Technological advancements for the detection of and protection against biological and chemical warfare agents

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There is a growing need for technological advancements to combat agents of chemical and biological warfare, particularly in the context of the deliberate use of a chemical and/or biological warfare agent by a terrorist organization. In this *tutorial review*, we describe methods that have been developed both for the specific detection of biological and chemical warfare agents in a field setting, as well as potential therapeutic approaches for treating exposure to these toxic species. In particular, nerve agents are described as a typical chemical warfare agent, and the two potent biothreat agents, anthrax and botulinum neurotoxin, are used as illustrative examples of potent weapons for which countermeasures are urgently needed.

Introduction

Throughout recorded history, numerous incidences of biological and/or chemical warfare tactics have been chronicled.[1] Evidence has been found of the use of curare and amphibian-derived toxins as arrow poisons by aboriginal South Americans. Additionally, fomites, or objects that harbor and can transmit pathogenic agents, have been utilized since antiquity in military scenarios; generally, these include the employment of filth, cadavers, or animal carcasses as weapons. One of the earliest recorded uses of biological agents was during the 14th century siege of the city of Kaffa (Theodosia, Ukraine).[2] The attacking Mongol force catapulted dead and dying victims of the plague into the city with the intent of initiating an epidemic. An outbreak was followed by the escape

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of the Kaffa defenders, who may have carried this disease to other parts of Europe, accelerating the subsequent pandemic. In a further example, smallpox was used as a biological weapon against Native Americans in the 18th century. One of the more popular stories surrounding this period details the gift of blankets and a handkerchief from a British smallpox hospital to Native Americans, followed by a subsequent epidemic of smallpox among Native American tribes in the Ohio River valley. There are also anecdotal references of attempts to spread smallpox *via* infected British soldiers during the American Revolutionary War and through contaminated clothing during the American Civil War. Finally, a modern example of relatively crude biological warfare can be found in the smearing of pungi sticks with excrement by the Viet Cong in the early 1960's.

During the first half of the twentieth century, numerous countries established extensive programs for the development of biological and chemical weapons. Indeed, in response to the horror of chemical warfare utilized during World War I, international diplomatic efforts were initiated to prevent the proliferation and use of weapons of mass destruction, including the 1925 Geneva Protocol for the Prohibition of the Use in War of Asphyxiating, Poisonous, or Other Gases, and of Bacteriological Methods of Warfare.[3] Yet, numerous countries continued research into and the stockpiling of biological warfare agents. During the late 1960's, the international community experienced increasing concern for biological warfare, as well as the ineffectiveness of the 1925 Geneva Protocol. As a result, the 1972 Convention on the Prohibition of the Development, Production, and Stockpiling of Bacteriological (Biological) and Toxin Weapons and on Their Destruction (BWC) was developed.[4] This treaty, ratified in April 1972 and signed by more than 100 countries, prohibits the development, possession, and stockpiling of pathogens or toxins in "quantities that have no justification for prophylactic, protective or other peaceful purposes." Nonetheless, several signatory nations, including Iraq and the former Soviet Union, have participated in activities specifically outlawed by the convention. This, coupled with the emergence of terrorist organizations capable of producing viable chemical and/or biological warfare agents, has lead to a surge in research aimed at detecting and treating exposure to these agents. Clearly, the need for new technologies for these goals has never been greater. This review highlights various chemical and biological warfare agents and technologies developed in our laboratory and others to counteract exposure to these toxic species, as well as new methodologies for sensitive detection of chemical and biological warfare agents in field settings.

Agents of chemical warfare

During the Second World War, a wide variety of potent nerve agents were developed for use in chemical warfare. Since this time, a tremendous deal of time and effort has been devoted to the study of the mechanisms of, symptomatic treatments for, and protective measures against these toxic species.[5] While this research has undoubtedly increased our understanding of these species by orders of magnitude, the treatments currently available for use in the field are not significantly different from those available at the end of World War II. Furthermore, all of these agents fail in many important aspects, namely, all pretreatment and treatment strategies are limited by the inherent constraints of human physiology and the properties of the nerve agents themselves. Ultimately, the basic limiting factor is the speed at which the treatment drugs can reach the critical nerve endings compared with the speed of action of the nerve agent. In light of the recent use of the nerve agents sarin and VX by the Aum Shinrikyo sect in terrorism attacks in Matsumoto, the Tokyo subway, and Osaka, Japan, the need for effective pharmacotherapeutics to treat nerve agent exposure has never been greater.

Organophosphate compounds

Certain types of organophosphorus compounds are commonly used as insecticides even though they display extreme toxicity to humans. These compounds and their structural variants also have the potential to be very effective weapons of chemical warfare and therefore have been stockpiled in various arsenals. The general chemical structure of these types of deadly organophosphorus compounds consist of a tetrasubstituted phosphorus(V) center, an oxygen or sulfur atom double bonded to the phosphorus, a leaving group, and two substituents that vary widely depending on the subclass (Fig. 1). The extreme toxicity of organophosphorus compounds can be attributed to the inhibition of the enzyme acetylcholinesterase (AChE).[6] This enzyme plays a crucial role in the body by regulating concentrations of the neurotransmitter acetylcholine within the synaptic cleft of the nervous system. When AChE is functioning properly, this serine esterase hydrolyzes acetylcholine yielding acetate and choline (Fig. 2A) and regenerating the active enzyme. This degradation process results in a lowered level of acetycholine, and ultimately the termination of nerve impulses. Organophosphorus compounds will covalently block the active site serine residue of AChE by undergoing nucleophilic attack to produce a serine–phosphoester adduct (Fig. 2B). This irreversible inactivation leads to an excess of acetylcholine causing cholinergic overstimulation, culminating in death caused by respiratory failure.

One particular class of potent organophosphorus compounds, termed nerve agents, are especially feared because their manufacture is relatively simple and the starting materials are both inexpensive and accessible. Molecularly, nerve agents are characterized as alkylphosphonic acid esters that contain at least one carbon–phosphorus bond. These deadly compounds can be further divided into two subclasses, V and G, based on additional functionalities that contribute to the unique properties of each individual member including persistence on

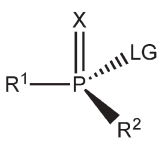

Fig. 1 The general chemical structure of deadly organophosphorus compounds (X = O or S; LG = leaving group; R^1 and R^2 = alkyl, O-alkyl, or OH).

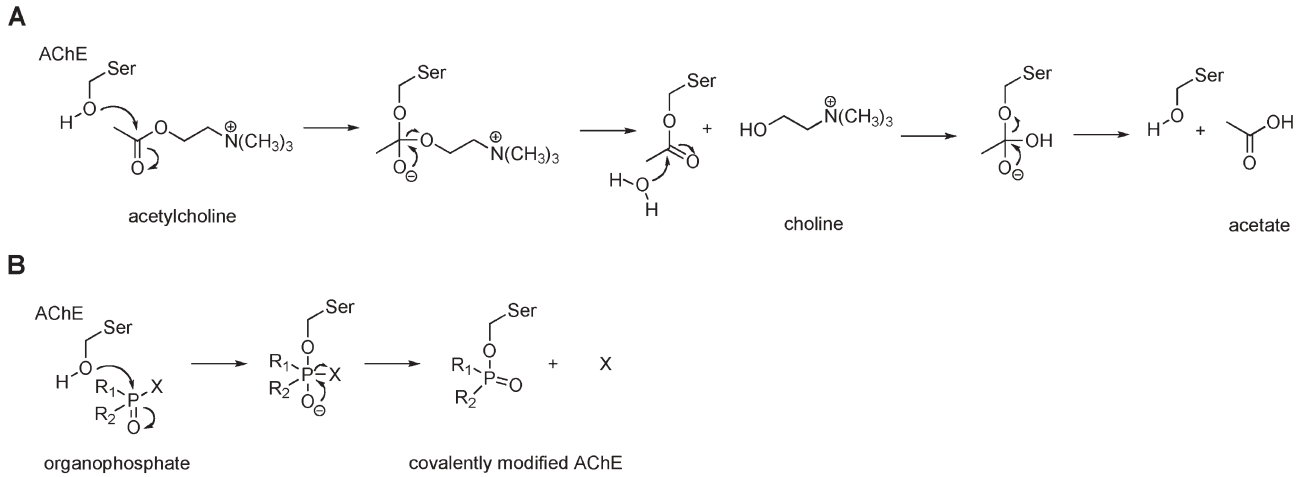

Fig. 2 A) The enzymatic reaction catalyzed by acetylcholinesterase. B) Inhibition of acetylcholinesterase by organophosphate compounds.

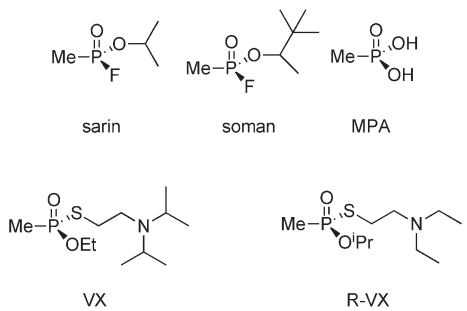

Fig. 3 Chemical structures of sarin, soman, VX, R-VX, and the degradation product common to all nerve agents, methylphosphonic acid (MPA).

surfaces, resistance to hydrolysis, solubility, and stability. VX and Russian VX (R-VX) are examples of V agents containing a thioester linkage; sarin and soman are two examples of G agents which contain fluorine substituents (Fig. 3). Despite several structural differences, all organophosphate agents are degraded either naturally or in the laboratory to methylphosphonic acid (MPA) (Fig. 3). For example, sarin first hydrolyzes to isopropyl methylphosphonic acid through the loss of fluoride, followed by subsequent hydrolysis of the isopropyl phosphonate ester to produce MPA (Fig. 4).[7] MPA is the final degradation product of many nerve agents and therefore serves as a universal downstream marker. This point is of particular interest as a strong argument can be made that in the event of a terrorist attack, the detection of MPA could serve as a critical tracking system for chemical exposure leading to expeditious diagnosis, containment, and decontamination.

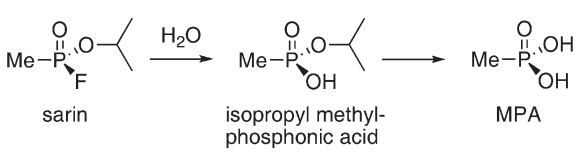

Fig. 4 Primary hydroysis pathway of sarin to methylphosphonic acid (MPA), a universal downstream marker for nerve agents.

Antibody detection of MPA

Antibody-based detection technologies have received considerable attention since the development of monoclonal antibody technology in the 1970's. These biomacromolecules have a number of particularly advantageous properties including robustness, specificity, and high affinity that make them good candidates for detecting a chemical substance such as MPA. Antibodies that bind specifically to a particular hapten can be generated by immunization techniques that take advantage of the seemingly limitless antibody repertoire found in nature. However, initial attempts to elicit an antibody response against MPA failed due to the low immunogenic properties of this compound.[8] In general, immunogenicity is conferred to a given molecule by the fulfilment of three criteria: foreignness, molecular weight greater than 6,000 Da, and chemical complexity. Consequently, small molecules with molecular weights generally less than 1,000 Da do not elicit a strong immune response. Furthermore, given the molecular simplicity of MPA, there are few elements present for antibody recognition. Therefore, in order to increase the immunogenicity of MPA, halogenated aromatic moieties were appended to the MPA structure as recognition elements while maintaining structural congruency.[8]

Phosphonic acids are known to react rapidly and quantitatively with diazomethane to form the corresponding methyl phosphonate esters. To exploit this type of reaction, the more complex diazomethane, 3,5-dichlorophenyldiazomethane (Fig. 5), was chosen because this compound is very stable and can be produced in high yield. The aromatic nature of this compound was speculated to provide MPA with two bulky groups that can be recognized by an antibody through π-stacking and/or cation–π interactions. MPA was reacted with 3,5-dichlorophenyldiazomethane in dioxane–0.5% water to afford bis(3,5-dichlorobenzyl) methylphosphonate in 67% yield after isolation and purification (Fig. 5). Conjugation to the carrier protein keyhole limpet hemocyanin (KLH) served to further increase the immune response due to the large size of the protein ($\sim 4.5 \times 10^5$–1.3×10^7 Da). Following the immunization of mice with the KLH-hapten conjugate, a panel of 11 monoclonal antibodies were isolated which bind the free

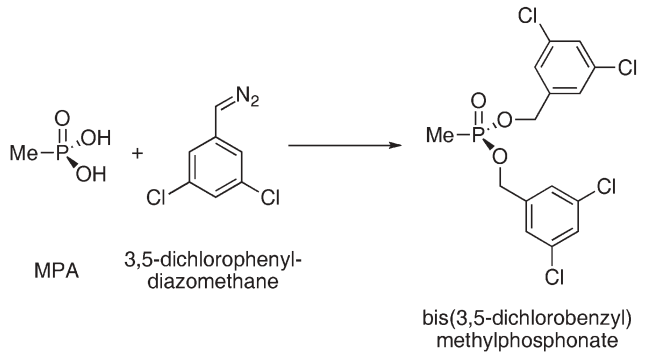

Fig. 5 The derivatization of MPA with the complex diazomethane compound 3,5-dichlorophenyldiazomethane to afford bis(3,5-dichlorobenzyl)methylphosphonate.

ligand bis(3,5-dichlorobenzyl) methylphosphonate with high affinity. The tightest binding antibody, CD27B4, displayed an affinity constant (K_d) of $\sim$1 μM.

In addition to affinity, specificity is another important feature for antibody–hapten recognition and is critical for any detection system. Cross-reactivity studies were conducted using antibody CD27B4 and a number of alternate ligands and MPA analogs (Fig. 6). At concentrations of 10 μM, no binding of compounds **1–6** was observed using an enzyme-linked immunosorbent assay (ELISA) protocol in a competition format. Given the presence of the 3,5-dichlorophenyl substituent as an anticipated strong haptenic determinant, the specificity was rather exquisite. Overall, the results suggest that both of the halogenated aromatic rings were recognized during the immune response and are required for CD27B4 binding. Additionally, CD27B4 was specific for the chemical nature and coordination sphere of the reactive phosphorus center as revealed by compounds **5** and **6**. Despite the presence of at least two 3,5-dichlorophenyl substituents, the hydroxyl group of **5** and the steric bulk of the extra aromatic ring of **6** preclude antibody binding. The estimated K_d for compounds **5** and **6** was $\sim$300 μM, two orders of magnitude higher than the immunized MPA analog bis(3,5-dichlorobenzyl) methylphosphonate.

Antibodies that bind MPA derivatives such as bis(3,5-dichlorobenzyl) methylphosphonate provide a valuable tool for detecting and tracking chemical agents. As in the case of antibody CD27B4, interference from other structurally related compounds present at test sites should be minimal. Correlation of MPA to the assayed derivative, provided an estimated detection limit for MPA of 170 $\pm$ 10 ppb. This sensitivity is comparable to many reported chromatographic and/or spectrometric techniques which require expensive instruments and/or special trained personnel.[9] Additionally, most of these approaches require a derivatization step prior to assessment, analogous to the proposed antibody–ELISA approach which is inexpensive, sensitive, convenient, and robust. Most importantly, this type of detection system can be applied to other chemical warfare agents and their degradation products.

Phosphotriester insecticide hydrolysis with catalytic antibodies

Another class of potentially life-threatening organophosphorus compounds is phosphate ester insecticides. Phospotriester insecticides such as paraoxon, parathion and diazinon share many characteristics with nerve agents including their overall general chemical structure and mode of toxicity (Fig. 7). While these phosphotriesters are important for insect control, they are also extremely toxic to humans and as a result, have been implicated as potential warfare agents along with their structural variants. Currently, there is considerable interest in developing catalysts capable of degrading such lethal chemicals into less harmful, or ideally, non-toxic forms. Critical features of a good catalyst include specificity, efficiency, stability, and in some cases biodegradability. Overall, this approach will not only be useful for the decontamination of affected areas but also serve as a potential therapy for the treatment of human exposure.

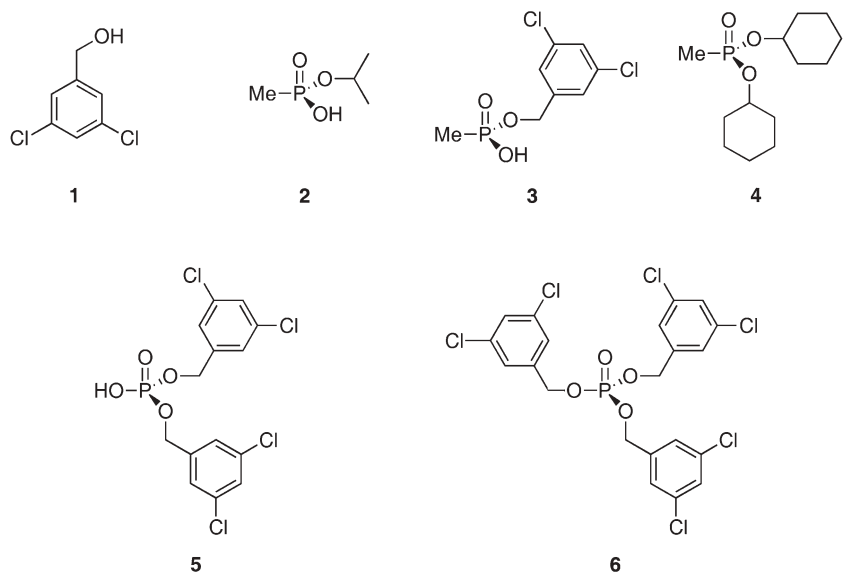

Fig. 6 Compounds analyzed for the binding specificity of antibody CD27B4 selected against the MPA derivative bis(3,5-dichlorobenzyl) methylphosphonate.

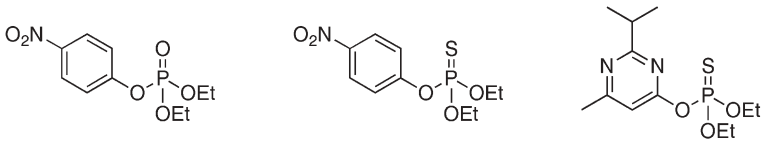

Fig. 7 Chemical structures of phosphotriester insecticides.

Over the last century, antibodies have been shown to prevent the deleterious effects of several toxins, such as bacterial exotoxins, snake venom, and insect venom, by binding and sequestering the foreign substance.[10] More recently, catalytic antibodies have been explored as a possible option for the treatment of toxin exposure because these biomacromolecules have the potential to not only isolate the toxin from susceptible tissue but also degrade the toxin into innocuous products. In general, catalytic antibodies can be thought of as enzyme-like proteins that catalyze a wide variety of chemical reactions by binding and stabilizing the transition state of a given reaction, thereby lowering the activation energy, and accelerating the reaction rate.[11] One of the most important issues to address when searching for catalytic antibodies among the immune system's repertoire is hapten design. The hapten must be able to elicit antibodies that can bind a substrate specifically and ultimately catalyze the desired transformation. Generally, there are two strategies that can be followed for generating suitable haptens.[12] First, a transition state mimic, an analog that maintains both geometry and charge distribution with respect to the transition state, may be used as a hapten. In a second strategy, a point charge is placed on the hapten in close proximity to, or in direct substitution for, a chemical functional group that is thought to be involved in the transformation of substrate to product. This haptenic charge is expected to recruit a complementary charge within the antibody active site. The charged amino acid residue can thereby contribute to catalysis by serving as a general-acid/base or a nucleophile. Since the haptens serve as the "bait" for eliciting antibody catalytic function during the immunization process, which is then "switched" for the original substrate, the strategy has been coined "bait and switch". Both of these strategies have been utilized successfully to generate tailored antibodies capable of catalyzing a wide variety of chemical reactions including acyl transfer reactions, Diels–Alder reactions, and isomerization reactions.

Antibody-mediated hydrolysis of paraoxon was chosen as a model system for the hydrolysis of organophosphorus insecticides and nerve agents.[13] In a large majority of cases, natural enzymatic reactions provide a starting point for the design of suitable haptens. Unfortunately for phosphotriesterases, only a few bacterial and fungal proteins have been isolated which are capable of degrading organophosporous compounds. The bacterial enzyme from *Flavobaterium* sp. is the most thoroughly studied and has been shown to require two zinc divalent cations for catalysis.[14] While previous work with antibodies has successfully demonstrated the use of metal cofactors for catalysis, the design of a hapten requiring such precision and complexity is not trivial. Therefore, a different route of hapten design relying on base-assisted catalysis was pursued.[13]

Phosphotriester hydrolysis is first order with respect to hydroxide ion and is thought to proceed through an in-line displacement mechanism. This places the nucleophile (OH^-) and leaving group on opposite sides of a triganol bipyramidal pentacoordinate transition state.[15] With this base-assisted hydrolysis in mind, an *N*-oxide hapten **7** was designed which contained elements of both the "bait and switch" and transition state strategy (Fig. 8).[13] In the "bait and switch" approach, the positive charge on the tetrasubstituted nitrogen atom was anticipated to induce a general base that would position a hydroxide group in the appropriate location to attack the phosphorus center, while the partial negative charge on the *N*-oxide oxygen could induce a residue capable of protonating the leaving group. In the transition state approach, this same partial negative charge could also induce a residue such as an arginine or lysine that would stabilize the developing negative charge on the phosphoryl oxygen (P=O) as the reaction proceeds. Importantly, hapten **7** maintains the nitrophenyl group of paraoxon as well as the overall molecular shape of the parent molecule.

Immunization of mice with hapten **7** linked to KLH resulted in the production of 25 monoclonal antibodies (mAbs) that bind paraoxon, although only one that actually catalyzed paraoxon hydrolysis, termed antibody 3H5. Hapten **7** was shown to be a tight binding competitive inhibitor of mAb 3H5 with respect to paraoxon signifying that hapten **7** and paraoxon bind to the same or an overlapping site within the antibody structure. This also provides evidence that hapten **7** was a good mimic of paraoxon. Antibody 3H5 displayed standard Michaelis–Menten kinetics with a Michaelis constant (K_m) of 1.60 mM and a catalytic constant (k_{cat}) of 3.99×10^{-4} min^{-1} at pH 7.8. Over the pH range 7.8–9.1, the catalytic constant increased linearly with hydroxide ion concentration from 3.99×10^{-4} min^{-1} to 1.95×10^{-3} min^{-1}, implying that catalysis of paraoxon hydrolysis by mAb 3H5 occurs primarily through transition state stabilization rather than general acid or base catalysis.

In this initial study, it was demonstrated that catalytic antibodies capable of hydrolyzing organophosphorus compounds

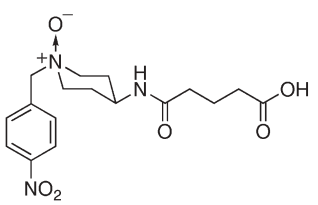

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Fig. 8 *N*-oxide hapten **7** designed for the isolation of catalytic antibodies capable of hydrolyzing the organophosphorus insecticide paraoxon.

such as paraoxon can be selected if a suitably designed hapten is immunized. Consequently, monoclonal antibodies such as 3H5 may be an appropriate means for decomposing toxins and therefore decontaminating affected areas. Unfortunately, to be fully useful as a clinical therapy for the treatment of human exposure to paraoxon, higher catalytic activity and lower binding affinities are necessary. Nonetheless, this study provided an important first step in the development of antibodies capable of treating organophosphorus poisoning.

Constrained hapten *versus* flexible hapten design

Conformational constraint of a hapten has been proposed as a technique for increasing antibody titer, defined as the number of antibodies elicited against a particular hapten. However, this does not imply that simply having a greater number of antibodies will lead to better catalysts. Indeed, in the case of the photo-Fries reaction, direct comparison of a conformationally constrained hapten against a flexible hapten yielded equally efficient catalysts, although the antibody panel resulting from immunization of the constrained hapten was larger.

The selection of a catalytic antibody capable of hydrolyzing paraoxon was successfully achieved with the constrained hapten **7**.[13] This rigid hapten contained a 4-nitrophenyl aromatic moiety and two alkyl groups constrained within a six-membered piperidine-*N*-oxide ring while maintaining the geometry of the reactive phosphorus center of paraoxon. Antibody 3H5 selected against hapten **7** displayed a rate enhancement of approximately 500 which is defined as the antibody catalyzed hydrolysis rate over the non-catalytic hydrolysis rate (k_{cat}/k_{uncat}) of paraoxon. In an attempt to obtain catalytic antibodies with improved rates of catalysis, a more flexible *N*-oxide hapten **8** was designed (Fig. 9).[16] This second generation hapten contains a 4-nitrophenyl group, a propyl group, and a linker that are all steric mimics of the ethyl esters of paraoxon. The most dramatic change in this hapten is the absence of the six-membered ring which is predicted to increase the overall flexibility of the structure. It was thought that this new set of features would allow for the binding of multiple hapten conformations and potentially lead to a more optimized catalytic paraoxon–antibody complex, thereby increasing the rate of hydrolysis.

After immunization with the KLH-conjugate of hapten **8**, a total of 20 monoclonal antibodies were obtained that bound specifically to hapten **8**, and seven were found to increase the hydrolysis rate of paraoxon with varying degrees of efficiency. The best catalyst (1H9) displayed a k_{cat} of 1.21×10^{-4} min^{-1} to 1.25×10^{-3} min^{-1} in the pH range 8.0–10.0. Analogous to the results with hapten **7**, there was a linear relationship between pH and the rate of hydrolysis suggesting transition state stabilization. Unfortunately, the rate enhancement (k_{cat}/k_{uncat}) provided by mAb 1H9 was only 56, an order of magnitude lower than that of mAb 3H5 selected against the constrained hapten **7**. The large difference may be attributed to differences in hapten design, that is, constrained *versus* flexible haptens. In this particular case, the constrained hapten **7** generated fewer, but more proficient catalytic antibodies than its flexible counterpart.

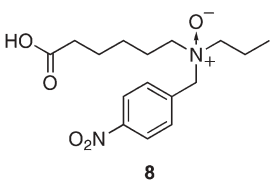

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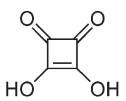
Fig. 9 Flexible hapten **8**, designed for antibody mediated paraoxon hydrolysis.

Reactive immunization approach

A more recent approach, termed reactive immunization, has been exploited for the generation of efficient catalytic antibodies and relies on the *in vivo* covalent modification of antibodies upon immunization with an appropriate hapten. This approach forgoes the natural maturation process usually employed by the immune system and relies solely on the ability of an antibody to catalyze a desired chemical transformation elicited by a specific hapten and/or substrate. Ultimately, this process covalently traps potential catalytic antibodies, producing a superior catalyst from the start thereby eliminating the affinity maturation process normally associated with antibody production.[12]

Application of this method to the hydrolysis of organophosphorus compounds was conducted based on the well-characterized chemistry of squaric acid and its derivatives (Fig. 10). This class of molecules has very unique chemical properties, wherein the reactivity of squaric acid is due to its two acidic hydroxyl groups which have a pK_a of 0.54 and 3.48.[17] Diester derivatives of squaric acid can undergo nucleophilic substitution with nucleophiles such as amines, producing the corresponding squaramides. To exploit this type of reactivity, a monoester monoamide derivative, hapten **9**, was used in a reactive immunization strategy.[18] This derivative has the potential to trap a nucleophilic amino acid residue within the antibody binding site through a substitution reaction, ultimately producing a covalently linked hapten–antibody complex (Fig. 11).

Immunization with KLH-hapten **9** resulted in the generation of 17 antibodies that bound hapten **9**. Generally, phosphotriesters are more susceptible to nucleophilic hydrolysis than their diester and monoester counterparts, therefore the highly reactive phosphotriester paraoxon was chosen as a suitable substrate for determining if these antibodies were capable of mediating hydrolysis. Two antibodies were able to accelerate the hydrolysis of paraoxon with moderate enhancement over the non-catalyzed reaction ($k_{cat}/k_{uncat} \sim 2{,}000$). Further kinetic studies suggested that these catalytic antibodies were covalently and irreversibly modified at an active residue by formation of an unreactive phosphoryl-antibody intermediate. Most importantly, this is the greatest rate enhancement for this reaction observed to date using a catalytic antibody.



squaric acid

Fig. 10 Chemical structure of squaric acid.

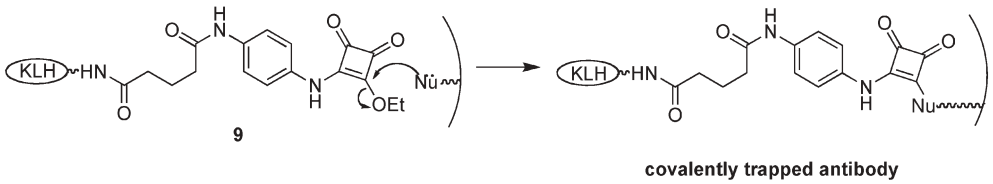


Fig. 11 Reactive immunization strategy using a monoester monoamide derivate (hapten **9**) of squaric acid.

Biological warfare agents

Anthrax

The potential threat of bioterrorism has become an increasing concern for national security and public safety. The U.S. Centers for Disease Control and Prevention (CDC) has categorized many biological agents into three classes, A, B, and C, with A being of highest priority. Category A agents, such as *Bacillus anthracis* and *Clostridium botulinum* toxin, are easily disseminated, may result in high mortality rates, and require planning and preparedness in order to protect the public's health. Indeed, anthrax, the disease caused by *Bacillus anthracis*, was most recently used by bioterrorists in the United States in 2001, resulting in the death of five individuals. To date, anthrax remains one of the most likely candidates for a biological assault.

Anthrax is an acute infectious disease caused by the aerobic, spore-forming, Gram-positive bacterium *Bacillus anthracis*.[19,20] Spores from this bacterium are commonly found in soil around the world where they may lie dormant, but viable, for years. Under the correct environmental conditions and with certain nutrients present, the spores germinate and return to a vegetative or active state. Human infection with anthrax spores most often occurs from exposure to infected animals and their products. In general, there are three types of infection: cutaneous (skin), ingestion (intestines), and inhalation (lungs). Cutaneous and intestinal are the least fatal forms of anthrax infection with mortality rates of 20% and 28–60%, respectively. Inhalation is the most deadly type with a mortality rate of nearly 100% if treatment does not begin almost immediately after exposure. The lethal dose (LD_{50}) estimated for inhalation anthrax in humans is approximately 3,000–50,000 spores. Fortunately, despite the extreme virulence of anthrax, there are no known cases of human to human transmission.

Once anthrax spores have entered the body, regardless of the route, toxicity proceeds through a similar sequence of events.[19,20] Therefore, the following discussion will focus only on inhalation anthrax, the most severe case of infection. After inhalation, the spores are located and engulfed by macrophages, a particular type of white blood cell that not only plays an important role in the immune response but also plays a simpler role by cleaning up cellular debris. Normally these phagocytic cells digest foreign microbes, yet anthrax spores are resistant to destruction. Inside the macrophages, the anthrax spores germinate, multiply, and eventually lead to macrophage lysis, releasing bacilli into the blood stream. Anthrax toxins are secreted by these rapidly replicating bacterial cells which can reach concentrations of as high as 10^7–10^9 cells per mL of blood.

Anthrax toxin is comprised of three polypeptides: protective antigen (PA), edema factor (EF), and lethal factor (LF).[19,20] PA is a 83 kDa protein (PA_{83}) responsible for the delivery of EF and LF into target cells, such as endothelial cells and macrophages. After binding to its cell surface receptor, PA undergoes proteolysis to remove a 20 kDa portion of the protein (PA_{63}) followed by rapid oligomerization into a heptameric pore-like species. EF (89 kDa) and LF (90 kDa) bind the heptameric PA, the entire complex is internalized by endocytosis, and subsequently EF and LF cross into the cytosol, where they produce toxic effects. EF and LF are both enzymes that attack different signaling pathways within the cell. EF is an adenylate cyclase that catalyzes the conversion of ATP into cyclic AMP, thereby raising the intracellular levels of this important signaling molecule. This increase in cyclic AMP levels eventually leads to edema or swelling. Additionally, EF is thought to play a role in the prevention of *Bacillus anthracis* phagocytosis by macrophages. LF is a zinc-dependent protease that cleaves mitogen-activated protein kinase kinases (MAPKK) and results in the disruption of critical signal transduction pathways. LF has also been implicated in the lysis of macrophages. Ultimately, the combined assault of EF and LF on their cellular targets results in vascular leakage, septicemia, and eventually death.

Anthrax detection

The dissemination of *Bacillus anthracis* spores by terrorist organizations continues to be of grave concern among government officials, as well as the general public. A rapid, facile, and sensitive means of detecting spores would provide an invaluable tool for determining whether anthrax is the causative agent of a biological assault. Currently, a number of methods have been developed for *B. anthracis* detection including immunohistochemistry, polymerase chain reaction (PCR), and immunoassays.[21] As demonstrated for nerve agents, antibody-based detection systems have the potential to be very specific for compounds or proteins, as well as provide an adequate limit of detection. In contrast to nerve agent studies that relied on immunization of animals with hapten analogs of a toxic species, antibodies for the detection of anthrax spores have been selected by phage display.[22] Due to the virulent nature of biological substances, such as bacterial spores and toxins, immunization of animals with the fully intact species is not always feasible. Therefore, many harmful bacteria or toxins are chemically or physically inactivated prior to immunization, thereby changing the overall conformation. This often leads to the production of antibodies that cannot recognize a viable form and consequently, may lead to the recognition of an altered or denatured epitope. Additionally, the selection and identification of

antibodies by phage display can be completed in a matter of weeks instead of months for immunization.

Phage display technology is an extremely powerful tool for selecting peptides and proteins from a large combinatorial library that bind targets with high affinity and specificity.[23,24] Generally, most phage display systems have made use of the filamentous bacteriophage M13 or the closely related phage *fd*. Bacteriophage are viruses that infect bacteria and are distinct from plant and animal viruses because they lack intrinsic tropism for eukaryotic cells. M13 phage, and similarly *fd*, are approximately 895 nm in length and 9 nm in diameter. Its single-stranded DNA genome is comprised of 6,407 bases that encode for 10 different proteins. This circular DNA is encapsulated in a protein coat consisting of approximately 2,800 copies of the gene VIII protein (pVIII or major coat protein) (Fig. 12). Additionally, at one end of the phage particle there are five copies of the minor coat proteins pIII and pVI and at the opposite end there are five copies of pVII and pIX. Unlike most other bacteriophage, M13 does not produce a lytic infection in *Escherichia coli*, but rather induces a state in which infected host cells produce and secrete phage particles without undergoing lysis. Fusion of cDNA encoding a protein to the gene encoding one of the five surface proteins, allows for the transcription, translation, and ultimately the display of the fusion protein on the phage surface. Large pools or libraries of phage containing upwards of 10^9 individual members, each displaying a unique peptide or protein, can be generated. These large libraries can then be used for selection of a protein that contains a desired property, such as binding to a particular antigen or catalysis of a specific reaction. Most importantly, there is a direct physical link between phenotype and genotype, that is, every displayed protein has an addressable tag *via* the DNA encoding that protein. Therefore, after several rounds of selection called panning, the DNA can be sequenced and the amino acid residues that make up the protein can be determined. All five of the aforementioned capsid proteins have been utilized for displaying a wide variety of proteins, antibodies, peptides, and even enzymes.

A naïve, phage display library consisting of human single-chain variable fragment antibodies (scFvs) displayed on the pIII coat protein of bacteriophage M13 was used for panning against live, native spores from the genus *Bacillus*.[22] These scFv antibodies are comprised of the light chain and the heavy chain variable regions of the immunoglobulin class G (IgG), joined by a polypeptide linker. This portion of an antibody is responsible for binding to antigens and their binding properties can be maintained within this scFv format. The strain *Bacillus subtilis* was chosen for panning because it is known to form encapsulated vegetative cells crucial to anthrax virulence; it is classified into the same morphological group as *Bacillus anthracis*. Most importantly, *Bacillus subtilis* is an avirulent strain that is not regulated by government agencies and does not require any special handling procedures. Four rounds of panning against *Bacillus subtilis* spores in solution resulted in twelve clones that were able to bind the spores by phage-ELISA. After determining the amino acid sequences of the displayed antibodies, two unique antibodies were further investigated for potential cross-reactivity with eleven other spore strains. Ultimately, only one phage antibody was specific for *Bacillus subtilis* spores while the other recognized all of the tested spore types including *B. licheniformis*, *B. cereus*, *B. thuringiensis*, *B. megaterium*, *B. pumilus*, *B. polymyxa*, *B. circulans*, *B. sphaericus*, and *B. globigii*. The binding of the antibody-phage to *Bacillus subtilis* spores was also visualized by fluorescence microscopy and it was demonstrated that a single spore could be observed using the antibody-phage in combination with two common fluorescent dyes, FITC and rhodamine.

Overall, this study serves as a model system for the isolation of antibodies against spores from the genus *Bacillus*. Within this genus, all species of *Bacillus* are very closely related and spore morphology throughout the genus is highly conserved. Yet despite the many similarities, antibodies that recognize one strain *versus* another can be selected. This type of distinction is critical for detection systems and could ultimately be applied to antibodies against anthrax spores.

Protection from anthrax infection

Due to recent bioterrorist events, the need for effective prophylaxes to prevent anthrax poisoning, as well as, theraupetic agents to treat exposed and infected humans is of great importance. In the early 1900's, the immunogenic properties of the PA of anthrax were discovered and have since been exploited for combating anthrax. Currently, a vaccine (Anthrax Vaccine Adsorbed or AVA) that consists of a culture supernatant containing PA from an avirulent non-capsulated strain of *Bacillus anthracis* is available in the United States and United Kingdom.[25] This vaccine induces active immunity within the individual by stimulating the production of antibodies against the antigen, that is, PA. While this pre-exposure measure offers protection when administered in advance, it is only available for high-risk individuals, such as military personal, and not the general public. More recently, a vaccine based on a recombinant form of PA is under investigation and is thought to require fewer injections and produce reduced side effects.[26] The standard post-exposure

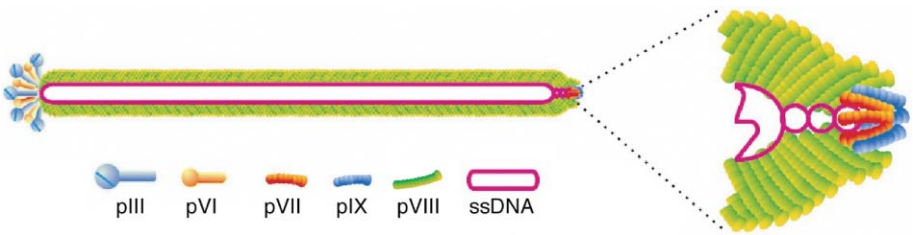


Fig. 12 The structure of filamentous bacteriophage *fd*.

therapy for anthrax poisoning is a regimen of antibiotics for a period of 60 days or more.[27] This prolonged drug treatment helps fight off infection while the anthrax-impaired immune system can recover and begin clearing the harmful bacterium. However, the ever-growing number of antibiotic-resistant anthrax strains is of major concern. Therefore, a variety of different approaches for post-exposure treatment are being explored which include the inhibition of PA proteolysis (PA_{83} to PA_{63}), blocking the binding of EF and/or LF to the PA heptamer complex, preventing the endocytosis of the PA/EF/LF complex, and inhibition of the EF and LF proteases.

Peptides that interfere with EF and LF binding to heptameric PA_{63} were isolated from a peptide phage-display library by Collier, *et al.*[28] Due to the weak binding of the synthetic versions of these peptides, a polyvalent inhibitor incorporating multiple copies of one of the peptides was necessary to efficiently disrupt binding. Additionally, antibodies against PA have been isolated from mice immunized with purified PA,[29] as well as, a phage-display library constructed from military donors vaccinated against anthrax with AVA.[30] In a recent study, scFv antibodies against cell-bound PA were isolated from a human, naïve phage-display library.[31] Unlike panning against immobilized PA, this method allows for the selection of antibodies that recognize the intact oligomeric PA on the cell surface. Whole cell panning was conducted with an adherent macrophage cell line that expresses the PA receptor and is sensitive to LF. Three antibodies that recognize PA were selected and shown to have dissociation constants (K_d) of $\sim$20–30 nM with respect to recombinant PA. Fluorescence microscopy studies with macrophages, PA, and the purified antibodies provided evidence that these antibodies bind to the oligomeric PA_{63} complex on the cell surface. Additionally, the ability of these antibodies to protect macrophages against the lethal effects of LF was investigated using a cytotoxicity assay measuring cell viability; in this system, the best antibody showed $\sim$50–80% protection.

Botulism

Another category A biological warfare agent is *Clostridium botulinum*, an anaerobic, spore-forming, Gram-positive bacterium responsible for the human disease botulism.[32] Analogous to *Bacillus anthracis*, spores of this bacterium are widely distributed in soil. Botulinum spores are also a common contaminant of many foods, such as honey, and are frequently ingested by humans. The spores cannot survive the gut and are therefore non-threatening, save in the case of infants under the age of one. Botulism usually results from the ingestion of foods contaminated with the toxin produced by the vegetative state of *Clostridium botulinum*. There are seven serologically distinct serotypes of the neurotoxin secreted by this bacteria (A–G), with types A, B, E, and rarely F responsible for human intoxication. Botulinum neurotoxin (BoNT) A is the deadliest of the seven toxins with potency approximately one hundred thousand times greater than sarin, one million times greater than cobra toxin, and one hundred billion times greater than cyanide;[33] the LD_{50} for humans is approximately 1 ng kg^{-1} of body weight.[34]

All BoNTs are synthesized as 150 kDa single chain propeptides that accumulate in the cytosol of the bacterial cell until they are released by cell autolysis.[35] This polypeptide is selectively cleaved by intra- or extracellular proteases to produce an active two-chain BoNT consisting of a heavy chain domain (100 kDa) and a light chain domain (50 kDa) joined by one or more disulfide linkages. Initially, BoNT is associated with nontoxic bacterial proteins in a large, stable complex known as the progenitor toxin ($\sim$900 kDa) that serves to protect the toxin from the denaturing environment of the stomach. After ingestion, the slightly alkaline pH of the intestinal tract causes the dissociation of the toxin complex allowing the free BoNT to enter the bloodstream. Subsequently, BoNT is specifically taken up by motor neurons at the neuromuscular junction where it inhibits the transmission of nerve impulses.

Consistent with other bacterial protein toxins including anthrax, BoNT intoxication occurs through a multi-step process involving binding, internalization, membrane translocation, and intracellular catalytic activity.[35] BoNT binds to a neuronal cell surface receptor through its heavy chain domain and is subsequently internalized by receptor-mediated endocytosis. Once internalized, a BoNT disulfide bond is reduced and the neurotoxin light chain is released from the vesicle into the cytosol. The light chain is a zinc-dependent metalloprotease that cleaves intracellular proteins essential for neurotransmitter-containing vesicle exocytosis, thereby preventing the release of acetylcholine at the neuromuscular junction causing flaccid paralysis. In contrast to an increase in acetylcholine levels caused by organophosphorus nerve agents, BoNTs lower the levels of acetylcholine thereby preventing cholinergic transmission. Symptoms of foodborne botulism may become apparent as early as two hours after ingestion of toxin-containing food and may include blurred vision, slurred speech, dropping eyelids, and general muscle weakness to name a few. In more severe cases, impaired respiratory function and autonomic dysfunction can occur, followed by respiratory failure and death.

Botulinum neurotoxin detection

Unlike anthrax, the direct threat of botulism poisoning in humans rarely comes from the spores of *Clostridium botulinum*, but rather from ingestion of neurotoxin already present in foods. Consequently, an aerosolized or foodborne BoNT deployment in the context of a terrorist weapon would require the detection of the BoNTs on surfaces and in food supplies. Due to its extreme potency, BoNT serotype A is the most likely of the seven toxins to be unleashed during an attack. The most accepted and reliable assay for confirming BoNT exposure is the mouse neutralization assay because of its high sensitivity, approximately 5–10 pg mL^{-1}.[36] However, this assay requires the injection of mice with extremely lethal substances, followed by observation to determine whether any symptoms related to botulism appear, including death. There are several disadvantages with this type of assay system including: 1) the time required for the assay can be as long as four days, 2) the sacrifice of animals is required, 3) the serotype of BoNT cannot be determined, 4) the overall expense of the

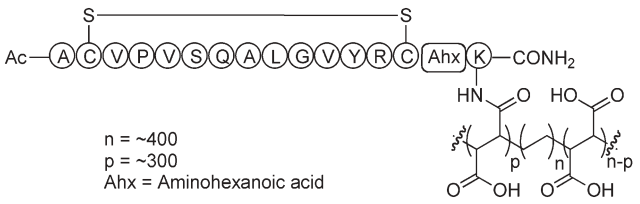


Fig. 13 Cyclic peptide–PEMA conjugate used in BoNT A detection. The letters *n* and *p* indicate the unit numbers of ethylene maleic anhydride and peptide in the final product, respectively.

assay. Therefore, a number of laboratories, including our own, have developed ELISA-based detection systems for BoNT as this type of assay can be efficient, fast, and relatively inexpensive.[21]

In our system, a phage-display library consisting of cyclic peptides ranging from 3 to 12 amino acids in length was selected against immobilized BoNT A.[37] Displayed on the pIX coat protein of bacteriophage M13, this pool of cyclic peptides contained a disulfide linkage between two flanking cysteine residues (CX_nC, where C is a cysteine residue, X stands for any natural amino acid, and *n* stands for the length of the peptide). In comparison to linear peptides, cyclic peptides are conformationally constrained, often resulting in increased stability and greater selectivity. After four rounds of panning followed by further analysis, a phage-displayed, 11 amino acid cyclic peptide (C11-019) that specifically binds BoNT serotype A and not B–G was isolated. Unfortunately, the chemically synthesized version of this cyclic peptide showed only a weak binding affinity towards BoNT A. The cyclic peptide was then attached to a polymer matrix, poly(ethylene maleic anhydride) (PEMA), which allowed for the attachment of multiple copies of the peptide to the support. Based on the design of the polymer–peptide, it was estimated that approximately 300 peptide molecules were attached per polymer chain (Fig. 13). This type of polyvalency has been shown to enhance the interactions of biomolecules with their intended ligands[38] and in this case, the binding of BoNT A by the polymer–peptide conjugate was markedly improved with respect to a single peptide species. Based on ELISA experiments with purified BoNT A, the minimal detection level with a colorimetric substrate was 1 ng mL^{-1} and could be reduced to 1 pg mL^{-1} using a chemiluminescent substrate. Examination of contaminated food and fluids, such as human serum, milk, and honey, resulted in a detection limit of approximately 1–10 ng mL^{-1} depending of the matrix in question.

Botulinum neurotoxin therapeutics

Over the last several years, the amount of funding provided to researchers developing protection methods and treatments against biological warfare agents has dramatically increased in the United States, in part due to the event of September 11, 2001. To date, the most effective immunotherapy for protection against BoNT relies on vaccination.[39] Currently, a pentavalent toxoid vaccine consisting of chemically inactivated toxins of serotypes A–E is used as a pre-exposure prophylaxis. As in the case of anthrax, this vaccine is only available for high-risk individuals, such as scientists actively working with the bacteria strain *Clostridium botulinum* or the toxins. Other existing countermeasures are limited to the passive administration of equine antibodies elicited against toxoids. Two equine antitoxins are presently distributed by the CDC for the treatment of adult botulism. Although broadly effective, human administration of equine antibodies in the form of a polyclonal serum can cause adverse reactions, such as serum sickness or anaphylaxis. Additionally, antitoxins are capable of only sequestering freely circulating BoNT and consequently become useless once the toxin has entered the neuronal cell. It is critical to note that the onset of botulism is very rapid with symptoms appearing as soon as two hours after the ingestion of contaminated food, and that the effects attributable to BoNT intoxication can continue up to six months. Thus, therapeutic approaches dependent on the extracellular interaction with BoNT (*e.g.*, antibodies) have a very short window of application, limiting their clinical utility. Due to the long paralysis and necessity for intubation and mechanical respiration, the development of practical, cost-effective, and easily obtainable postexposure prophylaxis is essential for the treatment of botulism. This point is particularly important currently as there are no known drugs approved for the treatment of botulism.

Riboflavin-mediated photooxidation of BoNT

Photodynamic therapy (PDT) has emerged as an extremely promising treatment for a number of ophthalmic, dermatological, cardiovascular, and urological diseases, as well as a range of cancers.[40] In general, PDT requires three components: a photosensitizer (light activated compound), a light source, and oxygen. Following the absorption of light, the photosensitizer produces singlet oxygen ($^1O_2$) and/or superoxide ($O_2^{\cdot -}$), two compounds with potent reactivity towards biomolecules. Approved forms of this technology, including Photofrin and Visudyne, rely on sensitizer activation by a laser light source that can be delivered directly to the target site. Previous studies have reported that BoNT photooxidation with the non-native sensitizer methylene blue leads to a reduction of toxicity *in vivo*, attributable to a modification of critical tryptophan residues in the reactive domain of the toxin.[41] While methylene blue has some clinical relevance, the use of natural dietary compounds as potential inhibitors of BoNT toxicity would be more beneficial. Therefore, it was proposed that irradiation of BoNT in the presence of riboflavin, or vitamin B_2, would result in oxidation of the toxin, leading to inactivation by either disruption of the folded conformation and/or loss of catalytic activity.[42]

Riboflavin (Fig. 14) is an easily absorbed, water-soluble micronutrient and photosensitizer that is readily obtained from dietary sources including almonds, organ meats, whole grains, mushrooms, milk, eggs, broccoli, and spinach. Based upon the photochemical potential of riboflavin, it was speculated that riboflavin would be an appropriate photosensitizer and could both intra- and extracellularly oxidize BoNT A. A critical feature of the selection of riboflavin as a sensitizer resides in the fact that there are no known toxic side effects associated with large doses of this cell-permeant vitamin. Thus, it was felt that any concern of non-specific

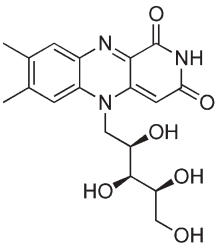


Fig. 14 Structure of riboflavin (vitamin B_2).

damage to native cellular proteins from riboflavin irradiation would be minimized. The ability of a potential treatment to function once toxin translocation has occurred is an enormous advantage over the use of either antitoxins or antibodies (*i.e.*, pre-exposure prophylaxis) as these agents simply bind and neutralize circulating toxin. Additionally, a therapeutic drug such as riboflavin would be effective against all BoNT serotypes, unlike current therapies that are only specific for one or two serotypes.

In order to determine the ability of riboflavin to photo-oxidatively protect against BoNT serotype A intoxication, a cell-based assay was conducted using two different cell lines, PC12 and Neuro-2a.[42] The intracellular target of the BoNT A light-chain protease within the neuroexocytosis apparatus is the soluble NSF-attachment protein receptor (SNARE protein), SNAP-25 (synaptosomal-associated protein of 25 kDa). The BoNT A protease cleaves the C-terminal nine amino acid residues of SNAP-25, thereby producing an approximately 24 kDa degradation protein. Analytical techniques have been developed that directly assess SNAP-25 cleavage in cell lysates using immunoelectrophoresis. Therefore, this method was used to analyze the amount of intact *versus* cleaved SNAP-25 allowing correlation to BoNT A activity within the cell. Initial studies incorporated a preincubation step of BoNT and riboflavin, similar to previous reports of BoNT inhibitors. Experiments in which a mixture of BoNT and riboflavin were first irradiated on a white light transilluminator and then added to cells, served to optimize the concentration of riboflavin and irradiation time necessary to neutralize the toxic effects of BoNT A in cells. After only 1 hour of irradiation in the presence of 10 μM riboflavin, intact SNAP-25 was visible, indicating toxin protection, and after 6 hours of irradiation a substantial amount of SNAP-25 remained. Gel electrophoresis analysis of BoNT before and after in the presence of riboflavin revealed that inactivation is likely to occur from destabilization of the protein structure, rather than proteolysis of the toxin. From the perspective of botulism treatment and possible clinical advancement, a more pertinent issue is whether or not BoNT A toxicity can be diminished when BoNT A and the photosensitzer are added directly to cells as *in vivo* preincubation of toxin and riboflavin would be impractical for treating BoNT intoxication. In this scenario, riboflavin was still able to provide a significant amount of protection. These data highly suggest that riboflavin is able to intracellularly mediate BoNT protection as it is anticipated that within the time of the irradiation, functional toxin would be taken up by the cells into endosomal compartments. The compilation of these results strongly suggests that the lethal effects of botulinum neurotoxin can be significantly blunted using PDT. The use of a dietary vitamin in botulism PDT is a clear strength of this method, however, further studies are required to determine if more efficient sensitizers are necessary for *in vivo* efficacy.

Small molecules inhibitors of BoNT a protease

As previously discussed (*vide supra*), BoNT proteases impair neuronal exocytosis through specific proteolysis of their target SNARE protein.[39] Assembly of the SNARE proteins into a stable ternary complex is thought to be intimately involved with vesicle membrane fusion and thus neurotransmitter release. These proteases comprise the 50 kDa light chain portion of the toxin (LC), provide an attractive target for the treatment of botulism and their intracellular inhibition may provide an effective post-exposure therapy. Due to its extreme toxicity and extended duration, BoNT A has been the major focus of many research efforts and therefore, will be the only serotype discussed further. Over the last decade, a large number of peptide based inhibitors have been thoroughly invested and have provided valuable information regarding substrate specificity, substrate recognition, and structural requirements thought to be important for inhibitor binding.[43] However, these molecules are unlikely to become leads for new drugs against botulism because of their short *in vivo* lifetimes. Therefore, cell permeable, low-molecular-weight molecules capable of inhibiting the BoNT A light chain intracellularly are of great interest.[44] Burnett and coworkers have identified several small molecule inhibitors through the screening of the National Cancer Institute Diversity Set containing 1,990 individual compounds.[45] Michellamine B and Q2–15 (Fig. 15) were the two most potent inhibitors displaying 62% and 60% inhibition, respectively, at a concentration of 20 μM. Additionally, a series of 4-aminoquinoline compounds, as well as, a number of currently approved quinoline-based anti-malarial drugs were tested although no improvement in inhibition was observed.[45]

Recently, our laboratory has initiated a multifaceted research program aimed at identifying novel small molecule inhibitors of the BoNT A light chain metalloprotease. Prior to the screening of large combinatorial libraries against BoNT A light chain, a high-throughput assay that is both reliable and robust was developed.[46] SNAPtide®, a 13 amino acid substrate originally developed by List Biologics as a tool for detection, is an optimized variant of a short fragment of the natural substrate SNAP-25 and contains the necessary properties for binding and catalysis. Upon cleavage of SNAPtide® by BoNT A light chain, an enhancement in fluorescence is observed which correlates to the rate of enzyme catalysis. Validation of this FRET high-throughput assay was achieved using a series of compounds containing the hydroxamate zinc-binding group (Fig. 16). Given the presence of a critical zinc ion in the BoNT A light chain active site, it was speculated that the hydroxamate moiety, when coupled to a suitable scaffold to impart specificity, would provide potent inhibitors. In general, hydroxamates have proven to be effective inhibitors of other zinc-dependent metalloproteases, such as anthrax LF,[47] and have also reached clinical trials in the case of several

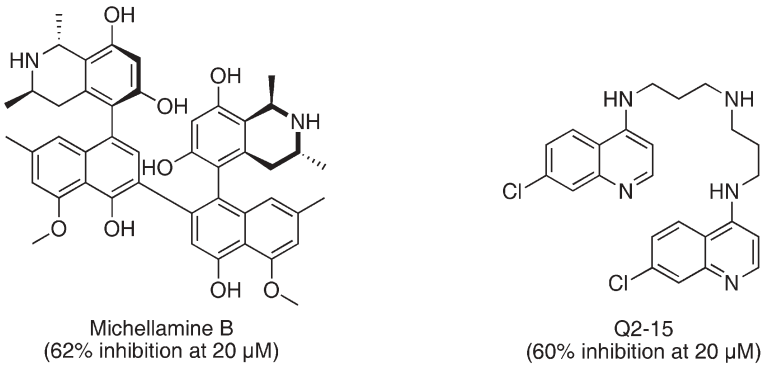


Fig. 15 Chemical structures of BoNT A metalloprotease inhibitors identified from a screen of the National Cancer Institute Diversity Set.

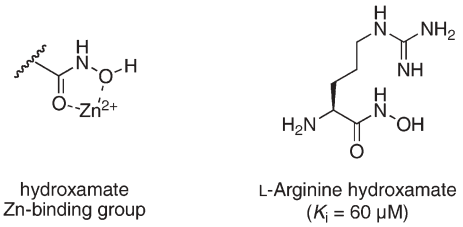


Fig. 16 Structures of the hydroxamate functionality and the BoNT A protease inhibitor L-arginine hydroxamate.

matrix metalloproteinases.[48] Since the cleavage site within the native SNAP-25 is between Gln197 and Arg198, both L-glutamine hydroxamate and L-arginine hydroxamate were chemically synthesized and shown to display 5% and 75% inhibition, respectively, at a concentration of 50 μM.[46] Subsequent kinetic studies demonstrated that L-arginine hydroxamate is a competitive inhibitor of BoNT A light chain with an inhibition constant (K_i) of 60 μM (Fig. 16).

In a second study, the *in situ* preparation of a diverse library of hydroxamates has been described.[49] Here, 150 commercially available carboxylic acids were converted to hydroxamates in a facile two-step procedure to allow for rapid lead identification. Based upon the initial screen, 4-chlorocinnamic hydroxamate (Fig. 17) displayed an IC_{50} value of 15 μM, and was considered a promising lead for further development. A small series of compounds were next synthesized to explore the structure–activity relationships of this lead. Amazingly, while substitutions of the chloro substituent were not tolerated, a 2,4-dichloro-substituted compound was found to be the most potent nonpeptidic inhibitor of BoNT reported to date (K_i = 0.30 ± 0.01 μM) (Fig. 17). Analogous to L-arginine hydroxamate, this compound was also found to be a competitive inhibitor of BoNT A light chain.

Prior to the advancement of any BoNT A light chain inhibitor into animal models of BoNT intoxication, efficacy in cellular models should be demonstrated. We recently reported the first demonstration of a BoNT A light chain inhibitor that shows protection from SNAP-25 cleavage in a cellular model.[50] Quite surprisingly, this compound is simply a protected amino acid used in peptide synthesis, Fmoc-D-Cys(Trt)-OH (Fig. 18). Kinetic analysis of this compound revealed it also competitively inhibits BoNT A (K_i = 18 μM). Additionally, computational docking studies were performed and it was found that the predicted binding constant for the inhibitor (10 μM) was in close agreement with the experimentally determined value. Structurally, this model also revealed that a significant amount of binding energy can be attributed to burying the Fmoc group in a hydrophobic pocket, while the carboxylic acid moiety was positioned in close proximity to several positively charged residues. Interestingly, while competitive inhibition was observed, the docking model predicted no interaction between the active site zinc-binding residues and inhibitor. In light of the potency of this compound combined with its ready availability, it was next tested in a cellular model of BoNT intoxication as previously employed for riboflavin protection. At a concentration of 30 μM, almost no SNAP-25 cleavage was observed in Neuro-2a cells, while complete protection was observed when the compound was added to cells at a concentration of 60 μM. The importance in the discovery of potent light chain protease inhibitors of BoNTs

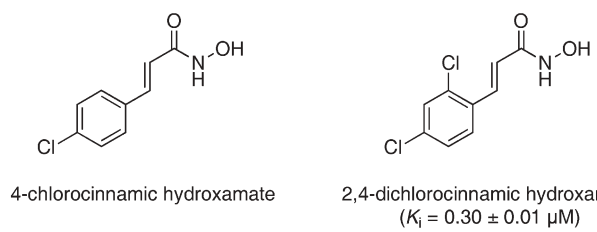


Fig. 17 Lead compound 4-chlorocinnamic hydroxamate and optimized inhibitor, 2,4-dichlorocinnamic hydroxamate, the most potent nonpeptide BoNT metalloprotease inhibitor reported to date.

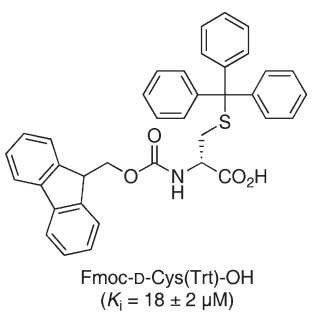


Fig. 18 Molecular structure of Fmoc-D-Cys(Trt)-OH, a compound shown to possess efficacy in the prevention of BoNT A-induced SNAP-25 cleavage in a cellular model.

could be a crucial step in rescuing nerve activity after toxin internalization. However, to date research has yet to advance any non-peptide molecules from enzymatic assays into the corresponding cellular and animal models.

Conclusion and outlook

The events of September 11, 2001 combined with the intentional release and dissemination of *B. anthracis* spores through the U.S. Postal Service has led to a heightened sense of awareness by the public of agents of biological and chemical warfare. Indeed, these horrific events have led to a dramatic increase in the levels of governmental funding for detection methods and prophylactic agents to protect from exposure to these toxic substances. Not surprisingly, this increase in support has led to an explosion in the number of publications in this area, with a wide variety of new technologies being introduced. Yet, while detection methods continue to be improved, an approved drug has yet to emerge that could combat an increasing threat from clandestine terrorist activities. Future work in this area will undoubtedly focus on this shortcoming, while continuing to advance detection technologies to allow for increasingly lower detection limits and greater field compatibility. New advances will likely be driven by a number of factors including the development of robust high-throughput assays for the rapid identification of compound efficacy in cellular and/or animal models. In total, current research studying chemical and biological warfare provides an excellent example of how to conduct studies at the interface of chemistry and biology.

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References

1 Stockholm International Peace Research Institute (SPIRI), *The Rise of CB Weapons: The Problem of Chemical and Biological Warfare*, Humanities Press, New York, 1971.
2 G. W. Christopher, T. J. Cieslak, J. A. Pavlin and E. M. Eitzen, Jr., *J. Am. Med. Assoc.*, 1997, **278**, 412–417.
3 E. Geissler, *Biological and Toxin Weapons Today*, Oxford University Press Inc., New York, 1986.
4 N. A. Sims, *The Diplomacy of Biological Disarmament*, Plenum Press, New York, 1983.
5 L. Szinicz, *Toxicology*, 2005, **214**, 167–181.
6 B. K. Singh and A. Walker, *FEMS Microbiol. Rev.*, 2006, **30**, 428–471.
7 N. B. Munro, S. S. Talmage, G. D. Griffin, L. C. Waters, A. P. Watson, J. F. King and V. Hauschild, *Environ. Health Perspect.*, 1999, **107**, 933–974.
8 J. A. Ashley, C.-H. Lin, P. Wirsching and K. D. Janda, *Angew. Chem., Int. Ed.*, 1999, **38**, 1793–1795.
9 D. Noort, H. P. Benschop and R. M. Black, *Toxicol. Appl. Pharmacol.*, 2002, **184**, 116–126.
10 V. P. Butler, Jr., *Pharmacol. Rev.*, 1982, **34**, 109–114.
11 P. Wentworth, Jr. and K. D. Janda, *Cell Biochem. Biophys.*, 2001, **35**, 63–87.
12 *Catalytic Antibodies*, ed. E. Keinan, Wiley-VCH, Weinheim, Germany, 2005.
13 B. J. Lavey and K. D. Janda, *J. Org. Chem.*, 1996, **61**, 7633–7636.
14 F. M. Raushel, *Curr. Opin. Microbiol.*, 2002, **5**, 288–295.
15 S. R. Caldwell, F. M. Raushel, P. M. Weiss and W. W. Cleland, *Biochemistry*, 1991, **30**, 7444–7450.
16 D. A. Spivak, T. Z. Hoffman, A. H. Moore, M. J. Taylor and K. D. Janda, *Bioorg. Med. Chem.*, 1999, **7**, 1145–1150.
17 L. M. Schwartz and L. O. Howard, *J. Phys. Chem.*, 1970, **74**, 4374–4377.
18 Y. Xu, N. Yamamoto, D. I. Ruiz, D. S. Kubitz and K. D. Janda, *Bioorg. Med. Chem. Lett.*, 2005, **15**, 4304–4307.
19 M. Mourez, *Rev. Physiol. Biochem. Pharmacol.*, 2004, **152**, 135–164.
20 D. N. Kyriacou, A. Adamski and N. Khardori, *Infect. Dis. Clin. North Am.*, 2006, **20**, 227–251.
21 D. V. Lim, J. M. Simpson, E. A. Kearns and M. F. Kramer, *Clin. Microbiol. Rev.*, 2005, **18**, 583–607.
22 B. Zhou, P. Wirsching and K. D. Janda, *Proc. Natl. Acad. Sci. U. S. A.*, 2002, **99**, 5241–5246.
23 R. H. Hoess, *Chem. Rev.*, 2001, **101**, 3205–3218.
24 J. W. Kehoe and B. K. Kay, *Chem. Rev.*, 2005, **105**, 4056–4072.
25 R. N. Brey, *Adv. Drug Delivery Rev.*, 2005, **57**, 1266–1292.
26 A. M. Friedlander, *Science*, 2002, **296**, 639–640.
27 J. G. Bartlett, *Clin. Infect. Dis.*, 2002, **35**, 851–858.
28 M. Mourez, R. S. Kane, J. Mogridge, S. Metallo, P. Deschatelets, B. R. Sellman, G. M. Whitesides and R. J. Collier, *Nat. Biotechnol.*, 2001, **19**, 958–961.
29 J. A. Maynard, C. B. Maassen, S. H. Leppla, K. Brasky, J. L. Patterson, B. L. Iverson and G. Georgiou, *Nat. Biotechnol.*, 2002, **20**, 597–601.
30 M. A. Wild, H. Xi, T. Maruyama, M. J. Nolan, P. M. Calveley, J. D. Malone, M. R. Wallace and K. S. Bowdish, *Nat. Biotechnol.*, 2003, **21**, 1305–1306.
31 S. C. Steiniger, L. J. Altobell, 3rd, B. Zhou and K. D. Janda, *Mol. Immunol.* in press.
32 E. A. Johnson and M. Bradshaw, *Toxicon*, 2001, **39**, 1703–1722.
33 B. R. Singh, *J. Toxicol. Toxin Rev.*, 1999, **18**, vii–x.
34 E. J. Schantz and E. A. Johnson, *Microbiol. Rev.*, 1992, **56**, 80–99.
35 C. Verastegui, G. Lalli, S. Bohnert, F. A. Meunier and G. Schiavo, *J. Toxicol. Toxin Rev.*, 2002, **21**, 203–227.
36 *Clostridium botulinum in FDA Bacteriological Analytical Manual*, ed. D. A. Kautter, R. K. Lynt, and H. M. Solomon, AOAC International, Arlington, VA, USA, 6th edn., 1984.
37 H. Ma, B. Zhou, Y. Kim and K. D. Janda, *Toxicon*, 2006, **47**, 901–908.
38 M. Mammen, S.-K. Choi and G. M. Whitesides, *Angew. Chem., Int. Ed.*, 1998, **37**, 2754–2794.
39 S. S. Arnon, R. Schechter, T. V. Inglesby, D. A. Henderson, J. G. Bartlett, M. S. Ascher, E. Eitzen, A. D. Fine, J. Hauer, M. Layton, S. Lillibridge, M. T. Osterholm, T. O'Toole, G. Parker, T. M. Perl, P. K. Russell, D. L. Swerdlow and K. Tonat, *J. Am. Med. Assoc.*, 2001, **285**, 1059–1070.
40 Z. Huang, *Technol. Cancer Res. Treat.*, 2005, **4**, 283–293.
41 D. A. Boroff, B. R. DasGupta and U. S. Fleck, *J. Albert Einstein Med. Cent.*, 1967, **15**, 22–34.
42 L. M. Eubanks, T. J. Dickerson and K. D. Janda, *FEBS Lett.*, 2005, **579**, 5361–5364.
43 R. P. Hicks, M. G. Hartell, D. A. Nichols, A. K. Bhattacharjee, J. E. van Hamont and D. R. Skillman, *Curr. Med. Chem.*, 2005, **12**, 667–690.
44 T. J. Dickerson and K. D. Janda, *ACS Chem. Biol.*, 2006, **1**, 359–369.
45 J. C. Burnett, J. J. Schmidt, R. G. Stafford, R. G. Panchal, T. L. Nguyen, A. R. Hermone, J. L. Vennerstrom, C. F. McGrath, D. J. Lane, E. A. Sausville, D. W. Zaharevitz, R. Gussio and S. Bavari, *Biochem. Biophys. Res. Commun.*, 2003, **10**, 84–93.
46 G. E. Boldt, J. P. Kennedy, M. S. Hixon, L. A. McAllister, J. T. Barbieri, S. Tzipori and K. D. Janda, *J. Comb. Chem.*, 2006, **8**, 513–521.
47 J. C. Burnett, E. A. Henchal, A. L. Schmaljohn and S. Bavari, *Nat. Rev. Drug Discovery*, 2005, **4**, 281–297.
48 B. G. Rao, *Curr. Pharm. Des.*, 2005, **11**, 295–322.
49 G. E. Boldt, J. P. Kennedy and K. D. Janda, *Org. Lett.*, 2006, **8**, 1729–1732.
50 G. E. Boldt, L. M. Eubanks and K. D. Janda, *Chem. Commun.*, 2006, **7**, 3063–3065.

