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# Biowarfare Pathogens. Is the Research Flavor Different Than That of Clinically Relevant Pathogens?

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## 1. INTRODUCTION

Significant progress has been achieved in the 20th century in the implementation of anti-infective therapies that have improved the quality of life of people throughout the world. These advances have also given both immuno-competent and immunosuppressed patients significant enhancements in life span. Numerous scientific and lay publications have focused with high emphasis on the problems of antibiotic-resistant clinical infections. However, there has been little balanced discussion on the etiological impact of the subgroups of pathogens called biowarfare agents [1–3].

In contrast to most clinically-relevant pathogens, biowarfare agents have intrinsic features which make their etiology and clinical treatment unique. These features range from production of exotoxins which promote rapid organ failure (anthrax) to simply a lack of small molecule therapeutics (smallpox and hemorrhagic fever viruses).

The events of October 2001, where dispersment of anthrax spores in key US government facilities crippled their function for weeks, highlighted the specter of societal traumas due to these agents. Aside from the medical challenges, effect on the public psyche is immeasurable [4]. Some of these agents have left their impact on the history of civilization through natural distribution. Notable examples are (i) smallpox (*Variola major*) since the early times and (ii) the Black Death (*Yersinia pestis*) in the Middle

Ages, which caused significant political shifts in Europe. In some cases, especially the hemorrhagic viruses, the originating loci fortuitously have been a Third World region where the epidemic self-extinguishes. However, transoceanic travel can facilitate the disbursement of some etiological agents as recently shown with Lassa Fever and Severe Acute Respiratory Syndrome (SARS)[1].

Biological weapons have been a societal problem since their first use in the sixth century BC. In the Middle Ages, during some military conflicts, these agents were intentionally dispersed by throwing diseased animal carcasses into an opposition’s encampments. In the 20th century, several notable incidences with severe consequences were: (i) Japanese dispersal of *Y. pestis* on Chinese population in Manchuria in late 1930s [5,6] and (ii) the accidental release of anthrax in Sverdlovsk, Russia in 1979 [7,8].

Weaponization further enhances the aerosoling of these agents, which Biopreparat (Soviet Union’s biological warfare program) undertook on industrial-scale. The Soviets weaponized anthrax, tularemia, brucellosis, plague, typhus, Q fever, smallpox, botulinum toxin, Ebola and Marburg virus, and Venezuelan equine encephalitis [9,10].

Intentional construction of multiply-resistant mutants (chimeric constructs) by genetic recombination of dissimilar agents with individual resistance mechanisms was also a cornerstone of the Biopreparat technology. Agents that were more virulent or multiply resistant to various classes of therapeutics were attained. Veepox, which is a combination of Venezuelan equine encephalitis and smallpox, is a frightening example of a chimeric construct [11].

**Table 1.** NIAID priority pathogens

Class A	Class B
<i>Bacillus anthracis</i> (anthrax)	<i>Burkholderia pseudomallei</i>
<i>Clostridium botulinum</i>	<i>Burkholderia mallei</i> (glanders)
<i>Yersinia pestis</i> (plague)	<i>Brucella</i> species (brucellosis)
<i>Francisella tularensis</i> (tularemia)	<i>Coxiella burnetti</i> (Q fever)
<i>Variola major</i> (smallpox), other pox viruses	Ricin toxin
Viral hemorrhagic fevers	Epsilon toxin of <i>Clostridium perfringens</i>
<b>Arena viruses</b>	Staphylococcus enterotoxin B
LCM, Junin Virus, Machupo Virus	<i>Rickettsia prowazekii</i> (typhus)
Lassa Fever	<b>Food and Waterborne Pathogens</b>
<b>Bunyaviruses</b>	<i>Clostridium perfringens</i>
Hantaviruses	<i>Vibrio cholerae</i>
Rift Valley Fever	<i>Cryptosporidium parvum</i>
<b>Flaviviruses</b>	Viral encephalitides
Dengue	
<b>Filoviruses</b>	
Ebola	
Marburg	

Starting in 1998, as part of coordinated effort by US government agencies for preparedness in the event of a biowarfare incident, a pathogen classification system was adopted. The more pertinent categories have been reproduced in [Table 1](#), with the most dangerous listed as Class A agents because of rapid death. The Class B agents cause debilitating diseases, slow death, or panic driven events [12].

The Class A pathogens have characteristics which are medically and operationally challenging: (i) high morbidity or mortality, (ii) inter-personal transmissibility (except anthrax and tularemia), (iii) lack of effective or safe vaccines, and (iv) lack of effective or available treatments. In the absence of pathogen speciation data, the initial clinical symptoms can be confused with those of more benign organisms. While early speciation is crucial, the lack of small molecule therapeutics is abundantly clear.

A recent survey of the Investigational Drugs Database (IDDB) for various research initiatives against Class A agents showed a dearth of small molecule leads (highlighted in grey in [Table 2](#)). The majority of the experimental therapeutics are either vaccines or monoclonal antibodies.

While the Class A agents (except for the hemorrhagic viruses) are the focus of this chapter, research and clinical knowledge obtained from the Class A and B agents could provide new treatment modalities for clinically relevant pathogens. Some examples are (i) the interplay between virulence factors and host immune systems which could lead to better understanding of the physiology of sepsis; and (ii) knowledge from *Burkholderia pseudomallei* and *Burkholderia mallei* could provide insights into treatments for *Burkholderia cepacia*, a pulmonary pathogen found in patients with cystic fibrosis.

Drug discovery efforts against the biowarfare agents listed above have historically been limited, either because the consequences of these pathogens were not high priority for policy-makers agenda, or because of technical difficulties. The technical difficulties in working with biowarfare agents include (i) inadequate knowledge of genomic sequences of some of these agents, (ii) the need for use of specialized bio-containment facilities, (iii) inadequate or lack of in-life models that approximate human circumstances, and (iv) also a lack of scientists with relevant research expertise.

Clearly, there is a need for new agents that are potent and minimally toxic against the biowarfare agents. This synopsis describes recent progress towards identifying small molecule inhibitors.

## 2. BACILLUS ANTHRACIS (ANTHRAX)

### 2.1. Background

Anthrax is a dimorphic bacterium that normally exists as spores. The clinical presentation can be as cutaneous, inhalational or gastrointestinal forms that are fortuitously not transmissible from person to person. As the October 2001 anthrax cases showed, the insidious nature of anthrax has both a vegetative and spore morphology. The vegetative state, being the growth phase, is typically responsive to most classes of antibiotics, while the spore phase is not. Thus, long treatment modalities with systemic antibiotics for

**Table 2.** R and D initiatives for modulators of biowarfare agents

Therapeutic	Company or organization	Status	Ref.
<b>Anthrax</b>			
Antitoxin antibodies	Alexion Pharmaceuticals	Discovery	NA
Anthrax vaccines	DynPort/AVANT	Phase I	[13]
Anthrax-plague vaccine	DynPort/AVANT	Discovery	[14]
Abthrax monoclonal antibody	Cambridge Antibody Technology	Phase I	[15,16]
Lethal factor inhibitor	Cengent Pharmaceuticals	Discovery	[17]
Anthrax vaccines	DOR Biopharmaceuticals	Discovery	NA
ETI-205 monoclonal antibody	EluSys Therapeutics	Discovery	[18]
Human monoclonal antibodies	IQ Corporation	Discovery	[19]
Anthrax vaccines	Medarex	Discovery	NA
Anthrax vaccines	Microscience	Discovery	NA
Anthrax vaccines	NIAID/USAMRIID/VaxGen	Phase I	NA
Anthrax vaccines	Vaxin Pharmaceuticals	Discovery	NA
Polyclonal anti-RcPA IgC	NEXT Therapeutics	Discovery	NA
NAD inhibitors, VDDI	U. Alabama	Discovery	[20]
DNA vaccine	Vical	Discovery	[21]
AVP-21D9 monoclonal antibody	Xenorex Biosciences	Discovery	NA
<b>Plague</b>			
Anthrax-plague vaccine	DynPort/AVANT	Discovery	[22]
Plague vaccine	DERA/Provalis	Discovery	[23–26]
Proteosome-formulated vaccine	ID Biomedical/USAMRMC	Discovery	NA
<b>Smallpox</b>			
CMX-001	Chimerix	Discovery	[27,28]
ACAM-2000 vaccine	Acambis	Phase III	[29]
MVA vaccine	Acambis	Discovery	NA
ACY-111 vaccine	Acceptys	Discovery	NA
Elstrein-BN vaccine	Bavarian Nordic	Phase I	NA
Smallpox vaccine	Kaketsuken/VaxGen	Launched	NA
Smallpox vaccine	DynPort	Phase I	[30]
Smallpox vaccine	NIH	Phase I	[31]
Smallpox vaccine	SIGA Technologies	Discovery	NA

NA, Primary technical reference(s) not available yet, but product theme has been discussed in government forums (cited in Investigational Drugs Database).

the deadly inhalation form are necessary in order to inhibit all of the organisms that convert from the spore state to the vegetative state [4]. This requires anti-infectives with safety profiles adequate for several months of administration. A major advance would be the discovery of antibiotics that are effective against the spore phase or stationary phase of these pathogens.

A second unique aspect of anthrax, which alters the clinical treatment, is the presence of anthrax toxin which is responsible for rapid on-set of organ and cardiac failure.

Cessation of anthrax growth through antibiotic action does not stop the downstream biological effects of the toxin components, especially when pathogen overload results in large titers of anthrax toxin release into the body.

After inhalation, *Bacillus anthracis* spores germinate in alveolar macrophages and then migrate to lymph nodes where they propagate. The vegetative bacteria secrete a tripartite toxin, which consists of three proteins: lethal factor (LF, 90 kDa), edema factor (EF, 89 kDa), and protective antigen (PA, 83 kDa), all of which work in concert to kill host cells. While the mode-of-action of anthrax toxin itself is not yet well understood, small molecule inhibitors or monoclonal antibodies to inhibit toxin assembly and/or function represent potentially useful approaches.

Lethal Factor (LF) is a zinc dependent protease which targets the Mitogen Activated Protein Kinase Kinases (MAPKK), that are involved in intracellular signaling pathways. The protease inactivates the MAPKK which cannot then signal the p38 MAPKs. Lack of P-38 activity causes lysis of macrophages, which then facilitates the propagation of the anthrax [32,33].

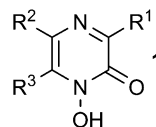
Edema factor (EF) is a calmodulin-mediated adenylate cyclase that impairs the host defenses through a variety of mechanisms inhibiting phagocytosis. These include interference with the host's immune response which facilitates the bacteria propagation, and induction of massive tissue necrosis, including pulmonary fluid retention. The EF-calmodulin complex is an exquisitely potent and hyperactive adenylyl cyclase. Cells activated by the adenylyl cyclase lose the ability to regulate their environment, release water, and die [34–36].

Protective antigen (PA) binds to the cellular receptor, Tumor Endothelium Marker-8 (TEM8). Upon binding to TEM8, PA is cleaved into 20 and 63 kDa fragments (PA20 and PA63) by furin or furin-like proteases. The PA63 fragment re-associates and binds either LF or EF. The resulting complexes of PA63-EF or PA63-LF are internalized into endosomes followed by translocation of LF and OF into cytosol of the cells. PA receptor TEM8 (also known as Anthrax Toxin receptor, ATR1) is a glycoprotein with extracellular (1-321aa), cytosolic (343-564aa) and TM (322-342) domains [37,38]

Nearly half (5/11) of the pulmonary anthrax patients from the Oct 2001 incidents died, while the survivors have ongoing symptoms such as fatigue, shortness of breath and memory loss [4]. This highlights the need for more effective therapies [39].

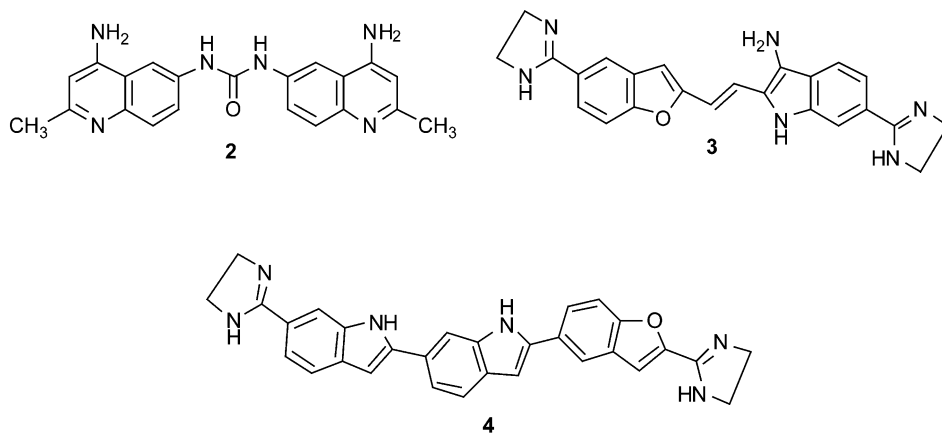
While various initiatives are ongoing to examine the efficacy of existing antibiotics against anthrax, this may not address the fears of a chimeric construct of anthrax which is multiply-resistant to various classes of existing antibiotics. Thus, parallel small molecule approaches for clinical use are needed [40]. There is a need for new antibiotics which function against new anthrax targets, and also agents to inhibit various toxin components.

While the multiplicative biological effects of the various toxin components is daunting, even inhibition of a single component would be beneficial. Several approaches have been reported. As a potential treatment for pulmonary indications, substituted 3-hydroxyhydropyrazine-2-ones **1** are being investigated as inhibitors of LF [17]. A small-molecule LF inhibitor demonstrated *in vivo* protection against anthrax toxin-induced disease processes, and also partial restoration



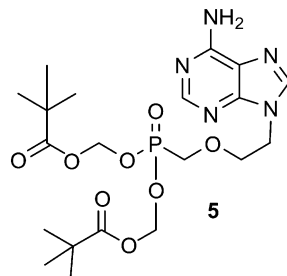
R<sup>1</sup> = benzyl, *i*-butyl, 4-phenylbenzyl  
 R<sup>2</sup> = alkyl, substituted phenyl,  
 2-thienyl, 2-naphtyl, biphenyl  
 R<sup>3</sup> = H, alkyl, phenyl

of anthrax lethal toxin-suppressed immunological function in mice. In addition, treating lethal toxin-exposed rats with the inhibitors increased the life span of the rats significantly.



In examining a subset of the NCI compound library, researchers have identified inhibitors 2–4 of LF with a common pharmacophore, which may be amenable to further structural modifications to provide more potent analogs [41,42]. Other earlier screening leads of peptidic origin have yet to evolve into more drug-like molecules [43–45].

The search for new drugs can also be serendipitous. Using *de novo* design strategies, researchers at the University of Chicago have identified adefovir dipivoxil (5, Hepsvera®), a hepatitis B antiviral, as an *in vitro* inhibitor of EF [46]. Adefovir fits 10,000 times better than the natural substrate into a pocket on the surface of EF. While *in vitro* success does not necessarily translate to *in vivo* success, a new use for a recently approved drug represents an attractive strategy.



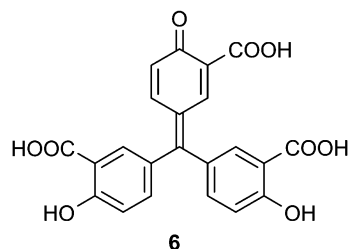
### 3. YERSINIA PESTIS (PLAGUE)

#### 3.1. Background

Plague is caused by a bacterium carried by a rodent flea [47–49]. While current antibiotics are effective against plague, the worry is the possibility of a bioengineered chimeric construct that would be resistant to all classes of antibiotics.

Wild rodents in certain areas around the world are infected with plague. Human plague in the United States occurs mostly in rural areas with an average of 10–15 cases/annum vs. 1000–3000 cases worldwide. Most human cases in the United States occur in two regions: (i) northern New Mexico, northern Arizona, and southern Colorado, and (ii) California, southern Oregon, and far western Nevada. Plague also exists in Africa, Asia, and South America [50].

Aside from citations on the use of older antibiotics for the treatment of plague, there is one key report on the molecular and cellular basis of plague's virulence [51]. An attempt to identify inhibitors of *Yersinia* protein tyrosine phosphatase (YopH) [52], one of the virulence factors, has been reported the activity of aurintricarboxylic acid **6** [53].



## 4. FRANCESELLA TULARENSIS (TULAREMIA)

### 4.1. Background

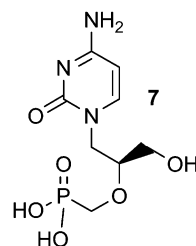
Tularemia is a zoonosis that occurs naturally in the United States, with animal (especially rodents, rabbits, and hares) transmission to man. Sometimes an insect vector may also be the primary route of infection. It is highly pathogenic and the inhalation of 10 organisms would be adequate for infection [54–55]. It is resilient to various environmental factors such as low temperatures and resides in the natural environment (moist hay, grass, water) when distributed. It exists in two subspecies: (i) *F. tularensis* biovar tularensis (type A) and (ii) *F. tularensis* biovar palaeartica (type B). Type A, the more virulent form, exists predominantly in North America whereas type B, a less virulent form, is found in Europe and Asia. Aerosol release of a virulent form of tularemia would be expected to lead to substantial morbidity and mortality. There are no reported approaches to a small molecule therapy.

## 5. VARIOLA MAJOR (SMALLPOX)

### 5.1. Background

Smallpox, which originated in northern Africa, has been known for *ca.* 10,000 years, and the last known case occurred in Somalia in mid-1970s. It was declared eradicated in 1977 by the WHO. Historically it has caused more deaths than any other cause of mortality. Smallpox is the most feared of all biowarfare pathogens, primarily due to its high transmissibility versus other pathogens whose etiologic affects are episodic (e.g., *Y. pestis* or *M. tuberculosis*) [1,56].

This area has prompted substantial interest in the last several years, especially in the use of vaccinia viruses as surrogates for smallpox. A number of nucleoside analogs were identified that inhibited various stages of viral reproduction by various mechanisms, including (i) inosine monophosphate (IMP) dehydrogenase inhibitors (EICAR), (ii) *S*-adenosylhomocysteine (SAH) hydrolase inhibitors (*5'*-noraristeromycin, 3-deazaneplanocin A and neplanocin A analogs), (iii) orotidine





monophosphate (OMP) decarboxylase inhibitors (pyrazofurin), (iv) cytidine triphosphate (CTP) synthetase inhibitors (cyclopentenyl cytosine), (v) thymidylate synthetase (TS) inhibitors (2'-deoxyuridines), (vi) DNA synthetase inhibitors (Ara-A), (vii) acyclic nucleoside (cidofovir), and (viii) polyacrylic acid.

Cidofovir 7, an approved drug for the treatment of cytomegalovirus (CMV) infections, is a potent inhibitor of the smallpox virus [57–59]. However, cidofovir must be administered by intravenous infusion making it impractical for broad and rapid distribution in the event of a smallpox outbreak. Further, in some patients, the parenteral formulation shows nephrotoxic events. Lipophosphate prodrugs of cidofovir have been reported to be orally bioavailable [27,28]. CMX-001 (structure undisclosed) is being studied for the potential treatment of smallpox infections and complications resulting from smallpox vaccination. It is more potent and less toxic than cidofovir. CMX-001 is 100 times more potent than cidofovir, as shown by antiviral assays, and it is active against a variety of pox viruses including smallpox and monkeypox. When given orally, it was fully effective in mouse models of pox virus infection. The development of CMX-001 for smallpox therapy is compelling since earlier clinical data, from the regulatory approval for CMV indications, would facilitate its use for smallpox.

## 6. CONCLUSION

While few small molecular entities were discussed in this review, the increased research activity along with enhanced government support of biowarfare agents is encouraging. We can hope that within a few years there will be significant developments in small molecule discovery to fill this void.

The events from 9/11, in conjunction with expanded government funding for biowarfare research, has led to shifts in anti-infectives research operations. These include:

- Interest in microbiology, molecular microbiology, and adjunctive technologies has increased in academic institutions. Especially evident is the use of comparative genomic and post-genomic strategies to identify pathogen targets.
- There is an increase in collaborations between private industry, academics, and government labs. As more 3D crystallographic data is obtained on target proteins from biowarfare pathogens, massive parallel virtual screening efforts are being encouraged through government-sponsored programs [60]. This will foster a better utilization of government-sponsored BL3 facilities.
- There is the opportunity for researchers to work creatively by using clinically-relevant pathogens as surrogates for biowarfare pathogens: (i) *B. cereus* or *B. subtilis* for *B. anthracis* activity or (ii) *Yersinia pseudotuberculosis* for *Y. pestis*.
- In contrast to the more traditional drug development sequence that requires Phase I–III studies, the FDA has created a new paradigm for approval of drugs for use against biowarfare agents [61].
- A new paradigm for funding of biotech start-ups has occurred. Several companies have been started with minimal levels of venture support, but with substantial non-dilutive government funding.

Ironically, one of the anti-infective successes in 20th century included the final eradication of smallpox in 1977 in Somalia. Within one generation, we now fear the possible use of smallpox as a biowarfare agent.

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