<td>myo-inositol-(1,2,3,5-tetrahydrophosphate)</td>
<td>Myo-InP₄(4,6) &gt;1000</td>
</tr>
<tr>
<td>myo-inositol-(2,3,4,5,6-pentahydrophosphate)</td>
<td>Myo-InP₅(1,4) 8.67</td>
</tr>
<tr>
<td>myo-inositol-(1,3,4,5,6-pentahydrophosphate)</td>
<td>Myo-InP₅(2) 10.47</td>
</tr>
<tr>
<td>myo-inositol-(1,2,4,5,6-pentahydrophosphate)</td>
<td>Myo-InP₅(5) 95.56</td>
</tr>
<tr>
<td>myo-inositol-(1,2,3,5,6-pentahydrophosphate)</td>
<td>Myo-InP₅(4) &gt;1000</td>
</tr>
<tr>
<td>myo-inositol-(hexakisphosphate)</td>
<td>Myo-InP₆ 14.79</td>
</tr>
<tr>
<td>myo-inositol-(1,2,3,5,6-phosphatehexa-</td>
<td>Myo-InP₆(5) 1.99</td>
</tr>
<tr>
<td>(1/3)-phosphate-myoinositol</td>
<td>Myo-InP₇(1/3) 3.04</td>
</tr>
</tbody>
</table>

[0070] It is proposed that an NO adduct is capable of forming with InsP₃, which confers antimicrobial activity towards C. difficile (without significantly altering the toxin autocleavage efficiency). Studies in an experimental CDI model demonstrate that intra-colonic targeting of nitroso-InsP₃ enhances the efficacy of InsP₃ to 90% survival rates following administration of the antimicrobial derivatives. Because of the current uncertainty of the nitroso-InsP₃ chemistry, there may be potential limitations to oral nitroso-InsP₃ delivery as this molecule may show poor colonic bioavailability due to degradation and absorption in the small intestine.

[0071] Any patents or publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. Further, these patents and publications are incorporated by reference herein to the same
extent as if each individual publication was specifically and individually incorporated by reference.

[0072] The present invention is well adapted to attain the ends and advantages mentioned as well as those that are inherent therein. The particular embodiments disclosed above are illustrative only, as the present invention may be modified and practiced in different but equivalent manners apparent to those skilled in the art having the benefit of the teachings herein. Furthermore, no limitations are intended to the details of construction or design herein shown, other than as described in the claims below. It is therefore evident that the particular illustrative embodiments disclosed above may be altered or modified and all such variations are considered within the scope and spirit of the present invention.

1. An inositol hexakisphosphate analog having a chemical structure of Formula I:

```
   OR1
  /
R2 O
  /
   OR3
  /
R4 O
  /
   OR5

R6 O
```

wherein R₁, R₂, R₃, R₄, R₅, and R₆ independently are —PO(OH), —PS(OH), or —PSe(OH); at least one of R₁, R₂, R₃, R₄, R₅, and R₆ is —PS(OH)₂ or —PSe(OH)₂ or —AsO₃; and R₁ and R₂ are not both —PS(OH)₂ or —PSe(OH)₂ when R₂, R₅, R₆, and R₆ are —PO(OH)₂.

2. The analog of claim 1, wherein R₁ and one of R₂, R₃, R₄, or R₆ are —PS(OH)₂.

3. The analog of claim 1, wherein R₁, R₂, and one of R₃, R₄, R₅, or R₆ are —PS(OH)₂.

4. The analog of claim 1, wherein R₁, R₃, and one of R₂, R₄, R₅, or R₆ are —PS(OH)₂.

5. The analog of claim 1, wherein R₁, R₄, and one of R₂, R₃, R₅, or R₆ is —PS(OH)₂.

6. The analog of claim 1, wherein R₁, R₅, and one of R₂, R₃, R₄, or R₆ are —PSe(OH)₂.

7. The analog of claim 1, wherein R₁, R₂, R₃, R₄, R₅, and R₆ are —PSe(OH)₂.

8. The analog of claim 7, wherein the inositol analog is a myo-inositol analog.

9. The analog of claim 1, wherein R₁ and one of R₂, R₃, R₄, or R₆ are —PSe(OH)₂.

10. The analog of claim 1, wherein R₁, R₂, and one of R₃, R₄, R₅, or R₆ are —PSe(OH)₂.

11. The analog of claim 1, wherein R₁, R₄, and one of R₂, R₃, R₅, or R₆ are —PSe(OH)₂.

12. The analog of claim 1, wherein R₁, R₅, and one of R₂, R₃, R₄, or R₆ is —PSe(OH)₂.

13. The analog of claim 1, wherein R₁, R₆, and one of R₂, R₃, R₄, or R₅ are —PSe(OH)₂.

14. The analog of claim 1, wherein R₁, R₂, R₃, R₄, R₅, and R₆ are —PSe(OH)₂.

15. The analog of claim 14, wherein the inositol analog is a myo-inositol analog.

16. The analog of claim 1, wherein nitric oxide (NO) is bound to at least one of R₂, R₃, R₄, R₅, R₆, and R⁶.

17. The analog of claim 16, wherein NO is bound to —PO(OH)₂ or —PSe(OH)₂.

18. The analog of claim 16, wherein NO is covalently bound.

19. The analog of claim 16, wherein NO is ionically bound.

20. A pharmaceutical composition comprising the analog of claim 1 and a pharmaceutically acceptable carrier.

21. A method for neutralizing a Clostridium difficile bacterial toxin, comprising contacting the C. difficile bacterial toxin with the analog of claim 1.

22. The method of claim 21, wherein the Clostridium difficile bacterial toxin is TcdA, TcdB, or TcdA and TcdB.

23. A method for treating a subject having a Clostridium difficile infection, comprising: administering to the subject an effective dose of the analog of claim 1.

24. The method of claim 23, wherein the analog is administered orally.

25. An inositol hexakisphosphate analog having a chemical structure of Formula I:

```
   OR1
  /
R2 O
  /
   OR3
  /
R4 O
  /
   OR5

R6 O
```

wherein R₁, R₂, R₃, R₄, R₅, and R₆ independently are —PO(OH)₂, —PS(OH)₂, or —PSe(OH)₂, and at least one of R₁, R₂, R₃, R₄, R₅, and R₆ is —PO(OH)₂, —PS(OH)₂, or —PSe(OH)₂