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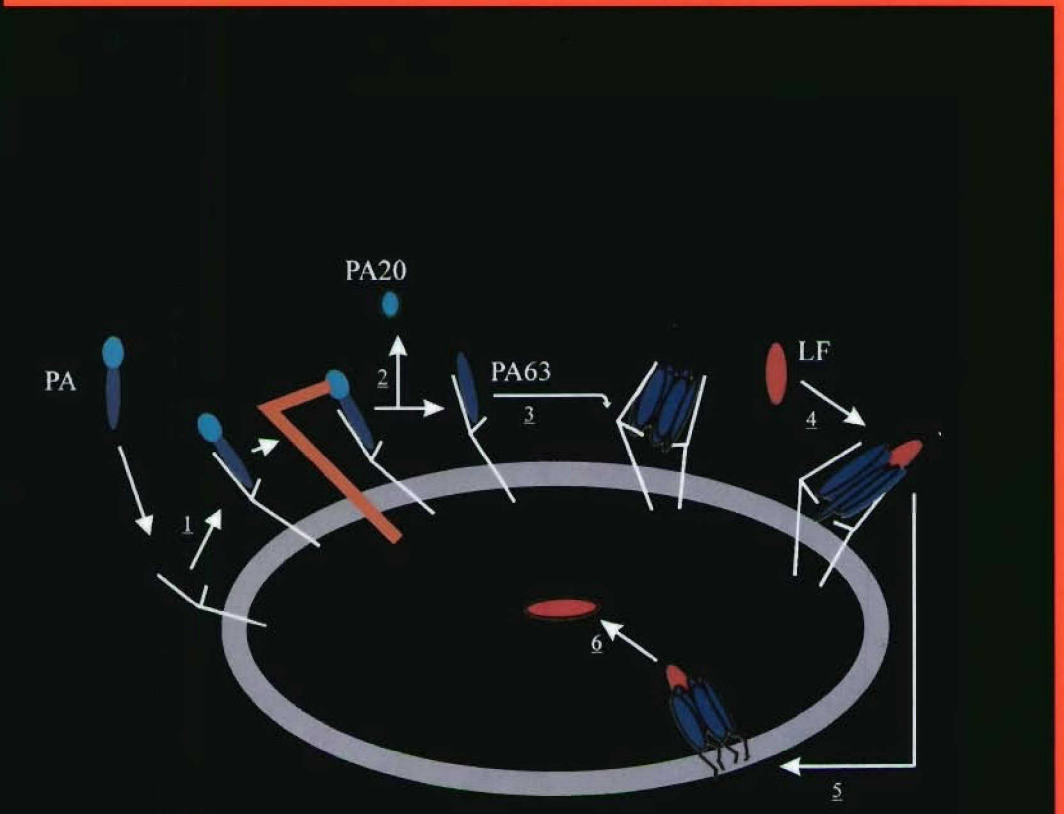

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The potential use of weapons of mass destruction (nuclear, biological or chemical) by terriorist organizations represents a major threat to world peace and safety. Only a limited number of vaccines are available to protect the general population fro mthe medical consequences of theses weapons. In addition there are major health concerns associated with a pre-exposure mass vaccination of the general population. To reduce or eliminate the impact of these terrible threats, new drugs must be developed to safely treat individuals exposed to these agents. A review of all therapeutic agents under development for the treatment of the illnesses and injuries that result from exposure to nuclear, biological or chemical warfare agents is beyond the scope of any single article. The intent here is to provide a focused review for medicinal and organix chemists of three widely discussed and easily deployed biological warefare agents, botulinum neurotoxin and ricin toxins and the bacteria Bacillus anthracis. Anthrax will be addressed because of its similarity in both structure and meachanism of catalytic activity with botulinum toxin.

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The Medicinal Chemistry of Botulinum, Ricin and Anthrax Toxins

Rickey P. Hicks*, Mark G. Hartell, Daniel A. Nichols, Apurba K. Bhattacharjee, John E. van Hamont and Donald R. Skillman

Department of Medicinal Chemistry, Division of Experimental Therapeutics, Walter Reed Army Institute of Research, Washington, DC 20307-5100, USA

Abstract: The potential use of weapons of mass destruction (nuclear, biological or chemical) by terrorist organizations represents a major threat to world peace and safety. Only a limited number of vaccines are available to protect the general population from the medical consequences of these weapons. In addition there are major health concerns associated with a pre-exposure mass vaccination of the general population. To reduce or eliminate the impact of these terrible threats, new drugs must be developed to safely treat individuals exposed to these agents. A review of all therapeutic agents under development for the treatment of the illnesses and injuries that result from exposure to nuclear, biological or chemical warfare agents is beyond the scope of any single article. The intent here is to provide a focused review for medicinal and organic chemists of three widely discussed and easily deployed biological warfare agents, botulinum neurotoxin and ricin toxins and the bacteria *Bacillus anthracis*. Anthrax will be addressed because of its similarity in both structure and mechanism of catalytic activity with botulinum toxin. The common feature of these three agents is that they exhibit their biological activity via toxin enzymatic hydrolysis of a specific bond in their respective substrate molecules. A brief introduction to the history of each of the biological warfare agents is presented followed by a discussion on the mechanisms of action of each at the molecular level, and a review of current potential inhibitors under investigation.

Rickey Hicks would Like to Dedicate this Manuscript in Loving Memory of his Mother Marian Hamilton Hicks, 1918-2004

HISTORY OF BIOLOGICAL WARFARE

The use of biological toxins, infectious agents and poisonous chemicals to kill one's enemies is not a new phenomenon [1-3]. For untold centuries, native populations in various regions of the world have employed toxins isolated from a variety of plants and amphibians to increase the lethality of their weapons. Man recognized several thousand years ago that infectious diseases could be used to reduce the fighting capabilities of an enemy army, as well as reduce the will of the civilian population to endure the hardships of a prolonged siege. Known examples of these unconventional approaches to warfighting include the use of the remains of infected animals and humans to contaminate the water supply of your enemies [3]. George Christopher *et al.* [2] relate the events surrounding the 14th century siege of the city of Kaffa (now Feodossia, Ukraine) by the Tatars. During the siege of the city, the Tatars experienced an epidemic of plague. They used their own misfortune to infect the population of Kaffa by catapulting the bodies of their dead into the city, thereby bringing about an epidemic of plague within the city. The plague so weakened the civilian and military population that the city soon fell. Evidence suggests that the Russian army may have used similar tactics against Sweden in 1710 [4]. In 1763, during the French and Indian War, Sir Jeffery Amherst [2] of the British army provided smallpox-contaminated blankets to native Americans in the Ohio River Valley, resulting in a smallpox epidemic that killed many hundreds of native Americans [2].

The last century has seen a dramatic expansion of all areas of biotechnology. Unfortunately, a less publicized expansion of the lethality of biological warfare agents has also occurred. Evidence suggests that the German military during World War I developed a biological weapons program involving covert attempts to contaminate livestock and animal feed in neutral countries prior to export to the Allied Nations [2,5]. Further reports suggest that attempts were made to contaminate horses and mules in France, Argentina and the United States to reduce the mobility of the Allied Armies in the field [2,6,7]. However, no direct evidence exists to indicate any attacks against human targets were carried out.

The most infamous biological weapons program known to date involves the Japanese research program directed by Shiro Ishii and Kitano Misagi, conducted at Unit 731 near Dingfan, Manchuria from 1932 to 1945 [8]. Unit 731 was a very large operation consisting of over 150 buildings that housed more than 3,000 technical personnel spread across a main facility and five satellite camps [9]. Japanese personnel who served with Unit 731 and were later captured by the Soviets Union after the war, reported that 12 large-scale field tests of various biological agents were conducted [2,8,9]. Hundreds of thousands of prisoners and Chinese civilians

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were exposed to anthrax spores and plague-infected fleas. It is believed that at least 10,000 prisoners of war, mainly Chinese, died as a result of these experiments [9]. Evidence presented at a war crimes trial conducted by the former Soviet Union in 1949 suggested that Unit 731 had produced as much as 660 lbs of plague bacteria per month, and it had the capability to produce 1,400 lbs of anthrax bacteria [8,10,11]. The intent of the Japanese to use biological weapons seems clear, as Unit 731 stockpiled approximately 900 lbs of anthrax to be used in special fragmentation munitions [10,12,13].

The first known plan to conduct a biological attack against the United States was by the Japanese Army during 1944 and 1945. Leaders at Unit 731 considered two different types of attacks on the United States with biological weapons. One such attack plan, codenamed CHERRY BLOSSOM, proposed using kamikaze attacks on major cities in California using seaplanes launched from submarines to deliver fleas infected with plague in hopes of starting major epidemics within the United States [13]. The use of large balloons, similar to those used to deliver fire-bombs across the Pacific, was also considered to deliver anthrax or plague [10,13]. Fortunately, the war ended before such attacks could be successfully carried out.

The United States military began in 1943, at Camp Detrick (now Fort Detrick) in Maryland, to investigate the offensive use of biological agents and toxins in response to a perceived German threat to develop and use such agents [4]. The United States continued to develop and produce biological weapons until 1969 when, by executive order [14,15], President Nixon terminated all offensive biological and toxin research and production in the United States [4]. As a result of this order, the United States Army destroyed all biological munitions in its inventory during the period of May 1971 to May 1972.

The former Soviet Union is believed to have developed the world's largest biological weapons program. The former Soviet Union maintained eight major biological weapons production and storage facilities at Zagorsk, Pokrov, Strizhi, Kirov, Omutninsk, Sverdlovsk, Stepnogorsk and Berdsk [16]. It is still unclear whether the Soviet Union used biological weapons during World War II. However, as pointed out by Mangold and Goldberg [13] in their book *Plague Wars* [13], there were two unexplained outbreaks of Tularemia and Q fever among German troops in Stalingrad and the Crimea during 1942 and 1943. No outbreaks of either disease has ever been reported in these areas of Russia, in addition, it is now known that the Soviets were developing both agents as biological weapons [13]. Dr. Kanatjan Alibekov, First Deputy Director, Biopreparat 1988-1992, reported that the Sverdlovsk facility manufactured anthrax biological weapons on a continuous basis, producing hundreds of tons of weapons grade anthrax [16].

The United States, and many other nations including the former Soviet Union, signed in 1972 the Biological Weapons Convention. This treaty prohibits research, development and production of offensive biological warfare agents. In 1998, then Secretary of Defense William S. Cohen responding to the question "How real is the threat from biological warfare?" stated, "It's very real. It's not only the threat of tomorrow, but it's the threat of today. More and more countries are developing both chemical and biological weapons. That number is bound to increase as technology information continues to be shared on the Internet and other means of communication. So, the threat is real both on the battlefield scenario and also direct towards civilian populations" [17]. We must be prepared to meet the medical challenges that these weapons of mass destruction present to the civilized world.

Pathogens and Toxins as Biological Weapons

"Biological Warfare is defined as the employment of pathogens or toxins to produce casualties in humans or animals and damage to plants or material" [1]. Pathogens are living, disease producing, microorganisms such as bacteria, viruses, rickettsia and fungi [3,4]. On a weight comparison, pathogens such as bacteria and viruses are many times more lethal than a comparable amount of chemical agent. This is because pathogens are self-replicating and they exhibit their toxicity by a specific and selective intervention into a physiological process of the targeted organism [3,4]. Chemical agents, on the other hand, are not self-replicating and generally exhibit their toxic effects by general disruption of physiological mechanisms. A wide range of biological organisms has been developed as potential biological warfare agents. However, to be useful militarily the agent must posses several of the properties listed in Table **1**. Toxins are chemical substances produced by various organisms that are toxic to humans or other species, and fall somewhere in between the pure definitions of a chemical weapon and a biological weapon [1]. Toxins are different from other more traditional chemical and biological agents in several ways (see Table **2**). Toxins are obtained from natural sources and are not synthesized in the laboratory. These compounds are non-volatile and usually are not absorbed through the skin as are many chemical agents. Finally, toxins are generally orders of magnitude more lethal per unit weight than common chemical agents such as sarin or soman [18]. A comparison of the LD_{50} dosage for selected toxins and chemical agents are summarized in Table **3**.

Table 1. To be Useful as a Weapon, a Biological Agent Must Posses Several of the Properties Given Below [1]

1)	Produce a well defined outcome in a given target.
2)	Manufacturability and stable to production, storage and transport conditions.
3)	Able to be disseminated (aerosolized etc.)
4)	Stable to the environment after dissemination.
5)	Difficult to detect in the environment.
6)	Short and predetermined incubation period.

The major difference between classic biological agents such as anthrax or *Clostridium botulinum* and toxins are: 1) toxins do not reproduce, 2) toxins generally do not demonstrate a long delay until the onset of symptoms, and 3) toxins are not contagious [1,3,4]. In this review, we have chosen to focus on the two most well studied toxins, botulinum neurotoxin and ricin. We have included anthrax in this discussion because of the great similarity in the mechanism (Table **4**) of action of the toxins produced by *Bacillus anthracis* to botulinum toxins and ricin.

Table 2. Comparison of the General Characteristics of Chemical Agents and Toxins [4]

Physical Parameter	Toxin	Chemical Agent
Source	Natural-isolated from various plants and microorganism	synthetic
Manufacturing ease	very difficult	large industrial scale
Volatility	non-volatile	generally very volatile
Relative Toxicity*	very high, 3-orders of magnitude or more greater than chemical agents	lower toxicity
Odor/Taste	odorless/tasteless	noticeable odors and taste
Immunogenicity	many are immunogens	most are poor Immunogens
Method of Delivery	can be aerosol	can be aerosol
Dermal Activity	not dermally active	dermally active

* on a per weight basis

All three toxins consist of two sub-units, both of which are required for transport across the cell membrane. Once inside the cell, the toxins are cleaved into their two respective sub-units. The catalytically active sub-unit causes cell toxicity by enzymatically cleaving a specific bond in its biomolecular target. Because these toxins act as enzymes, methodologies used to develop inhibitors of other enzymes may be employed to develop specific toxin inhibitors. A well established concept in enzyme chemistry is that compounds which resemble the enzyme-bound substrate transition state will bind very tightly to the active site of the enzyme, thus inhibiting the catalytic activity of the enzyme [19-21]. This approach has been used successfully to develop specific enzyme inhibitors such as human cathepsin L [22] and carboxypeptidase A [23]. The same methodology is currently being used to develop inhibitors of botulism neurotoxin A (BoNT/A), ricin and anthrax lethal factor (LF).

BOTULINUM NEUROTOXINS

Reviews on the therapeutic uses of botulinum toxins [24,25] and complications [26] associated with their use may be found be found in the literature.

As Biological Warfare Agents

The disease botulism, which is caused by the release of botulinum neurotoxins (BoNTs) by the bacteria *Clostridium botulinum*, has been well documented since the 1700's [27]. The name "botulism" in Latin means "sausage poisoning" and in most cases it is associated with "food poisoning" [27]. *Clostridium botulinum* spores germinate into vegetative bacteria that, under large-scale fermentation conditions produce large amounts of toxin [3]. The BoNTs, of which there are seven different serotypes, are some of the most toxic substances known (Table 3). Specifically, the serotype botulinum neurotoxin A (BoNT/A) is the most toxic substance known to man. A single gram of crystalline BoNT/A properly dispersed under ideal weather conditions could potentially kill over one million people [28]. The BoNTs inhibit acetylcholine release at the neuromuscular junction and induce paralysis that can lead to death by respiratory failure [29-31]. The BoNT's and the tetanus toxin are both members of the clostridal neurotoxin family and as such exhibit similar modes of action [31,32].

Despite their high level of toxicity, the technical requirements associated with weaponization of BoNTs on a large scale greatly reduces the potential success of such a terrorist attack. Additionally, sub-optimal environmental factors such as temperature, humidity, and wind will denature these proteins, thereby reducing their effectiveness. Exposure to air, or direct sunlight, will denature the toxin within a few hours, rendering it biologically inactive [3]. Heating at 80°C for 30 minutes will completely denature and inactivate the toxic effects of the protein [3]. The BoNTs are also denatured by exposure to free available chlorine

Table 3. Lethal Dose in Laboratory Mice of Selected Biological Toxins and Chemical Agents [4]

Agent	LD_{50} (μg/kg)	Molecular Weight(AMU)	Source
Botulinum toxin	0.001	150,000	bacterium
Tetanus toxin	0.002	150,000	bacterium
Diptheria toxin	0.10	62,000	bacterium
Ricin	3.00	64,000	plant
VX	15.0	267	chemical agent
Soman (GD)	64.0	182	chemical agent
Sarin (GB)	100.0	140	chemical agent

(FAC) in water. The BoNTs toxin is inactivated by approximately 84% after exposure for 20 minutes to 0.4% mg/L, FAC in water, conditions similar to those found in standard municipal water treatment plants [3].

Table 4. Common Features Shared by Botulinum Neurotoxins, Ricin and Anthrax Toxins

1.	Transport across cell membrane is required before intoxication
2.	Enzymatic activity results in cell death
3.	Toxins produced by bacteria
4.	Protein-protein interactions control the biological activity of these toxins

The application of the BoNTs as potential biological warfare agents began over sixty years ago. The Japanese biological warfare group, Unit 731, conducted experiments using prisoners of war to determine the lethal effects of force feeding *Clostridium botulinum* [2]. It has been reported that the United States first produced BoNT/A during World War II as a response to suspected possible German use of weaponized BoNT/A [2]. It is well known that the Soviets developed large stockpiles of weaponized BoNT/A. Additionally Iran, the former Iraqi government, North Korea and Syria are believed to be actively involved in the development of BoNT/A as a weapon [2]. The former Iraqi government admitted to the United Nations in 1995 that it produced 19,000 liters of concentrated BoNT/A, which is sufficient to kill the world's human population three times over [33]. It is believed that over 10,000 liters of BoNT/A were weaponized and placed into some type of delivery system [33].

It's commonly believed that even with the difficulties associated with aerosol dissemination of BoNT/A, an attack on the civilian population would cause large numbers of casualties. A point source aerosol release of BoNT/A is estimated to cause a casualty rate of 10% within 0.5 Km downwind of the point source. For a large city such as New York, this would effect tens of thousands of people [28]. The lethal inhaled dose for humans, estimated from primate studies, is in the range of 0.70- 0.90 μg (or 0.001 μg/kg) [28]. On a per weight basis, BoNT/A is reported to be 15,000 times more toxic than the nerve agent VX and 100,000 times more toxic than the nerve agent Sarin, two of the common organophosphate nerve agents [3]. The mechanism of toxicity of the BoNTs is the opposite of that of nerve agents. Nerve agents inhibit the action of acetylcholinesterase, thus increasing the concentration of acetylcholine to a high level. The BoNTs, on the other hand, inhibit the release of acetylcholine into the synapse [3]. Therefore, treatment methods that are effective for nerve agents, such as the administration of atropine, would most likely increase the damage done to individuals exposed to BoNTs [3].

Signs and Symptoms

The symptoms associated with *C. botulinum* intoxication may begin as soon as 24 - 36 hours after ingestion or inhalation. They may be delayed for several days if the source is a wound infection or wound contamination. The diagnosis is based solely on clinical observation and history, with symmetrical, descending, flaccid paralysis as a sentinel sign [4]. In outbreak settings, where others are known to be infected, botulism is relatively easy to recognize. Food borne, or ingestional botulism, often presents with nausea, a dry mouth, and diarrhea. Wound botulism lacks these gastrointestinal prodromal signs and symptoms. Botulism usually comes to medical attention when the victim(s) develop cranial nerve dysfunction, which typically starts with the eyes. Blurred vision, diplopia, dysarthria develops, and then generalized weakness spreads to the upper extremities, the trunk, and to the lower extremities. Respiratory failure may result from upper airway obstruction or paralysis of the diaphragm [4]. Laboratory procedures to include anaerobic cultures and toxin assays of serum, stool, and implicated food will confirm a diagnosis of botulinum intoxication in almost 75% of cases. Very uncomfortable electrophysiological nerve conduction studies will be characteristically abnormal in 85% of cases, although all muscle units may not demonstrate it. While initial symptoms may be attributed to a variety of diagnoses, the cranial nerve dysfunction followed by symmetric, descending progression of neuromuscular symptoms should alert the astute clinician that botulism is the accurate diagnosis. Similar entities such as Guillain-Barre syndrome, acute inflammatory polyneuropathies, and tick paralysis all have features that distinguish them from botulism. Patients with polio have fever and asymmetric weakness. Magnesium poisoning can be difficult to discern from botulism.

Civilian inhalation cases of botulinum intoxication are particularly rare. However, it is this mode of action that is the most relevant to a military or domestic terrorist threat. A rare example of clinical manifestations of inhalation botulism was reported in 1962 [34,35]. Within three days of conducting post-mortem examinations of animals exposed to BoNT, laboratory workers experienced a series of progressive neuromuscular and respiratory pathologies that now stand as a template for inhalation botulism diagnosis. By the third day post-exposure, the unique appearance of a "mucous plug" in the throat was the most notable observation. Difficulties swallowing and non-feverish cold symptoms were also apparent. The progression expanded to neuromuscular pathologies at the fourth day with the onset of mental fatigue, indistinct speech, moderately dilated pupils with slight nystagmus, retarded ocular motions and extreme weakness. In the instance of this 1962 exposure, the laboratory workers were treated on the fourth and fifth days with antibotulinum serum resulting in recoveries within 2 weeks. Resolution of botulism is much more difficult to achieve if treatment is delayed. Inhalational botulism may not respond if treatment is delayed until symptoms appear.

Mechanism of Action

As previously stated the BoNTs are produced by the bacterium *Clostridium botulinum* and are the most toxic chemicals known [36-38]. The seven immunologically different serotypes (A-G) of BoNTs are similar in structure to other biological toxins such as ricin, tetanus and cholera. The substrates and cleavage sites for the seven BoNT serotypes are given in Table **5** [39,40]. However, not all

seven serotypes have been associated with human toxicity. While species differences in toxicity remain unclear, serotypes A, B, E and F have been more commonly identified in human toxicity cases [35].

Table 5. The Seven Serotypes of Botulinum Neurotoxins Natural Substrates and Enzymatic Cleavage Sites [39,40]

Serotype	substrate	amide bond cleavage site* P_3---P_2---P_1----P_1'--P_2'--P_3'
A	SNAP-25	Ala-Asn-**Gln**---**Arg**-Ala-Thr
B	VAMP	Ala-Ser-**Gln**---**Phe**-Glu-Thr
C	Syntaxin	Thr-Lys-**Lys**---**Ala**-Val-Lys
D	VAMP	Asp-Gln-**Lys**---**Leu**-Ser-Glu
E	SNAP-25	Ile-Asp-**Arg**---**Ile**-Met-Glu
F	VAMP	Arg-Asp-**Gln**---**Lys**-Leu-Ser
G	VAMP	Thr-Ser-**Ala**---**Ala**-Lys-Leu

*The amide bond in the substrate cleaved by the botulinum neurotoxin serotype is shown in bold face type.

The structures of the BoNTs are reasonably well characterized. All are expressed as 150 kDa proteins [41]. The seven serotypes share a common zinc-binding motif of HExxH [42]. Each protein consists of three regions: the binding domain, the translocation domain and the catalytic domain [41,43]. These three regions are shown on a ribbon representation of the X-ray crystal structure of BoNT/A reported by Stevens and Lacy (Fig. **1**) [44,45]. BoNTs are characterized as AB toxins consisting of two protomers, A the activating and B the binding, that remain connected via a disulfide linkage after proteolytic cleavage [41]. In the case of BoNT/A and B, post-translational tryptic cleavage generates a 50 kDa N-terminal light chain (LC) and a 100 kDa C-terminal heavy chain (HC) [41]. The LC is the activating protomer and the HC is the binding protomer [41]. The LC possesses the toxic, zinc-endopeptidase catalytic domain [28,46]. The complete toxin is required to inhibit acetylcholine release in whole cells [46].

The process of acetylcholine inhibition is believed to involve three separate steps. The first two steps involve the binding of BoNT to a receptor located on the cell membrane of the nerve cell followed by membrane translocation of the toxin into the cytosol [46,47]. The final step is inhibition of exocytosis. The first two steps require the active participation of the HC while only the LC is required to inhibit exocytosis. A cartoon description of this process is presented in (Fig. **2**) [28].

BoNT/A inhibits acetylcholine release by site-specific cleavage of a Gln-Arg peptide bond in the synaptosomal protein SNAP-25 [27]. BoNT/B inhibits acetylcholine release via zinc-mediated proteolytic cleavage of a site-specific Gln-Phe peptide bond at positions 76-77 [48] of synaptobrevin isoform 2 [27]. Synaptobrevin-2 is a 19 kDa vesicle associated membrane protein (VAMP) located in the synaptosomes and is believed to be involved in the fusion of the plasma membrane with synaptic vesicles [47,49,50]. The BoNT toxins exhibit the highest substrate selectivity of all known proteases [40]. Substrate size specificity is critical to the maintain hydrolysis activity. The minimum substrate size varies within the BoNT family, BoNT/A requires a minimum size of 17 amino acid residues [51], BoNT/B requires 35 residues [57], BoNT/D and F require 42 residues [53]. The substrate specificity for each serotype is believed to be related to the three-dimensional relationship of the two

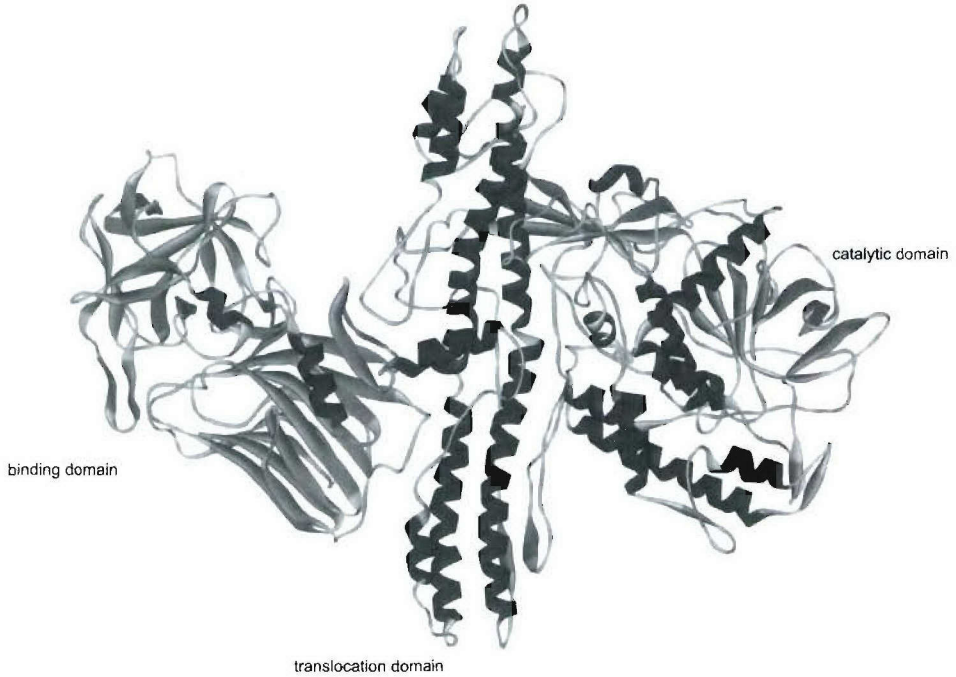

Fig. (1). The three regions of BoNT/A: 1) the binding domain, 2) the translocation domain and 3) the catalytic domain are shown on a ribbon representation of the X-ray crystal structure of BoNT/A reported by Stevens and Lacy [44,163].

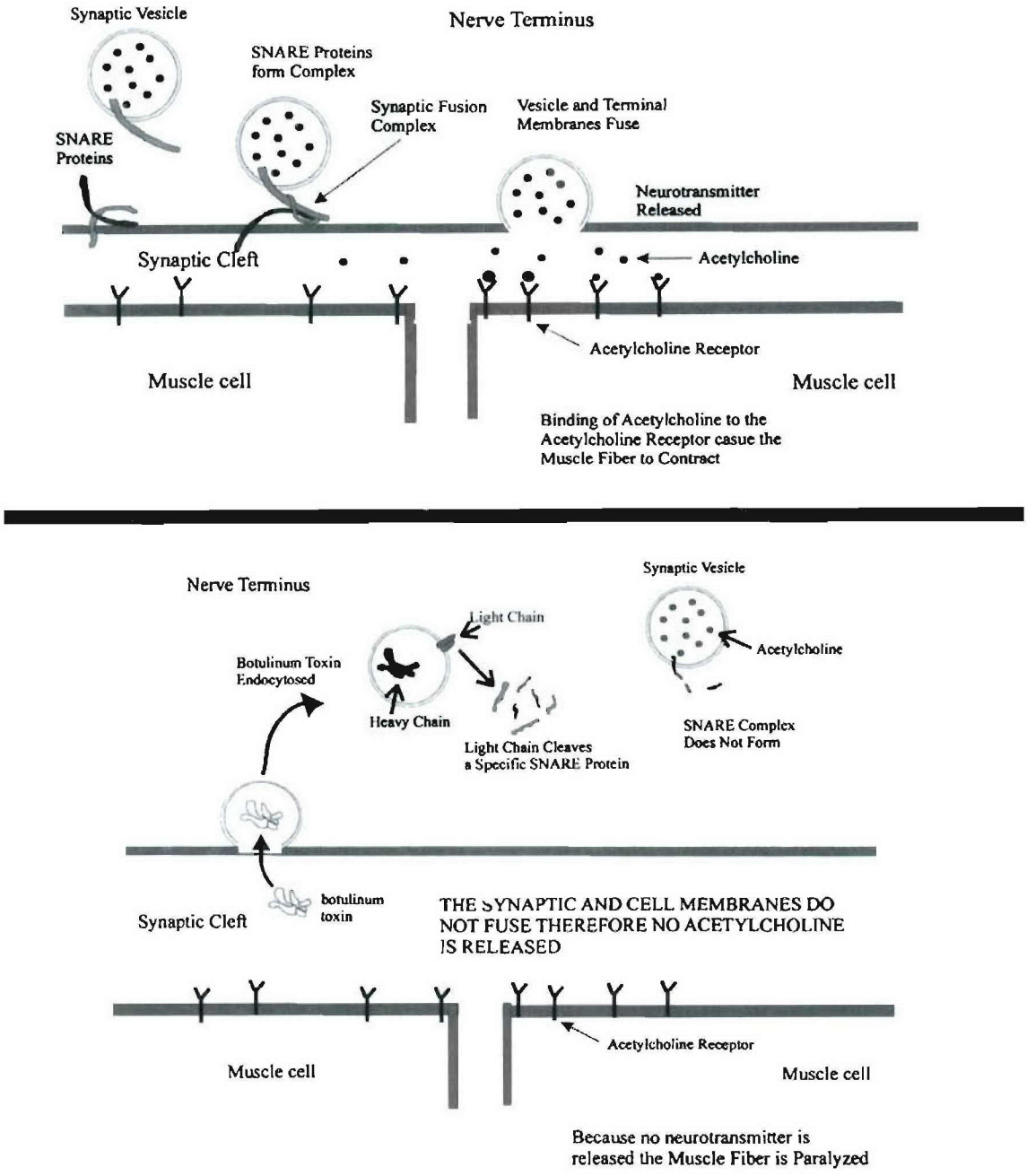

Fig. (2). Mechanism of action of BoNT. Top) In a normal nerve cell the fusion of the synaptic vesicle with the neuronal cell membrane induces the release of the neurotransmitter acetylcholine into the synaptic cleft. The process of fusion of the synaptic vesicle with the neuronal cell membrane is controlled by the assembly of the synaptic vesicle with the SNARE proteins forming a synaptic fusion complex that facilitates the unification of the two membranes. Bottom) Exposure to BoNT results in binding of the toxins to the neuronal cell membrane followed by endocytosis of the toxin into to the cytosol. The light chain of BoNT/A cleaves at a specific site of the SNAP-25 protein and prevents assembly of the SNARE proteins with the synaptic vesicle. Thus the synaptic fusion complex is not formed and no acetylcholine is released, resulting in the paralysis of the muscle fiber [28].

recognition sites on the toxins [46, 47]. The catalytic sites of the seven BoNT serotypes each contain two separate substrate recognition sites. One site on each serotype contains a homogenous nine amino residue recognition site, or SNARE (*s*oluble *N*-ethylmaleimide sensitive-*a*ttachment protein *re*ceptors) [40] secondary recognition (SSR) sequence [54]. The binding of the SSR is as expected, nonspecific for the seven serotypes [54]. For cleavage to occur, the substrate must be of specific length and amino acid composition to bridge the SSR and the active site where specific bond cleavage occurs [47,54].

The X-ray crystal structures of BoNT/A and BoNT/B reported by Steven and co-workers [44,45] and Swaminathan and co-workers [41] indicate that the areas within 8 Å of the zinc binding site of these two serotypes are "virtually

identical" in structure [41,44,46]. Yet significant variation in structure is observed at a distance of 15 Å [41,44,46]. However, the zinc-binding pocket is buried more deeply in the protein of BoNT/A (20-24 Å) than in BoNT/B (15 Å). These differences may be responsible for the observed substrate specificity of each serotype. An additional difference between the two serotypes is the role played by the N-terminal residues of the HC, which forms a "belt-like" polypeptide chain wrapped around the LC. In BoNT/A, the "belt" covers the active site and inhibits substrate binding [41,44,47]. Thus in BoNT/A, the LC is catalytically active only after reduction of the disulfide bond and separation of the HC from the LC. In BoNT/B, this belt does not cover the active site, leaving it fully accessible to the substrate. Therefore, BoNT/B-LC is catalytically active prior to reduction of the disulfide bond.

The X-ray crystal structure of BoNT/A also indicates that the Zn^{2+} atom is coordinated to His^{222} and His^{226} of the Zn^{2+} binding motif His-Glu-X-X-His [44,55]. Replacement of His^{222} with a Tyr residue completely inactivates the enzyme [56]. In addition, the Zn^{2+} atom is coordinated with Glu^{261}. Mutation of Glu^{261} greatly reduces the catalytic activity of the enzyme [57]. Glu^{223} is coordinated to a water molecule that is necessary for substrate hydrolysis. By coordinating the Zn^{2+} atom in this way the BoNTs are classified as being members of the gluzincin super family of metalloproteases [58]. Thermolysin is the prototypical member of this super family and serves as a model for a proposed base-type mechanism of hydrolysis [58]. The active site of BoNTs are very similar in structure to the active site of thermolysin and thus a similar mechanism of action for both classes of enzymes has been assumed, but the exact mechanism of action of the BoNTs is yet to be determined [40,59]. In the ground state of thermolysin a water molecule is bound to the zinc atom in the active site [40,60,61]. This water molecule is critical for enzymatic activity [40]. The process of enzymatic hydrolysis of thermolysin may be described in five steps [40,59]. Step 1, the ground state enzyme with the water molecule bound to the zinc atom binds to the substrate. Due to steric interaction with the bound ligand the position of water molecule changes and moves closer to the carboxylate side chain functionality of Glu^{143}. Step 2 the carbonyl of the amide bond that is to be cleaved is activated toward nucleophilic attack by hydrogen bonding with the imidazole side chain of His^{231}while at the same time the coordinated water molecule is activated by hydrogen bonding to the carboxylate of Glu^{143}. Thus the lone pair of electrons on the oxygen atom of water molecule attacks the electron deficient carbon atom of the carbonyl. These leads to step 3, the generation of a tetrahedral intermediate. In step 4 this tetrahedral intermediate or oxyanion intermediate is stabilized by coordination with the zinc atom and by hydrogen bonding to the side chains of His^{231} and Tyr^{157}. The atoms contributing to this stabilization form what is termed the "oxyanion hole" [40]. Collapse of the oxyanion hole results in amide bond cleavage and regeneration of the enzyme ground state [59]. The X-ray structures of BoNT/A [62] and BoNT/B [63] indicate that these two enzymes don't process the "oxyanion hole" [40]. The standard HExxH motif for zinc coordination is found in the BoNTs and thermolysin, however the corresponding T^{157} and H^{231} residues required to form the "oxyanion hole" [40] are not found in BoNT/A or BoNT/B. Using site directly mutagenesis Binz and co-workers reported that after substrate binding the side chains of the amino acid residues Tyr^{365} and Arg^{362} are involved in stabilizing the tetrahedral transition states formed during hydrolysis [58]. Hanson and Steven [64] proposed that Arg^{369} and Tyr^{372} are involved in stabilization of the transition state in BoNT/B. This leads to the question of whether or not functional groups could be incorporated into the inhibitor and interact with these side chains in such a way as to stabilize the enzyme-bound inhibitor. This approach was successfully employed on another biological toxin, ricin, by Tanake and co-workers [65] to develop a series of inhibitors of ricin A-chain. These inhibitors were designed to mimic the oxacarbenium ion transition state formed during depurination of ribosomal RNA [65]. Based on the similarity of BoNT/A to thermolysin [50], Binz and co-workers [58] have proposed a mechanism for hydrolysis of SNAP-25 by BoNT/A incorporating the roles played by individual residues on BoNT/A as shown in (Fig. **3**).

Development of Potential Inhibitors

Current therapy for BoNT intoxication involves "passive immunization" with equine antitoxin [28]. Treatment must begin as soon as possible after clinical diagnosis. Very little literature is available concerning the safety of the equine antitoxin, however several health concerns exist as a result of using antitoxins in the general population [28]. "*Currently no effective chemical antidote is available against botulism, primarily due to the lack of knowledge about the molecular structure and activity of BoNTs*" [46]. Clearly, there is a critical need for the development of specific potent inhibitors of BoNT that do not require pre-exposure immunization and can be used as a prophylaxis treatment. The angiotensin converting enzyme (another zinc-metalloprotease) inhibitors, captopril and phosphoramidon, do not inhibit the catalytic activity of the BoNTs [66,67]. This suggests that the BoNTs are significantly different from this class of zinc-metalloproteases [68].

The first competitive inhibitors of the proteolytic activity of BoNT/A were reported by Schmidt and co-workers [69]. Their approach to the discovery of these inhibitors employed a substrate peptide derived from residues 187 to 203 of SNAP-25 (acetyl-SNKTRIDEANQRATKML-NH_2). Replacement of residues at positions P_4, P_3, P_2', and P_3' along the polypeptide backbone by Cys yielded peptides that were hydrolyzed by BoNT/A [69]. A model binding site and the definitions of sub-sites on the enzyme and the substrate are illustrated in (Fig. **4**). The same research group also reported analogs of the SNAP-25 substrate analogs in which the first nine residues were removed, and the residue at what would have been either position 10 or 11 (corresponding to positions P_1 and P_2) was replaced by Cys, exhibited inhibitory activity. These peptides exhibit K_i (concentration of the compound needed to inhibit 50% of the catalytic activity of the enzyme) in the micro-molar range [69]. The most potent inhibitors were found to be shorter peptides containing 7 to 8 amino acid residues. Removal of the N-terminal nine amino acid residues in the SNAP-25 substrate peptide produced analogs with increased inhibitory potency. In addition, reducing the size of the N-terminus negates the

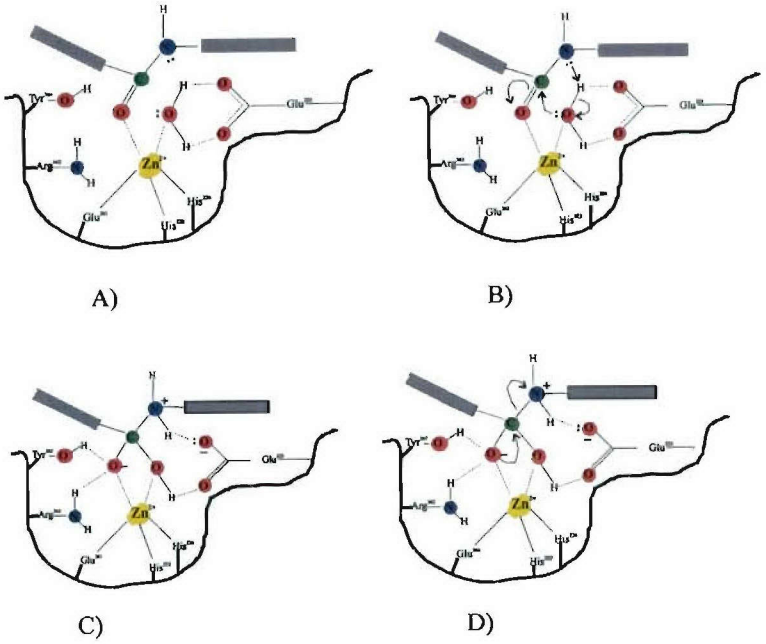

Fig. (3). Binz and co-workers [58] proposed a concerted mechanism of BoNT/A catalyzed hydrolysis. In order to understand this concerted mechanism we have developed this stepwise representation. **A)** This illustrates the substrate peptide bound to the Zn active site and the polarized water molecule bound to the carboxylate group of Glu^{223} prior to the beginning of the catalytic process. **B)** The carbonyl carbon of the substrate undergoes nucleophilic attack by the water molecule that is coordinated to and polarized by Glu^{223}. This is followed by proton transfer *via* the carboxylate of Glu from the attacking water molecule to the substrate amide nitrogen atom. This results in two tetrahedral intermediates at the carbonyl carbon and the amide nitrogen of the peptide bond undergoing hydrolysis. **C)** The developing positive charge on the tetrahedral nitrogen is stabilized by electrostatic interactions with the negatively charged carboxylate group of Glu^{223}. The developing negative charge on the carbonyl oxygen is stabilized by intermolecular hydrogen bonding with the side chain functionalities on Tyr^{365} and Arg^{362}. **D)** Amide bond hydrolysis is completed.

effect of the chirality of the Cys residue on inhibition [69]. A comparison of K_i for peptide inhibitors of varying length are summarized in Table **6**.

The structural requirements for inhibitory activity obtained from this investigation conducted by Schmidt and co-workers [69] may be summarized as follows: 1) The sulfhydryl functional group of the Cys is required for inhibitory activity. This is most likely due to the binding of the sulfhydryl functional group to the active site Zn^{2+}. Substitution at positions P_1 and P_2 yielding inhibitors and

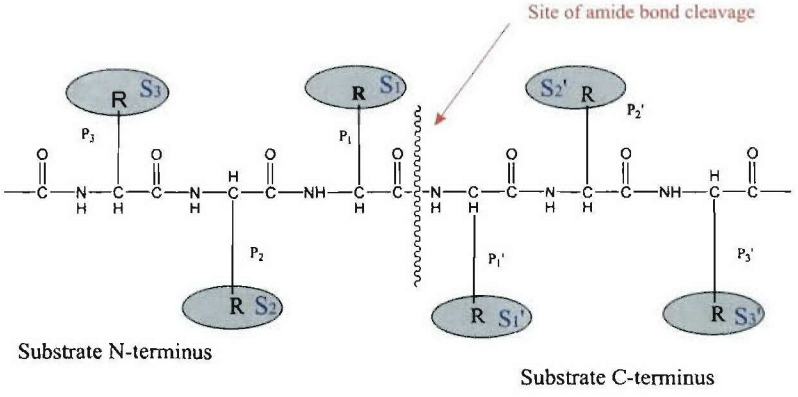

Fig. (4). Illustration of nomenclature used to describe the position of the amino acid side chains and enzyme binding sites as a function of the position of the enzymatic cleavage site. Residues located on the substrate to the left (N-terminus) of the cleavage site are termed P_1, P_2 increasing in number as you move further away from the cleavage site (toward the N-terminus of the substrate). The binding locations on the enzyme are number S_1, S_2, etc. Residues located on the right (C-terminus) of the cleavage site are termed P_1' and P_2' increasing in number as you move further away from the cleavage site (toward the C-terminus of the substrate). The binding sites on the enzyme are numbered S_1', S_2', etc.

substitution at positions P_4, P_3, P_2', and P_3' yield substrates. 2) D- or L-Cys are almost equally effective. 3) Shorter peptides particularly those containing the last five residues (Ala-Thr-Lys-Met-Leu) of the SNAP-25 substrate peptide are more effective.

Table 6. Peptide Inhibitors of BoNT/A [69]

Peptide	K_i (mM)
Acetyl-SNKTRIDEANQRATKML-NH_2	1.7
Acetyl-SNKTRIDCANQRATKML-NH_2	0.59
Acetyl-SNKTRIDEAN(d)CRATKML-NH_2	0.0040
Acetyl-CRATKML-NH_2	0.0019
Acetyl-(d)CRATKML-NH_2	0.0018
Acetyl-A(d)CRATKML-NH_2	0.026

The specific active site interactions required for enzymatic inhibition may be summarized as follows. The S_1 sub-site of BoNT/A is similar to those of other zinc metalloproteases in the fact that many different amino acid side chains can be tolerated at this site without loss of catalytic activity [69]. However, replacement of the native Gln residue found at P_1 with a Cys residue produces a peptide that is no longer a substrate and is not hydrolyzed by BoNT/A. These peptides exhibited inhibitory activity in the 2 to 4 μM range [69]. This observed activity suggests that the sulfhydryl group of the Cys residue interacts with the Zn^{2+} atom in the active site. This theory is supported by what has been observed with other metalloproteases containing P_1 zinc binding sites, which indicate that high inhibitory affinity is due, to a large extent, to the binding to the active-site zinc atom [70,71].

Replacement of the native Asp residue at the P_2 sub-site (interacting with S_2 site on BoNT/A) with either Ala or Gln residues results in a large decrease in the rate of hydrolysis with little effect on substrate binding [69]. Replacement of the Arg residue at position P_1' with a Cys residue yields a compound that exhibited poor binding characteristics and did not function as a substrate or inhibitor [69]. The Asp residue at position P_2 is required for catalytic activity, but seems to play no role in the initial steps of ligand binding [69]. However, the S_1' site on BoNT/A exhibits a very high degree of selectivity for an Arg residue at position P_1' of the substrate. Replacement of the native Arg residue with a Lys or even a D-Arg residue results in a significant loss of catalytic activity and functionality [69]. The S_1' site of other zinc metalloproteases, as determined by crystallographic and structural investigations, are buried deep into the hydrophobic core of the metalloprotease and are not solvent accessible [71,72]. These results suggested the opposite for the S_1' site of BoNT/A where the site is solvent accessible and hydrophilic in nature [69]. The substrates for the other BoNT serotypes all contain a hydrophobic residue at position P_1', indicating that the S_1' site for these serotypes are hydrophobic and similar to those found in other zinc metalloproteases [69].

Schmidt and Stafford continued to modify the active peptide N-acetyl-CRATKML-amide by replacing the Cys residue with different sulfur containing moieties (Fig. **5**) [73]. Two different approaches were employed: (1) The α–amino functionality was removed and the sulfhydryl group was moved to the α-carbon and (2) the α-amino functionality was replaced with a hydrogen atom and the sulfhydryl group remained on the β-carbon [73]. Replacement of the Cys with 2-mercapto-3-phenylpropionyl resulted in an inhibitor with a K_i of 330 nM, the most potent BoNT/A inhibitor reported to date [73]. In addition, these results suggest that the angiotensin-converting enzyme is not a good model to use for BoNT/A inhibitor development [73]. Results obtained in this study support the earlier observation that only the P_1' arginine sub-site is specific for a particular amino acid side chain [73].

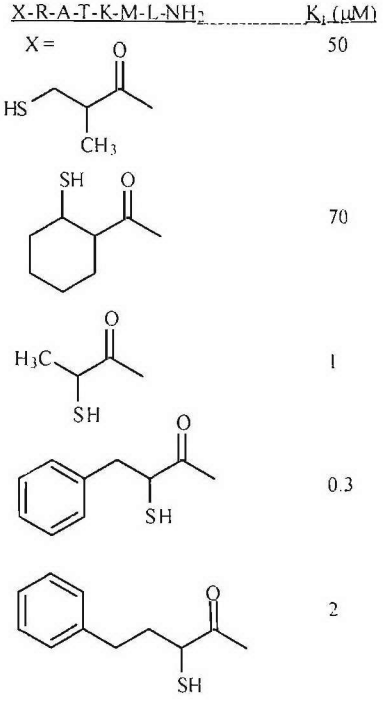

Fig. (5). Sulfhydryl moieties employed by Schmidt and Stafford [73] to replace the Cys residue in the active peptide N-acetyl-CRATKML-amide to develop more potent inhibitors of BoNT/A.

Burnett and co-workers reported the discovery of several small molecules that inhibit at low μM concentrations the protease activity of BoNT/A [74]. These compounds were identified by screening the NCI Diversity Set of 1990 compounds in a high-throughput fluorence-based bio-assay. From the NCI Diversity Set, 21 compounds were identified that a concentration of 20μM exhibit percent inhibition of protease activity of 14-100%. Several examples of these compounds are given in (Fig **6**). In addition a series of N,N-bis(7-chloroquinolin-4-yl)alkanediamines [75] and N,N-bis(7-chloroquinolin-4-yl)heteroalkanediamines [76] were screened also at concentrations of 20 μM some exhibited percent inhibition of protease activity of the BoNT/A in the range of 5-60% [74]. Based on these results the approved antimalrial drugs of the bisquinoline class, choroquine, amodiaquine, quinacrine, quinine and quindine were screed at a concentration of 50 μM exhibited percent inhibition of protease activity of 3 -30% [74].

Structural similarities, molecular docking investigating were conducted to a pharmacophore defining the binding requirements of these compound to the BoNT/A active site

[74]. These results suggest that the BoNT/A active site can accommodate two large aromatic ring systems (containing two to three fused aromatic/heteroaromatic rings). These two ring systems form a skeleton defining the three-dimensional relationship to other functional groups. One of these ring systems must contain a heteroatom that serves possibly one of two functions, coordination with the zinc or displacement of the water molecule from the active site. In addition to these aromatic rings two hydrophobic groups are also required [74].

Eswaramoorthy and co-workers [42] reported a novel mechanism for inhibition of the BoNT/B catalytic activity by the known zinc chelator, bis(5-amidino-2-benzimidazolyl)methane (BABIM). Crystallographic studies determined that upon complexation with BABIM, the active site of BoNT/B rearranges and the zinc atom is gradually removed from the active site by the BABIM and is then transported to another region of the protein. The IC_{50} for BABIM in the presence of BoNT/B light chain is in the range of 5 to 10 μM [42], and it is yet to be determined for the holotoxin (holotoxin is the term used to refer to the complete toxin, i.e. the HC attached to the LC).

Zdanovsky and co-workers [77] have reported the application of phage display libraries as a source for inhibitors of clostridial neurotoxin. Based on the results obtained, three peptides: Ser-His-Ser-Ala-Arg-Met-Thr-Gln-Thr-Ala-Ala-Phe-Leu-Tyr-Thr,(Z1): Ser-His-Ser-Asp-Arg-Thr-Pro-Trp-Pro-Arg-Trp-Leu-Leu-Ser-Tyr (Z2): Ser-His-Ser-Arg-Leu-Lys-Lys-Glu-Leu-Arg-Leu-Pro-Leu-Ala-Pro (Z3) were synthesized and evaluated for their ability to inhibit the enzyme activity of BoNT. All of these analogs exhibited inhibition in the low μM range [77].

Hayden and co-workers [78], using the approach they termed "hinge peptide mini-libraries" (PMLs), designed and synthesized a library of peptides that exhibited BoNT/A inhibitory activity. These peptides have the sequence acetyl-X(1)-X(2)-linker-X(3)-X(4)-NH_2 or acetyl-X(1)-X(2)-linker-X(3)-NH_2. 4-Aminobutyric acid is used as the very flexible linker moiety while the residues X(1) to X(4) are mixtures of the amino acids, Glu, Asp, Arg, Gln, His and Cys [78]. "Deconvolution of these libraries" [78] should provide leads for the design of peptide mimetics with BoNT/A inhibitory activity.

Garcia and co-workers[68] reported that the natural peptide buforin I, isolated from the stomach of the Asian toad *Bufo. bufo* gargarizans, inhibits BoNT/B activity at very low μM concentrations. Buforin I consists of 39 amino acid residues, has a single Q-F cleavage site (AGRGKQGGKVRAKA-KTRSSRAGL**QF**PVGRVHRLLRKGNK, cleavage site shown in bold) and exhibits an 18% homology with VAMP-2 surrounding the Q-F cleavage site. The presence of a Q-F peptide bond does not insure substrate or inhibitor binding to BoNT/B. High doses of substance P, a neuropeptide containing 11 amino acid residues with a single Q-F site, does not bind with BoNT/B [68]. The size and spatial requirements for ligand binding to BoNT/B seem to be different from those of BoNT/A. BoNT/B requires a longer amino acid sequence on both sizes of the cleavage site than does BoNT/A [66,68]. VAMP-2 analog peptides with a single Q-F site containing as few as 25 amino acid residues

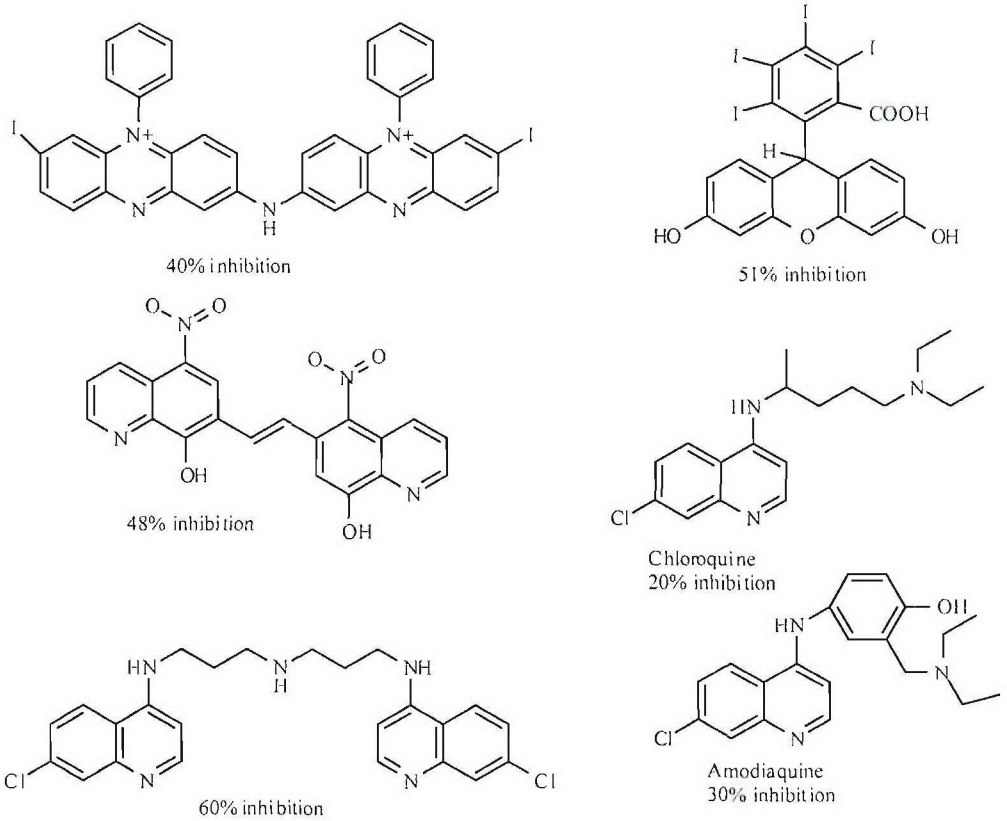

Fig. (6). Example BoNT/A inhibitors identified by Burnett and co-workers [74]. Examples taken from the NCI Diversity Set, the chloroquinolines and the antimalarial drugs amodiaquine and chloroquine.

are still cleaved by BoNT/B, but at a much reduced rate [66].

Secondary structure prediction calculations predict that VAMP-2 will exhibit two helical regions, one on either side of the cleavage site [79]. Similar analysis of buforin I suggest that it exists as a helix-turn - cleavage site - helix structure similar to that predicted for VAMP-2 [68]. In summary, this helix - cleavage site - helix conformation is believed to be important for substrate recognition and is required for efficient substrate cleavage [68].

Anne and co-workers [31,80] reported the development of a series of tripeptide consisting of non-natural amino acid residues as BoNT/B inhibitors. Due to the common substrate, synaptobrevin, for both BoNT/B and tetanus toxin [31,47] valuable structural information used to develop inhibitors of the tetanus toxin [81] were applied in the initial development of these BoNT/B inhibitors. The first step in the development of these compounds was to investigate the S_1 subsite of BoNT/B that recognizes the Glu [76] residue of synaptobrevin [47]. The S_1 sub region of BoNT/B has been shown to preferentially interact with polar functionality [47] this interaction was exploited and investigated by development of a series of inhibitors containing a variety of β-amino thiol groups [31,80]. The second step of the process involved incorporation of "optimized S_1 synthon" [31] developed in step 1 into a tripeptide designed to bind with the S_1 to S_2' subsites of BoNT/B. Combinatorial methods were used to investigate the three-dimensional conformational space of the natural amino acids to identify

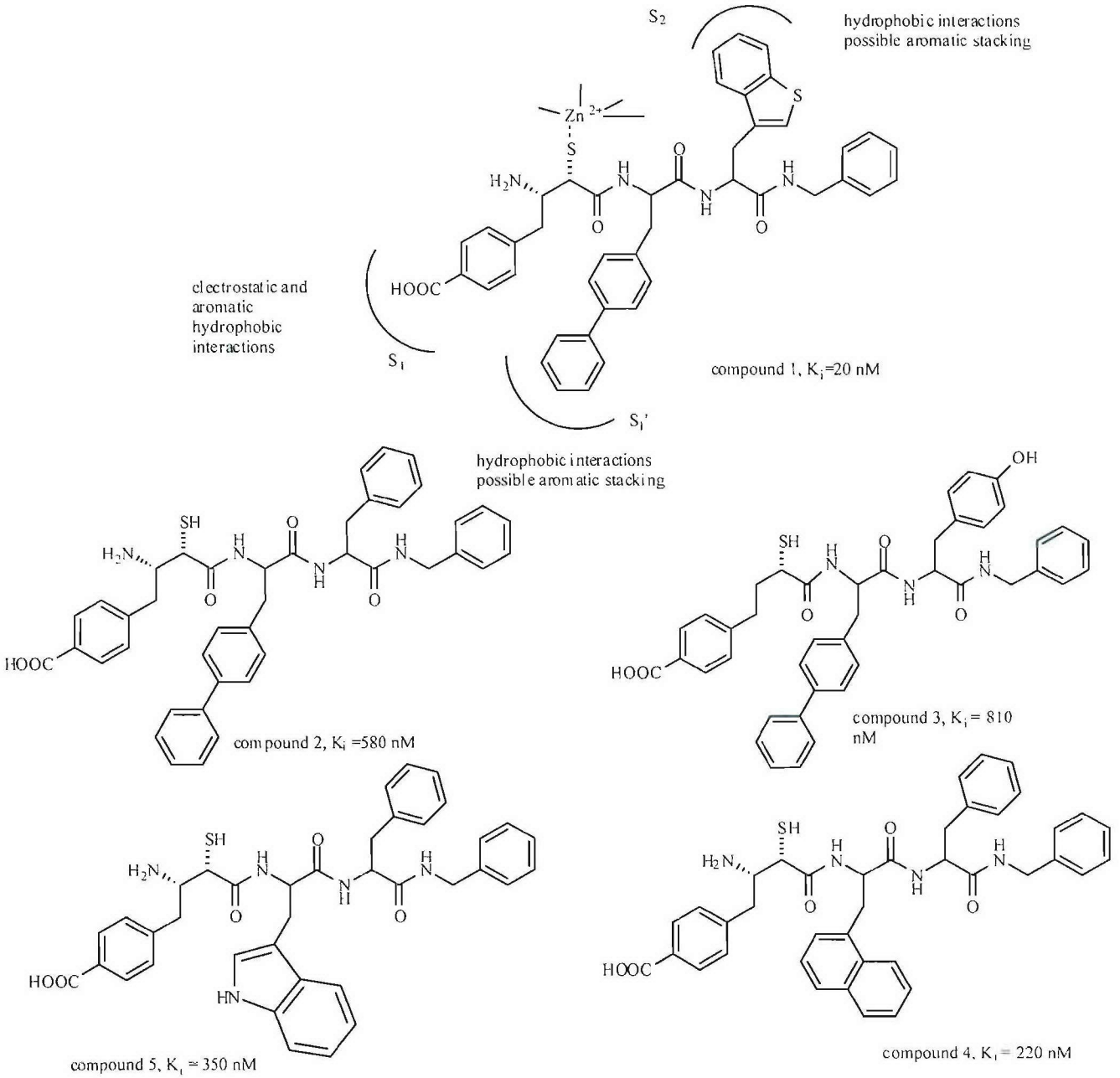

Fig. (7). Example tripeptide BoNT/B inhibitors developed by Anne and co-workers[31]. Ki values are given below each compound. At the top a cartoon representation showning how the most active analogs interacts with the BoNT/B, S_1, S_1' and S_2' binding pockets.

those that provided the most efficient non-covalent interactions with the three subsites [31]. Once determined these natural amino acids were replaced by non-natural amino acids to maximize the non-covalent interactions between the tripeptide and BoNT/B to increase the potency of the inhibitor [31]. Selected inhibitors obtained from this investigation and their K_i values are given in (Fig. 7) [31]. The best inhibitor compound 1, exhibited a K_i of 20 nM. These studies indicated that at the S_1 subsite a negatively charged functionality is required, a benzyl group substituted at the para position with a carboxylate moiety provided the optimal binding interaction [31]. The requirement for a negative charged functionality at the S_1 subsite of BoNT/B is different from that observed for the tetanus toxin were a meta-sulfonamide functionality provided optimal interaction [81]. The S_1' subsite clearly favor hydropbobic aromatic functionality which is consistent with the cleavage site of synaptobrevin where the P_1' site is a phenylalanine residue [31]. The importance of the S_1' position in ligand binding to BoNT/B [82,83] and to zinc metallopeptidases [84] in general are well documented. Large bulky aromatic functionalities were found to maximize the hydrophobic interactions between the inhibitor and the S_1' subsite [31]. This observation is consistent with crystallographic [41,47] data that indicates the S_1' subsite is a very deep hydrophobic gorge capable of accommodating very large hydrophobic groups.

Previous observations concerning BoNT/B enzymatic cleavage of synaptobrevin analogues substrates indicates that the roll played by the S_2' subsite is minimal [83], however in the case of these tripeptide inhibitors the S_2'subsite is critical. The work of Anne and co-workers [31] found that large heterocyclic aromatic groups give optimal binding to the S_2' subsite. This position in synaptobrevin is occupied by a glutamate residue [32] this suggested to Anne and co-workers [31] that this indicated that the binding interactions between the S_2' subsite of BoNT/B and synaptobrevin are not optimal.

Anne and co-workers used these pseudotripeptides as a model for a series of disulfides were prepared and evaluated for inhibitory activity. The most potent compound exhibited a K_i of 3.4 nM [85,86], the structure of this disulfide is given in (Fig. **8**).

The technique of designing transition state analog based on an enzymes natural substrate has been successful employed to develop protease inhibitors [87,88]. Oost and co-workers have used this strategy to developed inhibitors of BoNT/B based on modification of the 35-mer peptide VAMP-2 (60-94) a known substrate of BoNT/B [40]. The reduced amide and hydroxethylamine moieties were successfully employed to develop HIV-1 protease inhibitors [87,88] and therefore these groups were selected by Oost and co-workers [40] for incorporation into analogs of VAMP-2 (60-94). Based on literature reports that thiol and hydroxyl groups will coordinate with the active site zinc atoms and induce inhibitory activity [89], the 2-hydroxy-3-amino and 2-thiol-3-amino moieties were also selected from incorporation into analogs of VAMP-2 (60-94) [40]. The four transition atate moieties shown in (Fig. **9**) were used to replace even the residue Glu [76] of VAMP-2 (60-94) which corresponds to the S_1 site of the BoNT/B active site. Unfortunately all but the thiolamide analogs exhibited K_i values at 100 μM or above. The two thiolamide analogs exhibited K_i values of 1.1 and 3.6 μM [40].

RICIN

As a Biological Warfare Agent

Ricin, a 66 kDa enzyme, is a member of the broad family of toxic enzymes known as ribosome inhibiting proteins (RIP) and is easily isolated from the bean of the castor plant, *Ricinis communis* [90,91]. The enzyme consists of two polypeptide chains, A and B, linked by a single disulfide bond [90,91]. It is defined as an AB protein just like BoNT/A. The A-chain consisting of 267 amino acid

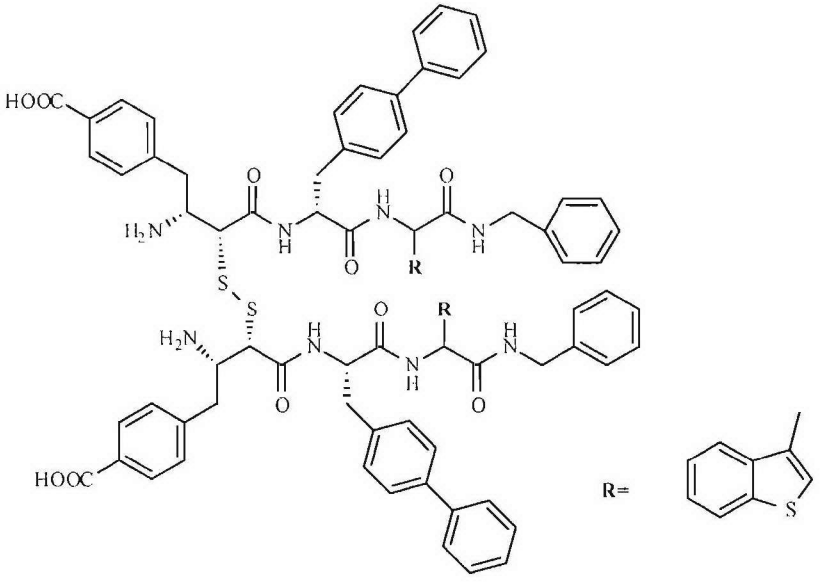

Fig. (8). The most active disulfide tripeptide analog developed by Anne and co-workers [85,86].

residues with 8 alpha helices and 8 beta sheets. The B-chain consists of 262 amino acid residues arranged in a barbell-like tertiary structure [92]. Ricin is a member of a class of biological toxins, the dichain ribosome-inactivating proteins, which selectively depurinates a single adenosine in ribosomal ribonucleic acid (RNA) [91]. In the case of ricin, the A-chain catalytically depurinates the 28S subunit of eucaryotic ribosomes to inhibit protein synthesis [91].

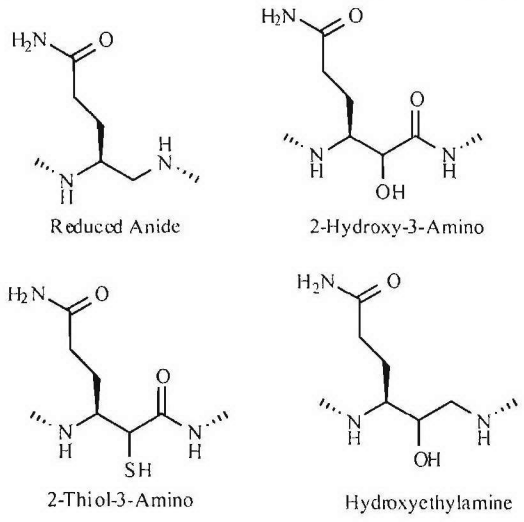

Fig. (9). The four transition state moieties used by Oost and co-workers to develop a substrate based inhibitors of BoNT/B [40].

The ancient Egyptians grew and processed *Ricinis communis* for its oil, which was used for lubrication and as a laxative [90]. Even today there are many reports from various parts of the globe of the use of both the seeds and the oil to treat a wide variety of health related conditions. Castor oil was employed extensively during World War I and World War II in aircraft engines as a lubricant [90]. Its use greatly diminished with the advent of synthetic lubricants.

While ricin is 1000 times less toxic than BoNT/A, with a LD_{50} for humans in the range of 0.1 to1.0 μg/kg, it offers other advantages as a biological weapon [91]. Ricin is heat-stable and is readily available worldwide as a by-product of castor oil production. The toxin remains in the castor meal after extraction and may be obtained by simple procedures [93]. Ricin, codenamed COMPOUND W by the United States, was considered for weaponization based on two major factors: its relatively high toxicity and its ease of production in large quantities [90]. The United States and Great Britain are reported to have developed and tested a W-bomb during World War II [94]. There are no known instances of the mass use of ricin as a biological warfare agent. It was, however, used in London by the KGB to assassinate the Bulgarian defector Georgi Markov [95]. Ricin, due to its ease of preparation and availability, has become the biological weapon of choice for individuals or terrorist groups [90]. In 1995, two American tax protestors were convicted, under the 1989 Biological Weapons Anti-Terrorism Act, of possessing ricin as a biological weapon [90,96]. Canadian authorities arrested an individual in 1995 attempting to transport a large quantity of ricin toxin into Canada from the United States [97]. In 1999, an individual threatened to poison two Colorado judges using ricin. He was subsequently arrested at which time the raw materials for making ricin were seized from his Tampa, Florida home [98]. On January 5th 2003, CNN reported the arrest of seven men in London charged with the possession of the necessary equipment and supplies to manufacture ricin in sufficient quantities to conduct a terrorist act.

Signs and Symptoms

As with BoNT, the route of entry relevant to a military and/or domestic terrorism application would be through the respiratory system. Also similar is the fact that human inhalation toxicity data for ricin is very limited. Perhaps the only well-documented information for review is a case of processing plant workers who were treated for an allergic syndrome after exposure to castor bean dust [99]. The clinical onset of symptoms appears to follow a course consistent with general respiratory allergic reactions: nasal and bronchial congestion, tightness of chest and itchiness of eyes culminating over time to more severe wheezing and bronchial asthma. Accidental sublethal aerosol exposures occurred in the 1940's. Affected persons presented with acute fever, chest tightness, cough, dyspnea, nausea, and arthralgias 4 – 8 hours after exposure [100]. Profuse sweating developed several hours later, and it was associated with termination of symptoms. While human inhalation toxicity data is very limited, oral and parenteral exposure data appear to correlate quite well with animal studies [101]. Therefore, it is believed that similar correlations may be suggested from examining animal respiratory studies. Rat [101] and nonhuman primate studies [102] have documented specific features consistent with a severe inflammatory response. Large aerosol exposures in experimental animals result in necrosis of upper and lower respiratory epithelium, and perivascular and alveolar edema. Aerosolized ricin binds to the ciliated cells of the bronchiolar lining, alveolar macrophages and the cells lining the alveolar spaces [101]. The entire exposed respiratory tract will necrose after exposure to a large dose. Ingestion of ricin leads to onset of symptoms at least 8-10 hours later. Nausea, vomiting, abdominal cramps, severe diarrhea, and vascular collapse develop. This is followed by necrosis of the gastrointestinal epithelium, local hemorrhage, and necrosis of the liver, spleen, kidneys, and lymph nodes. Intramuscular injection causes severe local muscle necrosis and visceral organ involvement [103].

General laboratory results for ricin intoxication are non-specific, diagnosis is difficult and dependent on clinical observations as with generalized forms of allergic responses. However, a more specific confirmation of ricin aerosol intoxication may be made based on specific ELISA analyses of nasal mucusal swabs taken within 24 hours of exposure [104]. It is important to note that while ricin is strongly immunogenic and thus would yield circulating antibody responses in survivors, it is also quickly metabolized and excreted making the analysis of blood and tissue samples more challenging [105].

Mechanism of Action

The ricin holotoxin consists of a heterodimer linked via a disulfide bond (Fig. **10**). This disulfide bond joins the ricin

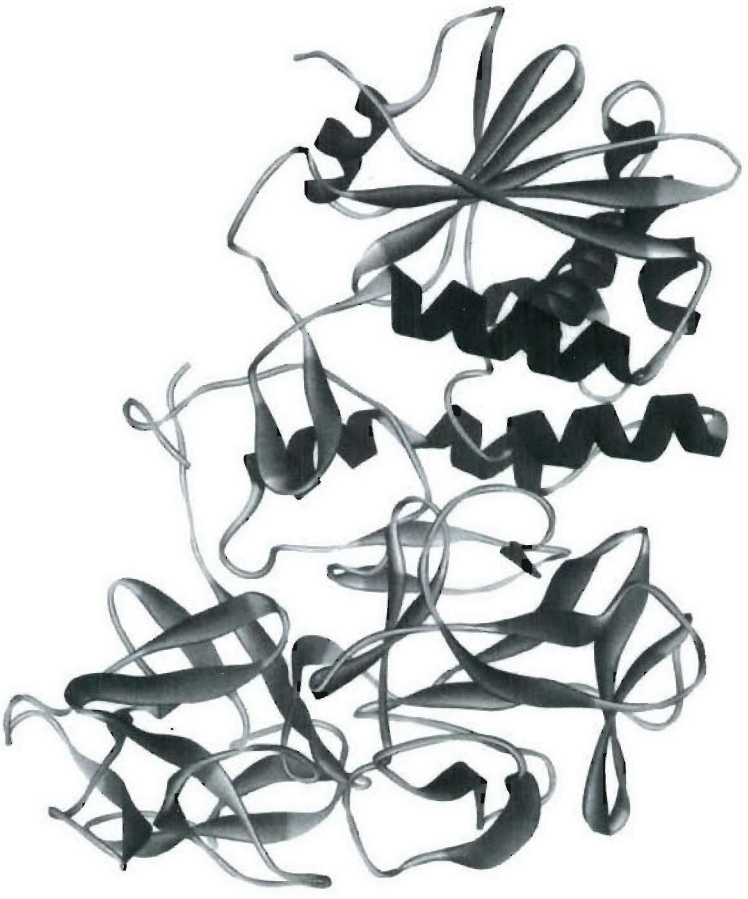

Fig. (10). A ribbon representation of the X-ray crystal structure of ricin holotoxin reported by Rutenber and co-workers [92,163].

A chain (rRNA N-glycosidase) and the ricin B chain (a galactose-specific lectin) [106,107]. The ricin B chain (RTB) is required for entry into the cytosol and binds to galactosides located on the surface of the cell [107,108]. The holotoxin is transported into the cytosol via endocytosis. Once the toxin enters the cell, the disulfide bond linking RTA and RTB is reduced [109]. The RTA must now pass through, *via* an unknown mechanism, an intracellular membrane to the ribosome [107]. The A-chain of ricin is a very specific N-glycosidase that catalyzes the hydrolysis of a specific adenosine residue (A^{4324}) [110] of rat 28S ribosomal RNA, thus inhibiting protein synthesis and resulting in cell death [65,109,111]. The specificity of the catalytic activity of RTA requires the presence of the sequence GAGA located in a stem-loop structure motif [112,113]. In this structure, RTA will catalyze the hydrolysis of the first adenine in the sequence GAGA [65,112,113]. It has been shown that short sequences of RNA that contain at least three base pairs and the GAGA sequence in a stem-loop also act as substrates for RTA [65,112-114].

The work of Schramm *et al.* [115] have shown via kinetic isotope studies that the catalytic activity of RTA proceeds via a $D_N{*}A_N$ mechanism involving an adenine oxacarbenium ion intermediate (Fig. **11**) [65].

The crystal structures of ricin, RTA and ricin-substrate analog complexes have been determined [109,116-119]. A ribbon representation of the x-ray crystal structure reported by Retenber and co-workers is shown in (Fig. **10**). The crystallographic data coupled with the results of mutagenesis studies conducted by Robertus and co-workers [109] have shown that the substrate, adenine, binds in the active site of the enzyme between two conserved tyrosine residues at positions 80 and 123. In the active site, a strong hydrogen bond is formed between the side chain of Arg^{180} and the N_3 of adenine [109]. Replacement of the Arg residue with a Gln residue reduces the activity of the enzyme by 250-fold, while replacement with a Lys reduce reduces the activity only 3-fold [109]. This indicates the presence of an ionizable charged group at position 180 is critical for enzymatic activity [109]. The Glu residue at position 177 is near to the active site and believed to play a role in hydrolysis of the substrate, however its exact role remains unclear [109]. There are several different possible mechanisms of hydrolysis, all of which involve the same residues on the enzyme. Robertus and co-workers [109] have summarized the major differences in the proposed mechanisms as which of the two residues Glu^{177} or Arg^{180} activates the attacking water molecule and the degree of protonation of the N_3 nitrogen of the adenine substrate by Arg^{180}.

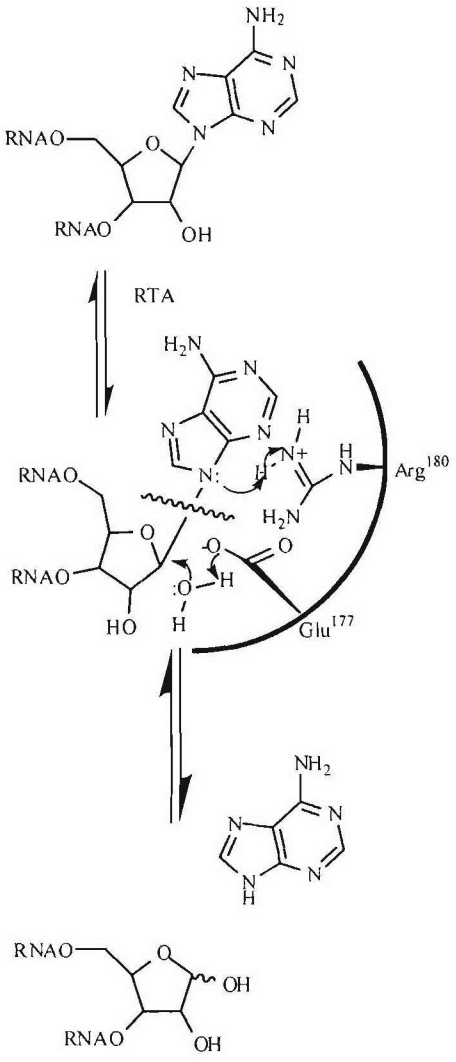

Fig. (11). The work of Schramm *et al.* [115] have shown *via* kinetic isotope studies that the catalytic activity of RTA proceeds *via* a D*A mechanism involving an adenine oxacarbenium ion intermediate [65].

Development of Potential Inhibitors

Yan and co-workers [120] studied the interaction of four small heterocyclic ring systems with the RTA binding site. Molecular mechanics, semi-empirical and *ab initio* quantum mechanics methods were employed to calculate the binding energies of formycin, adenine, quanine and pterin (Fig. **12**) to the x-ray crystal structure of the RTA binding site [92]. Based on this crystallographic data [92] and protein engineering experiments [121-123] a hypothetical active site involving residues Tyr^{80}, Val^{81}, Gly^{121}, Tyr^{123}, Glu^{177} and Arg^{180} was used as the docking site for these calculations [120]. This investigation indicates the order of binding affinity for these compounds as pterin > formycin > adenine > quanine [120]. The major interaction which determines substrate recognition, binding selectivity and affinity in this model is the formation of inter-molecular hydrogen bonds between the substrate and specific residues in the active site. Residues Gly [121] and Val^{81} (carbonyl oxygen atoms) act as hydrogen bond acceptors while the amide nitrogen atom of Val^{81} and the side chain nitrogen atoms of Arg^{180} act as

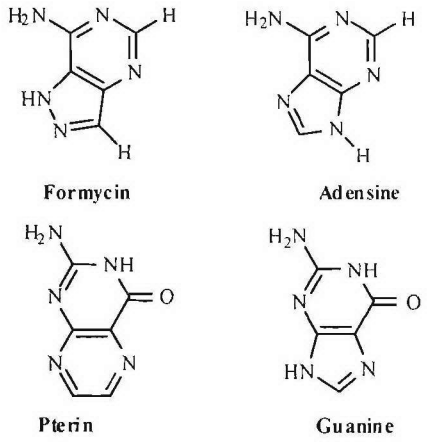

Fig. (12). The four small heterocyclic ring systems used by Yan and co-workers to study the interaction of small heterocyclic ring systems with the ricin A chain binding site [120].

hydrogen bond donators [120]. A very simple cartoon representation of the hydrogen bonding framework of this model is shown in (Fig. **13**). This information will be useful in the development of more selective and potent small molecule inhibitors of ricin.

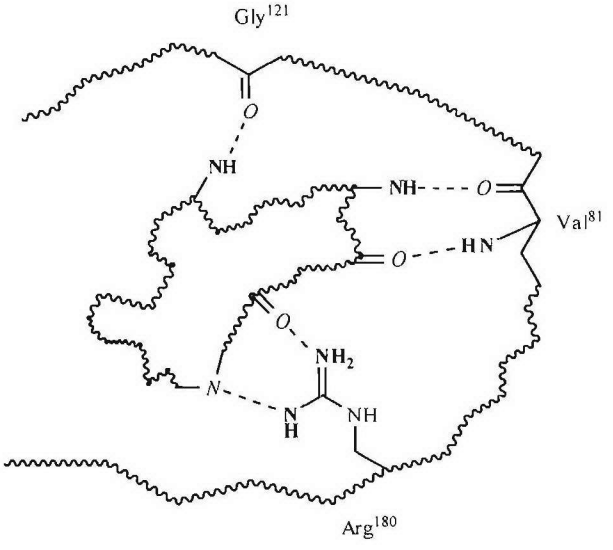

Fig. (13). A cartoon representation of the hydrogen bonding framework of the model proposed by Yan and co-workers [120]. This model was based on the crystallographic data [92] and protein engineering experiments [121-123] yielding a hypothetical active site involving residues Tyr^{80}, Val^{81}, Gly^{121}, Tyr^{123}, Glu^{177} and Arg^{180}. Residues Gly and Val (carbonyl oxygen atoms) act as hydrogen bond acceptors (shown in *italics face*) while the amide nitrogen of Val^{81} and the side chain nitrogen atoms of Arg^{180} act as hydrogen bond donators (shown in **bold face**) [120]. Hypothetical hydrogen bond acceptors (shown in *italic face*) and hydrogen bond donators (shown in **bold face**) on the bound ligand are also indicated.

The natural product brefeldin A, (Fig. **14**) is known to exhibit a wide and diverse array of biological activity including antibiotic, antimitotic and antiviral activities [124]. It was reported separately in 1991 by the groups of Yoshida and co-workers [125] and Hudson and co-workers

[126] that brefeldin A protects cells from the toxic effects of ricin by disrupting the structure of the Golgi apparatus. Unfortunately, the research conducted with brefeldin A has not evolved into a viable candidate for development as a treatment for ricin intoxication.

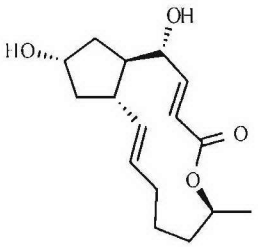

Fig. (14). The structure of the natural product brefeldin A [124].

Schramm and co-workers [65,115] have reported the synthesis and activity of a series of RTA inhibitors that resemble the proposed oxacarbenium ion transition state. The specific molecular subunits were incorporated into the one position of 10 or 14-base pair RNA stem-loop motifs as shown in (Fig. **15**). Binding coefficients (K_i) in the low μM to nM range were also observed for these compounds [115]. Additional derivatives are in development. The research group of Robertus [127] has also developed a series of ricin inhibitors based on the complementarities of critical hydrogen bond donator and acceptor functional groups on the inhibitor to the active site of ricin. Pteroic acid was found to have an IC_{50} of 0.6 mM, while other pyrimidine based analogs exhibited IC_{50} in the range of 0.4 to 3.6 mM (Fig. **16**).

ANTHRAX

As a Biological Warfare Agent

Anthrax, from the Greek word for coal (anthrakitis), was the name originally given to the cutaneous illness cause by the Gram-positive bacteria *Bacillus anthracis* due to the formation of black skin lessions [128]. The name now applies to all forms of anthrax. During the fall of 2001, an unknown person or persons terrorized the United States by sending anthrax spores through the mail system. During this attack, eleven people were infected with inhalation anthrax and five of these died (fatality rate of 45%) as a result of the infections [129]. A larger, more deadly example of the danger of inhalation anthrax occurred in 1979 at a former Soviet military bio-weapons production facility in Sverdlovsk [130]. It was reported that during routine maintenance of processing equipment, protective filters were not replaced and anthrax spores escaped from the containment building. This exposure resulted in 79 cases of inhalation anthrax with a fatality rate of 86% (68 deaths). The estimated human LD_{50} for inhalation anthrax, based on primate data, is from 3,000 to 50,000 spores [2,129,131-134]. The extrapolation of human LD_{50} estimates from animal challenge studies has not been a trivial issue.

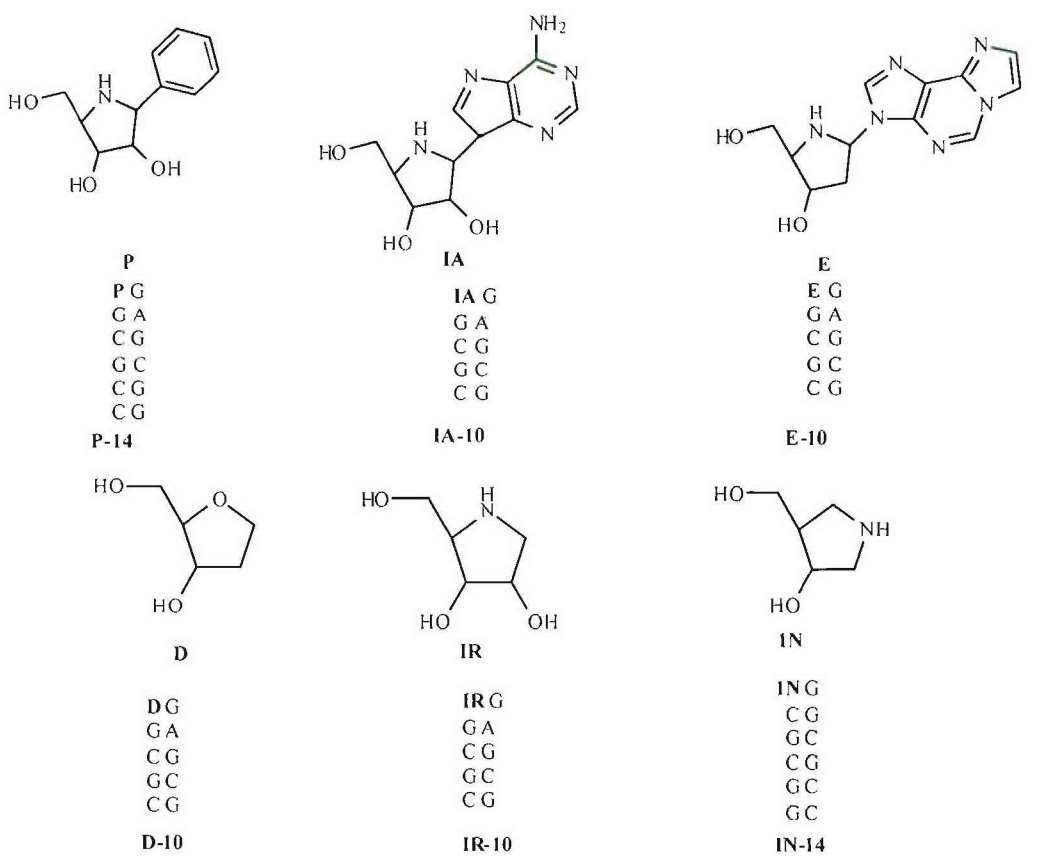

Fig. (15). Schramm and co-workers [115] have reported the synthesis and activity of a series of ricin A-chain inhibitors that resemble the proposed oxacarbenium ion transition state. The specific molecular subunits were incorporated into the one position of 10 or 14-base pair RNA stem-loop motifs.

Responding to media controversy over the licensed anthrax vaccine administered to members of our armed forces, the Institute of Medicine (IOM) took up a number of issues surrounding vaccine efficacy to include the fundamental correlation of human and animal pathophysiology [135]. After an extensive review of historical animal studies and human autopsy data, the IOM concluded that the pathophysiology in macaques best mimic the pathophysiology seen in humans after inhalation exposure to anthrax spores. Additionally, the IOM noted that in certain small animal models, particularly guinea pigs and rabbits, the model appeared to be more susceptible to aerosol challenge than in humans and monkeys.

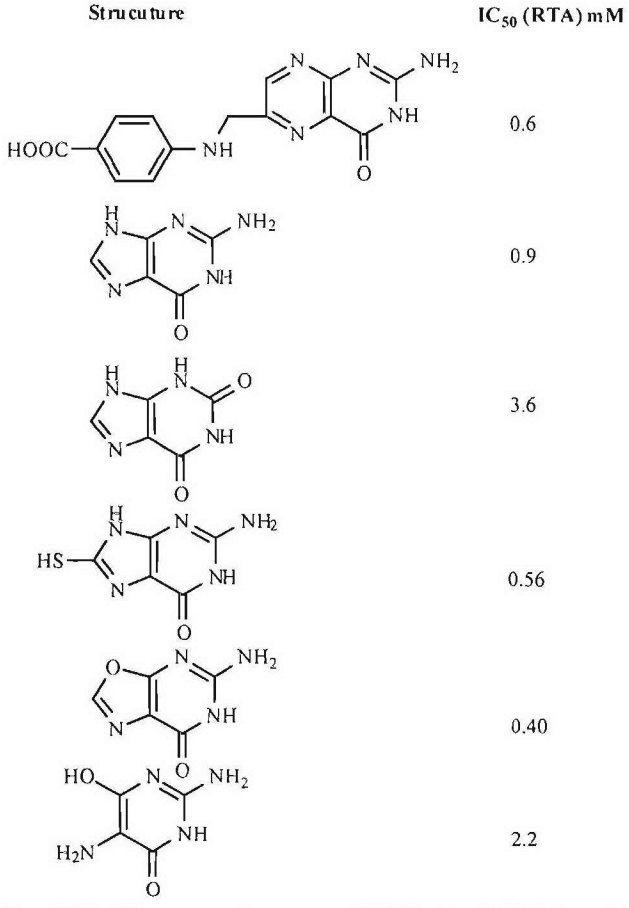

Fig. (16). The research group of Robertus [127] has also developed a series of ricin inhibitors based on the complementarities of critical hydrogen bond donator and acceptor functional groups on the inhibitor to the active site of ricin.

Two widely cited summaries of an anthrax threat to an urban population indicated 50 kg of anthrax released over an urban population of 5 million would yield 250,000 exposed persons and result in 100,000 untreated deaths [136]. 100 kg of anthrax spread properly over Washington, DC would yield between 130,000 and 3 million deaths [137]. The number of fatalities could be reduced by prophylactic treatment of the affected population within 24 to 48 hours of exposure with antibiotics such as penicillin, doxycycline, and ciprofloxacin [129]. Duration of prophylactic therapy, dosage required, and whether or not concomitant vaccination with the anthrax vaccine offers a survival advantage, are topics of intense debate.

Signs and Symptoms

The incubation period for inhalation anthrax is 1 to 6 days. Early symptoms include fever, malaise, fatigue, cough and mild chest discomfort, very much like the flu [4]. These symptoms are typically followed by a period of symptomatic improvement which lasts between several hours to three days. This is followed by acute, abrupt onset of severe respiratory distress with dyspnea, stridor, diaphoresis, and cyanosis. These symptoms represent the tremendous growth of bacteria in the mediastinal and bronchial lymph nodes ultimately leading to hemorrhagic mediastinitis. Bacteremia, toxemia, septic shock develop as disease rapidly progresses, with meningitis in 50% of victims. Death usually occurs within 24-36 hours after onset of the acute phase.

A potential diagnostic clue is a widened mediastinum on chest x-rays [138]. The disease is detectable by Gram stain of blood cultures during the late stages of the disease. Nasal swabs and respiratory secretions can be sent for PCR as important diagnostic specimens during the incubation period, which is normally hours to as long as seven days after inhalation. Most cases will present within 48 hours. A rapid diagnostic test can detect toxins in the blood during the acute phase. Early and precise diagnosis is difficult, and relies heavily upon the clinician's acumen and the clinical setting. Serology tests are primarily of use in retrospective diagnoses [139-142].

Mechanism of Action

Most toxins that act intracellularly, such as ricin and the BoNTs, are characterized as AB toxins consisting of two protomers after proteolytic cleavage of the holotoxin. Protomer A is the activating or enzymatic region and B is the binding protomer, which interacts with a toxin specific receptor located on the surface of the target cell [41,143]. *Bacillus anthracis* is also characterized as an AB toxin. However, the bacterium secretes three separate proteins: protective antigen (PA: 83kDa), edema factor (EF: 89kDa) and lethal factor (LF: 83 kDa). The combined actions of these proteins constitutes the anthrax toxins which induce cell death [129,143]. Each protein by itself is non-toxic [128]. The PA serves the role as the common binding protomer (protomer B), while EF and LF serve as independent A protomers, carrying out different enzymatic reactions within the host cell. The proposed mechanism for toxin self-assembly and endocytosis is given in (Fig. **17**).

An individual PA molecule consists of four structural domains existing mainly as anti-parallel β-sheets (Fig. **18**) [128,129]. Domain 1 contains the binding sites for two calcium ions as well as the cleavage site for the furin protease [128]. Domain 2 is involved in pore formation and has a large flexible loop that is believed to be involved in membrane insertion [128]. Domain 3 is involved in the formation of the heptamer. Lastly, domain 4 binds with the anthrax toxin receptor (ATR) [128]. The first step in anthrax intoxication is the binding of PA to the ATR. At the receptor, PA undergoes a proteolytic cleavage by furin, or a furin-like protease, at a surface loop within Domain 1 to yield two fragments [129], a 20 kDa N-terminal fragment

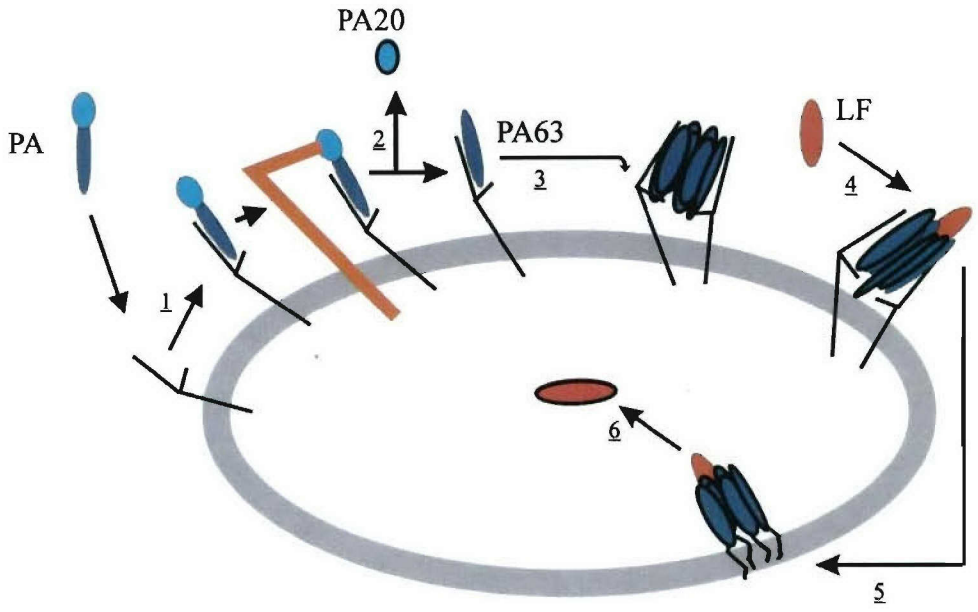

Fig. (17). A cartoon representation of the proposed mechanism for anthrax toxin self-assembly and endocytosis [157]. **Step 1**: PA binds to the anthrax toxin receptor located on the surface of the cell. **Step 2:** Proteolytic cleavage of PA by furan proteases to PA_{63} and PA_{2c}. **Step 3:** Self-association of monomeric PA_{63} to form heptamer bundle. **Step 4:** Binding of LF/EF to the prepaore stage of the PA_{63} heptamer bundle. **Step 5:** Endocytosis of the receptor-PA_{63} heptamer bundle-LF complex. **Step 6:** pH- dependent insertion of PA_{63} and translocation of LF/EF.

(PA_{20}) and a 63 kDa C-terminal fragment (PA_{63}). PA_{63} remains bound to the receptor and undergoes self-assembly forming a ring-shaped heptamer bundle that binds one to three molecules of EF or LF. The interaction of the seven PA_{63} molecules has been described as packing "pie wedges" with Domain 1'(the remainder of Domain 1 after proteolytic cleavage) and Domain 2 lining the inside of the bundle and Domains 3 and 4 aligned on the outside of the heptamer bundle [128]. This arrangement presents a large hydrophobic region for the binding of LF and EF [128].

The resulting complex is transported into the cell *via* a receptor-mediated endocytosis and subsequently carried to an acidic compartment within the cell. The low pH causes the PA_{63} heptamer/LF (or heptamer/EF) complex to cross the membrane into the cytosol. Here, in the case of LF, a metalloprotease cleaves mitogen-activated protein (MAP) kinase-kinases, Mek1 and Mek2, at their N-terminus hydrolyzing the Pro^8-Ile^9 amide bond of Mek1 and the Pro^{10}-Ala^{11} amide bond of Mek2 [144,145]. This most likely results in the overproduction of certain lymphokines by the macrophages, and thereby causes cell death through a type of septic shock [129].

Anthrax LF is a distant relative of other zinc metalloproteases [146,147]. A ribbon representation of the x-ray crystal structure of LF bound to MAP kinase 2 (Mek2) (Met-Leu-Ala-Arg-Arg-Lys-Pro-Val-Leu-Pro-Ala-Leu-Thr-Ile-Asn-Pro) reported by Pannifer and co-workers is shown in (Fig. **19**), divided into four separate domains [128,147]. Domain 1 is involved in binding to PA_{63}. Domain 2 is believed to play a role in substrate recognition. Domain 3 is inserted into Domain 2 and Domain 4 contains the large deep (approximately 40 Å) gorge containing the active site. This gorge is lined with regions of Glu and Asp residues that impart a negative electrostatic potential. In addition, a large number of Gln and Asn residues are also located in the gorge of the active site. The side chain amide functional groups may act as hydrogen bond donor and acceptors during the binding of the substrate. This hypothesis has yet to be evaluated. The x-ray crystal structure [147] of LF indicates that the Zn^{2+} atom is coordinated to a water molecule and to the side chains of His^{686}, His^{690} and Glu^{735}. The Glu^{687} residues is believed to activate the water molecule coordinated with the Zn^{2+} during hydrolysis [147]. Tyr^{728} is located opposite Glu^{687} and is believed to be involved in protonation of the leaving amino group after hydrolysis [147].

Development of Potential Inhibitors

Due to recent domestic terrorism events, there is a clear need for therapeutic agents to protect large populations of both military and civilian personnel from the toxic effects of anthrax exposure. Vaccines, if administered in advance, offer effective protection for healthy, high risk, military and civilian personnel. However, available vaccines are not an efficient protective measure for the general population [148-150]. In the case of the development of anthrax intoxication there are three potential targets: inhibition of the endocytosis, inhibition of the protease activity of lethal factor and inhibition of the protease activity of edema factor. For individuals that have not received pretreatment with vaccines, or for members of the general population, there is a clear and critical need for a therapeutic agent to inhibit, post-exposure, the toxic affects of the anthrax lethal factor. Inhibition of the catalytic activity of LF would be the major

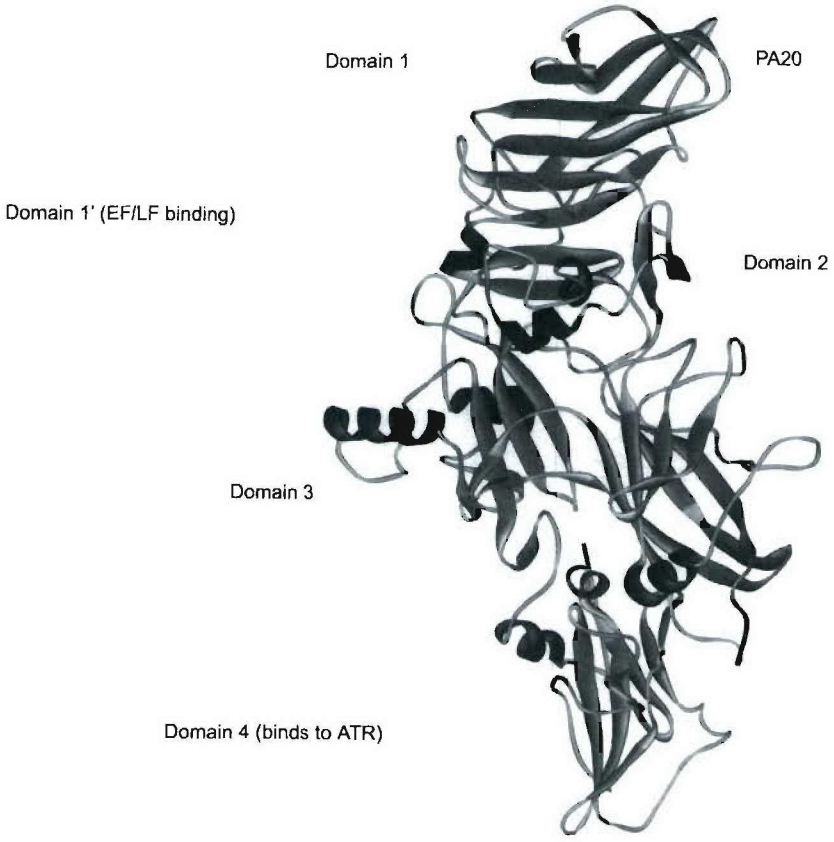

Fig. (18). A ribbon representation of the x-ray crystal structure of PA reported by Petosa and co-workers [156,163]. Individual PA molecule consists of four structural domains existing mainly as antiparallel β-sheets [128,129]. Domain 1, contains the binding sites for two calcium ions as well as the cleavage site for the furin protease [128]. Domain 2, which is involved in pore formation, has a large flexible loop that is believed to be involved in membrane insertion [128]. Domain 3, is involved in the formation of the heptamer. Domain 4, binds with the anthrax toxin receptor (ATR) [128].

desired treatment option for exposed individuals [128,151,152].

Collier and co-workers [153] reported the isolation via phage display of two peptides that bind to the PA_{63} heptamer and inhibit its interaction with LF and EF, thus preventing the transport of LF and EF into the cell. The two peptides [153] consist of the following amino acid sequences: peptide 1 (P1) His-Thr-Ser-Thr-Try-Trp-Trp-Leu-Asp-Gly-Ala-Pro and peptide 2 (P2) His-Gln-Leu-Pro-Gln-Try-Try-Trp-Trp-Leu-Ser-Pro-Gly. These two peptides are believed to interfere with step number 4 (Fig. **17**) of the process of binding and translocation of LF and EF. These compounds may interact with the PA_{63} heptameric bundle in a similar fashion to vaccine antibodies that distort the heptameric bundle, thus preventing binding and translocation of LF and EF into the cytosol. The approach taken by Collier and Whitesides [153] to develop inhibitors of LF binding to PA_{63} heptameric bundles involves the preparation of polyvalent inhibitors that incorporate multiple copies of P1 and P2 bound to a molecular spacer.

Hicks and co-workers [154] reported based on NMR and NOE restrained simulated annealing calculations that P1 adopts a helical structure involving residues 3-9 on binding to the PA_{63} heptamer bundle. Collier and co-workers [153] have suggested that the tetrapeptide sequence Try^5-Trp^6-Trp^7-Leu^8 plays an important role in binding to a hydrophobic pocket near the LF binding site of PA_{63}. The results of Hicks and co-workers [154] indicate that the tetrapeptide sequence exists in the bound conformation as a very well defined α-helical structure. (Fig. **20**) These four residues are on different faces of the helix, and are not interacting with PA_{63} in a linear fashion. Therefore, it must be concluded that the relative positions of the side chains in three-dimensional space is of critical importance in binding to PA_{63}. Based on examination of this structure, they speculated that the N-terminal His^1 residue (which is also common to P1 and P2), even though it is more conformationally flexible than residues 3 to 9, plays a critical role in binding by providing an electrostatic interaction on the same face of the helix as Tyr^5 and Trp^6 residues. The observation by Hicks and co-workers [154] is in contradiction to the structure proposed by Glick and co-workers [155]. This group investigated the binding interaction of the tetrapeptide Try^5-Trp^6-Trp^7-Leu^8 using conjugate gradient minimization computational methods with the crystal structure of the PA_{63} heptamer "pre-pore" reported by Petosa and co-workers [156]. Their results indicate that the tetrapeptide binds in an extended β-conformation to a hydrophobic pocket on PA_{63} - formed

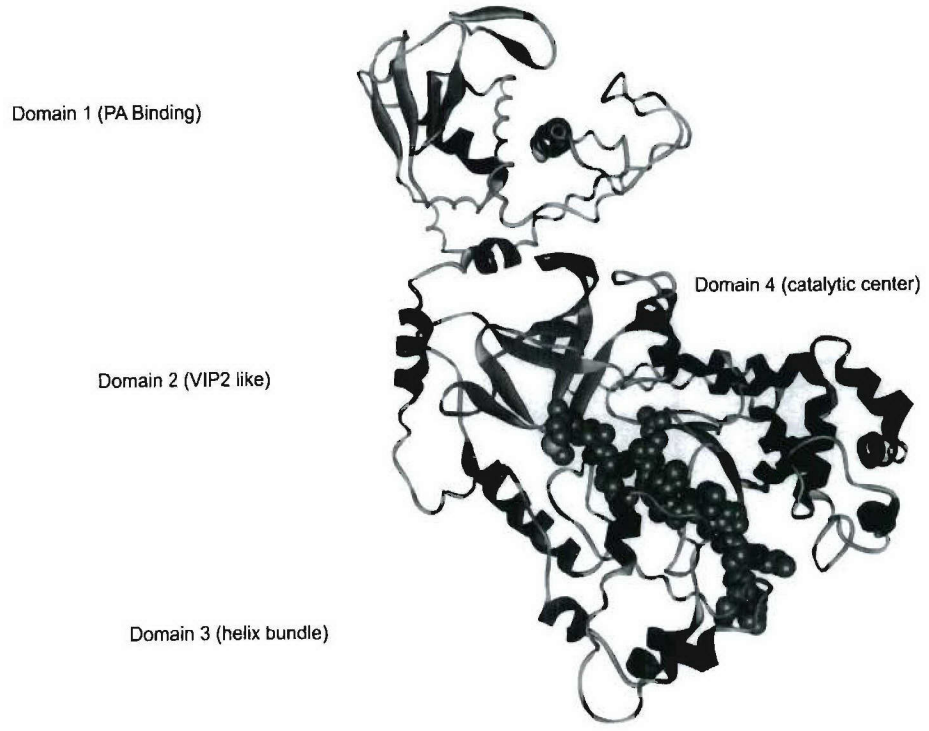

Fig. (19). A ribbon representation of the x-ray structure of LF bound to mitogen-activated protein kinase kinase 2 (Mek2) (Met-Leu-Ala-Arg-Arg-Lys-Pro-Ala-Leu-Thr-Ile-Asn-Pro,, shown as a space filling model) reported by Pannifer and co-workers divided into four separate domains [128,147]. Domain 1 is involved in binding to PA Domain 2 is believed to play a role in substrate recognition. Domain 3 is inserted into domain 2. Domain 4 contains the large deep (approximately 40 A) gorge containing the active site. This gorge is lined with regions of Glu and Asp residues that impart a negative electrostatic potential. In addition a large number of Gln and Asn residues are located in the gorge of the active site. The x-ray crystal structure [147] of LF indicates that the Zn atom is coordinated to a water molecule and to the side chains of His , His and Glu . Glu activates the water coordinated with the Zn during hydrolysis [147]. Tyr is located opposite Glu and is believed to be involved in protonation of the leaving amino group after hydrolysis [147].

residues Trp^{226}, Tyr^{462} and Phe^{464}. This discrepancy is not unexpected, since the computational methods did not involve the complete amino acid sequence of the peptide, particularly the N-terminal His^{1} and the Asp^{9} residues.

Additional work of Collier and co-workers [157] has shown that dominant-negative mutants (DN) of PA will assemble with wild-type PA to form heptameric bundles. However, these bundles do not translocate LF and EF into the cell. Collier reported that male rats treated with DN and injected with 10 times the lethal dose of LF or EF were protected from intoxication [157].

Mrksich and co-workers developed a matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) mass spectrometry based chemical screening protocol to identify potential inhibitors of anthrax lethal factor protease activity [158]. This technique employs the method "SAMDI (self-assembled monolayers for MALDI)" which combines the technique of self-assembled monolayers designed to measure enzymatic activity with MALDI-TOF mass spectrometry detection. Using this system the nonpeptide inhibitor of the protease activity of anthrax lethal factor shown in (Fig. **21**) was identified [158].

Turk and co-workers reported the application of "mixture-based" peptide library to identify optimal peptide substrates and inhibitors of anthrax lethal factor [159]. In this analysis two peptide libraries were prepared. The first library focused on investigation of the role played by the amino acids found at the N-terminal side of the LF cleavage site, the second library investigated the role played by the amino acids found at the C-terminal side of the LF cleavage site [159]. The results of this investigation indicated that the P1' position, the residue to the C-terminal of the cleavage site, is the most sensitive to substitution and requires a hydrophobic residue at this position [159]. Additional important observations indicate that the P_2 position also favors a hydrophobic residue. It was quite unexpected that the N-terminal region also favors multiple basic residues relative to the cleavage site [159]. It is well known that substrate-derived inhibitors can be developed by incorporation of functional groups, that will chelate with the metal ion found in the active site of the enzyme [160,161], into a known substrate on either side of the cleavage site. This methodology was employed by Turk and co-workers to develop the peptide inhibitor shown in Table **7** [159].

Turk and co-workers [159] also examined the crystal structure of the substrate peptide (MLARRKKVYPYPMEPTIAEG-amide) bound to LF. Several key structural features were identified. The basic lysine residues at positions P_5 and P_4 are located very close

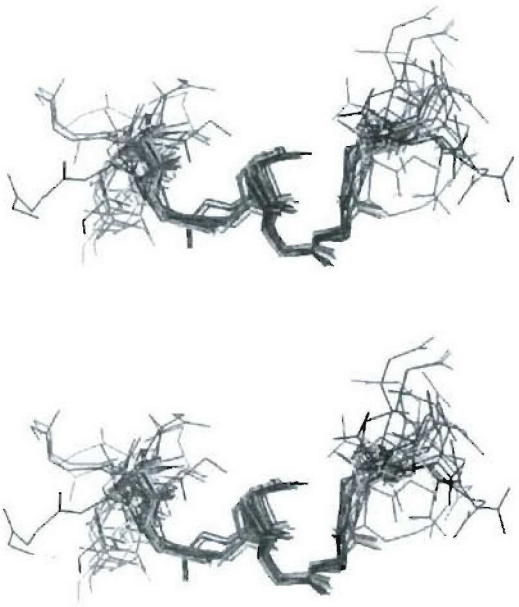

Fig. (20). Stereoview of the 30 structures of the PA_{63}-bound confomration of P1 superimposing the backbone atoms of residues 3 to 10 onto each other [154]. The rmsd for this superimposition ranges from 0.206 to 1.35Å (0.841Å, average). Clearly the mid region of the peptide consisting of residues 4-8 convergence to a α-helix. Residues 3 and 9 of the helix appear to be somewhat more flexible. The remaining N-terminal and C-terminal residues appear to be very flexible.

to an acidic patch located near the entrance of the active site [159]. The previous mentioned preference of basic residues on the N-terminal region of the substrate can now be explained based on this observation. The amino acid residues at positions P_3-P_1 interacts with strand 4β3 of LF forming a antiparallel β-sheet [159] like structure. The side chain of the tyrosine residue at position P_2 occupies a very small hydrophobic pocket, while the tyrosine residue at position P_1' is located in the very deep S_1' hydrophobic pocket of the enzyme [159]. It appears that his pocket undergoes significant expansion on peptide binding allowing for the accommodation of large hydrophobic groups at this site [159]. Turk and co-workers speculate that the long hydrophobic groove and the deep hydrophobic S_1' pocket located next to the zinc atom are the main determinants for binding affinity [159].

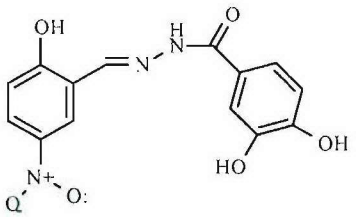

Fig. (21). The structure of the inhibitor of anthrax lethal factor reported by Mrksich and coworkers [158].

The crystal structure of the inhibitor SHAc-YPM was also determined [159]. Three amino acid residues found in this inhibitor are also found to the C-terminals of the cleavage site in the twenty amino acid substrate peptide discussed above.

Table 7. Peptide Based Inhibitors of Anthrax Lethal Factor Protease Activity Developed by Turk and Co-Workers [159]

Compound	K_i (μM)
GM6001 (3-(N-hydroxycarboxamido)-2-isobutylpropanoyl-Trp-methlamide)	2.1
SHAc-YPM	11
Ac-KVYP-hydroxamate	> 100
PLG-hydroxamide	>100
MKARRKKVYP-hydroxamate	0.0011

Panchal and co-workers [162] have report the identification of a series of small molecule inhibitors of the protease activity of anthrax lethal factor. The National Cancer Institutes Diversity Set of 1990 were screened and 19 compounds were identified that exhibited greater than 50% inhibition of the protease activity of anthrax lethal factor at a concentration of 20 μM [162]. Several of the more interesting compounds identified from this investigation are given in (Fig. **22**) [162]. Molecular docking studies using active and inactive compounds were conducted to develop a "first generation" pharmacophore and used to conduct 3D database searches of several databases including MayBridge, Available Chemicals, BioByte and the entire NCI repository. From this search approximately 60 additional lead compounds were identified, however only 6 of these compounds exhibited inhibitory activity [162]. These six compound were then employed to aid in the refinement of the pharmacophore [162]. The pharmacophore incorporates

two hydrophobic aromatic centers, and three polar centers acting as hydrogen bond donors or acceptors as well as neutral center linking the two aromatic centers [162].

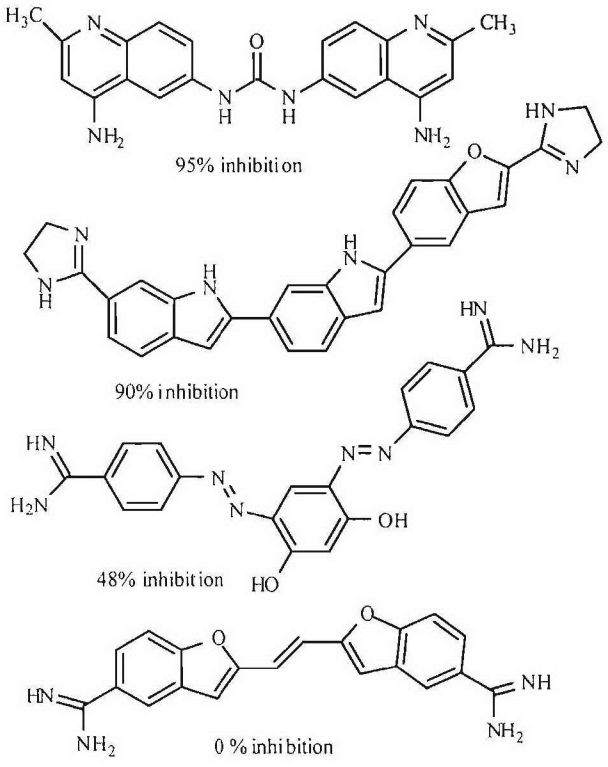

Fig. (22). Representative chemical structures identified by Panchal and co-workers that inhibit anthrax lethal factor protease activity at a concentration of 20 μM. Percent inhibition is given below each structure [162].

Development of inhibitors of anthrax edema factor has received a great deal less attention. However Shen and co-workers have reported adefovir dipivovir, a drug approved to treat infectious hepatitis B, inhibits adenylyl cyclase activity of edema factor *in vitro* at a concentration of 27 nM [11] (Fig. **23**).

CONCLUSION

In light of the increasing threats of a potential biological attack from hostile nations and/or terrorists groups, research into the development of treatments for exposure to these and other biological warfare agents must continue. It is up to the biomedical community to respond to these threats as quickly and as effectively as possible. The current methodologies used to develop drugs for the treatment of toxin exposure are listed in Table **8**.

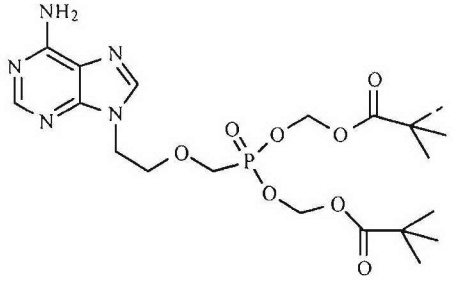

Fig. (23). Chemical structure of adefovir dipivovir, a drug approved to treat infectious hepatitis B, inhibits adenylyl cyclase activity of edema factor *in vitro* at a concentration of 27 nM [11].

Table 8. Approaches Used to Develop Drugs to treat Toxin Exposure [101]

1.	Design of drugs that will inhibit the binding of the toxin to its receptor
2.	Design of drugs that will bind to the active site of enzyme toxins that will inhibit enzyme activity
3.	For toxins that block the release of neural transmitters develop drug that stimulate the increased release of that neural transmitter
4.	For toxins that induce the release of secondary mediators, drugs are being designed to block the effects of these secondary mediators.

For the three agents discussed here, a great deal of progress has been made in developing an understanding of the biochemical mechanisms of intoxication. However a great deal of work is still needed in order to develop effective therapies for these agents. Of the three agents, ricin seems to be the best understood, with several known small molecule inhibitors. Still a well defined pharmacophore is lacking, and is needed for optimal drug development. In the case of BoNT/A and B the x-ray crystal structures are know. However, little is known about the structural and physicochemical requirements for inhibitory activity. It is therefore of critical importance to develop a pharmacophore describing these requirements in order to develop non-peptide inhibitors of the enzymatic activity of BoNT/A and BoNT/B. Currently there are no known inhibitors of the enzymatic activity of anthrax lethal factor. In addition, very little is know about the binding of LF to the PA_{63} heptamer bundle and how to prevent it from occurring. There is a critical need to develop an understanding of the physicochemical requirements necessary for LF to bind to the Heptamer bundle and the requirements for binding to the active site of LF. This information is needed to develop small non-peptide inhibitors of both the enzymatic activity of LF and of the binding of LF to the heptamer bundle.

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