COMMENTARY

Peptide inhibitors MAP the way towards fighting anthrax pathogenesis

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The pathogenesis of anthrax is such that unless antibiotic treatment is initiated at an early stage in the disease, it is ineffective against the bacteria-induced toxemia that subverts the immune response, inflicts massive tissue damage and is ultimately the major factor contributing to death during anthrax infection. As current events have demonstrated the feasibility of the use of anthrax as a bioterrorism agent, and exemplified the difficulty of treating the ensuing infection, inhibition of anthrax toxin has become a

major focus of research for the design of antitoxin therapeutics. In this issue of *Biochemical Journal*, Bracci and co-workers describe the discovery by competitive screening of a phage-display library of a peptide inhibitor of anthrax toxin assembly that shows great promise towards the treatment of anthrax.

Key words: anthrax, lethal toxin (LeTx), oedema toxin (ET), peptide, multiple antigen peptide (MAP), screening.

INTRODUCTION

The intentional release of anthrax which occurred in the United States during the autumn of 2001 exemplified the reality of bio-warfare in the 21st century and made research and development of antibiological therapeutics a global priority. Infection with *Bacillus anthracis*, the causative agent of anthrax, can rapidly evolve into a systemic infection against which antibiotic therapy has little recourse unless administered at a very early stage of the disease. However, initial symptoms are non-specific and general: therefore pathogen exposure may not be immediately evident.

Anthrax infection may occur through inhalation, ingestion or through abrasions in the skin. Of the three primary infection routes, inhalation of anthrax typically results in the most severe disease, with death occurring within 48 h of symptom onset. *B. anthracis* exists naturally in the soil as an endospore, which is heat-, drought-, and UV-tolerant, and retains infectious capacity for decades. Once within the body, anthrax endospores are engulfed by macrophages and transported to regional lymph nodes, with germination occurring *en route* [1].

Disease pathogenesis is attributed to two separate virulence factors: a poly(D-glutamic acid) capsule and the anthrax toxin, which is produced and secreted by vegetative bacteria. Both the capsule and anthrax toxin prevent phagocytosis and degradation of the bacteria by phagocytic leucocytes. Additionally, anthrax toxin inhibits signal-transduction pathways critical for innate and adaptive immunity and the production of pro-inflammatory cytokines, essentially crippling the host immune response.

Although vaccines for many microbial pathogens, including anthrax, are currently available, it is unreasonable and implausible for all individuals to be vaccinated for the wide variety of potential organisms that may be used in a bioterrorism event. For this reason, as well as the ineffectiveness of post-infection antibiotic therapy, it is imperative to generate novel toxin antidotes which can be used for post-exposure treatment of biological pathogens in conjunction with antibiotic therapy. As the primary and most threatening virulence factor produced by *B. anthracis*, inhibition of the anthrax toxin is one of the most promising areas of focus for anthrax therapeutic research.

ANTHRAX TOXIN

Anthrax toxin is a member of the bacterial AB-type exotoxin family, and shares functional and structural characteristics with the large clostridial cytotoxins, including *Clostridium botulinum* C2 toxin. Toxins in this family consist of a B subunit, which binds to the host cell and mediates cellular uptake of the enzymatic A subunit. In the case of anthrax toxin, PA (protective antigen) mediates the binding and internalization of two separate enzymatic subunits, namely LF (lethal factor) and EF (oedema factor).

Two receptors, CMG-2 and TEM-8 (tumour endothelial marker 8), have been identified as the binding site for PA on the host cell surface. Proteolytic cleavage by furin-family proteases removes the N-terminal 20 amino acids from full-length PA, converting the native 83 kDa protein into the 63 kDa mature form (PA₆₃). PA₆₃ heptamerizes to form the [PA₆₃]₇ pre-pore. Oligomerization redistributes the PA receptors into cholesterol and sphingolipid-enriched lipid raft domains, and allows binding of LF and EF. All three toxin subunits are then internalized by clathrin-mediated endocytosis.

Translocation of LF and EF from the endosomal compartment into the host cell cytoplasm requires a pH-induced conformational change in the [PA₆₃]₇ pre-pore structure, which drives its insertion into the lipid bilayer and forms the channel through which LF and EF pass. Once released into the cytoplasm, the LF metalloprotease interferes with MAPK (mitogen-activated protein kinase) signalling pathways by cleaving, and therefore inactivating, members of the MAPK kinase family [MKKs (MAPK kinases) 1, 2, 3, 4, 6 and 7]. EF inflicts tissue damage by increasing intracellular cAMP levels through its calmodulin-dependent adenylate cyclase activity. (For comprehensive reviews on anthrax pathogenesis and the anthrax toxin, see [2,3].)

FIGHTING ANTHRAX INTOXICATION: PREVIOUS STRATEGIES

As a testament to how current events affect trends in research, nearly all known inhibitors of anthrax toxin have been identified within the last 5 years. Within the same time period, significant advances have been made towards understanding the molecular

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structure of anthrax toxin, the mechanism of its internalization and intracellular trafficking routes, and the intracellular targets and functions of LF and EF. Several unique strategies have led to the discovery and design of different anthrax toxin antidotes [4,5], amounting to more than 40 publications since the year 2000.

Prevention of toxin assembly by absorption of subunits has been achieved with the use of the extracellular domain of TEM-8 as a receptor decoy, and with both primate and human antitoxin antibodies. The human monoclonal antibody AVP-21D9 has been shown to block formation of the [PA₆₃]₇ pre-pore *in vitro*, and, when used in conjunction with antibiotic therapy, prevents lethal anthrax infections in rabbits [6]. Inhibition of pore assembly by blocking furin-mediated cleavage of full-length PA₈₃, or by utilization of dominant-negative mutants of PA, has also been reported.

A second major strategy for combating anthrax toxin has been to develop inhibitors which specifically interfere with the catalytic activity of the EF and LF enzymes [7,8]. Many of these studies have identified such inhibitors via peptide and chemical library screens, coupled with structure-based computer modelling. Interestingly, 'natural' inhibitors of LF have also been discovered, such as EGCG [(–)-epigallocatechin-3-gallate], originally identified from green-tea extracts. Synthesis and screening of galloyl-(3,4,5-trihydroxybenzoyl) derivatives as well as tetrahydroquinolines have improved the specificity and efficacy of these compounds. The analysis of known, clinically approved drugs has resulted in the identification of previously unrecognized anthrax toxin inhibitors; for example, adefovir dipivoxil, a drug which is currently in clinical use for the treatment of hepatitis B viral infections, has been shown to be a potent inhibitor of EF [9]. These types of approaches appear to be increasingly promising as putative inhibitory molecules are identified and refined.

THE MAP (MULTIPLE ANTIGEN PEPTIDE) SYSTEM

MAPs were designed as a way to provide a chemically uniform carrier for antigen presentation as an alternate to protein adjuvants, such as keyhole-limpet haemocyanin, or synthetic polymers, such as polyacrylamide [10]. MAPs consist of a two-, three- or four-trifunctional-amino-acid core matrix to which the synthesized peptides are linked, resulting in multivalent presentation of the peptide antigen with a dendritic secondary branched structure. As they are significantly more immunogenic than the same peptide presented by 'traditional' carriers, MAPs are useful for vaccinations against pathogens and, potentially, for tumour antigens. In addition, these structures are chemically homogeneous and of low molecular mass, characteristics lacking in similar peptides linked to polyacrylamide backbones, and which are critical for reproducible and dependable therapeutic activity.

MAPs are also beneficial in that they more closely mimic peptides presented by phage display during the screening process in terms of multivalency and orientation than do monomeric peptides in solution. They thus more faithfully retain biological activities identified in the screening process. Additionally, MAPs are more stable in plasma and serum than monomeric peptides, an advantage that makes MAP peptide inhibitors useful for therapeutic applications *in vivo*.

IDENTIFICATION OF STABLE PEPTIDE INHIBITORS OF ANTHRAX TOXIN ASSEMBLY

In this issue of *Biochemical Journal*, Bracci and co-workers [11] describe the synthesis and characterization of peptide inhibitors for anthrax toxin assembly that block internalization of the LF and EF subunits by disrupting their ability to bind to the

pre-formed PA pore on the cell surface. In the past, this same group has successfully used a similar strategy to design peptide antidotes to α -bungarotoxin, a snake-venom neurotoxin [12]. Previous studies that identified peptide inhibitors for PA₆₃ and LF binding by phage display used PA to compete for peptide binding [13,14], whereas the study by Bracci and co-workers [11] utilized LF as the competitor. Surprisingly, the peptides identified using this approach, as with the two previous studies using different selection strategies [13,14], all contain a four-amino-acid YWWL motif.

Two peptides identified through this novel screening process were chosen for further study. These peptides, as with previously characterized peptides, have decreased efficacy as monomers in solution, and were therefore conjugated to a lysine backbone to create MAPs (MAP2: TLPYWWLTSPNP; MAP3: NVMTYWWLDPPPL). In addition, the authors also re-investigated a 12-mer peptide shown previously to inhibit PA–LF interactions when conjugated to a polyacrylamide backbone [13] in a MAP format (MAP1: HTSTYWWLDGAP). All three peptides were able to inhibit PA–LF binding, and also blocked cell death in macrophages in response to LeTx (lethal toxin) challenge.

The YWWL motif is not present in either LF or EF, and therefore the explanation as to why these peptides are effective inhibitors of toxin subunit binding to the PA pore was not immediately apparent. However, computer modelling using a clustering algorithm has indicated that the YWWL motif most likely binds to a hydrophobic pocket in the PA pore formed by the amino acids Trp²²⁶, Tyr⁴⁶² and Phe⁴⁶⁴ [15]. This hypothesis also explains why these inhibitors are able to effectively block EF binding to the PA pore as well, and why this series of peptide inhibitors provides one of the most promising therapeutic strategies for combating anthrax poisoning.

Since identification of specific binders from a phage library is limited by the screening agent (in this case, LF) having sufficiently high affinity to competitively elute high-affinity phages, it cannot be assumed that the selected peptides correspond to the optimal binding sequences. In an attempt to further increase the inhibitory affinity of the identified MAP peptides, the authors used alanine scanning and random substitutions, as well as modification of the peptide length. In doing so, the importance of the YWWL motif became evident: any substitution of these residues resulted in a complete loss of PA binding. A MAP3 with a Val → Ala substitution (MAP3V/A) exhibited an IC₅₀ that was at least 10-fold lower than that of any other anthrax-toxin-inhibiting peptide identified thus far. Interestingly, the minimal size of the peptide required for efficient inhibition of PA–LF binding is dependent on amino acid context. The MAP3 peptide could be shortened to a nonamer (MTYWWLDPP), whereas the MAP2 peptide required only six key residues (YWWLTP).

The authors then systematically added additional residues at either the N- or the C-terminus of the MAP2 hexamer. Whereas addition of residues at the N-terminus did not influence peptide activity, Bracci and co-workers [11] found that additional residues with the consensus sequence YWWLXPPX, where 'X' is any negatively charged or neutral residue, had an increased affinity for inhibiting PA₆₃–LF binding. In contrast, the presence of a positively charged residue in position 8 completely inhibited PA binding. Both positioning and inclusion of the proline residues in this sequence is also essential for maximal inhibition of PA–LF binding. Importantly, however, whereas the truncated consensus peptides were more effective at interrupting toxin binding as assessed by ELISA assay, the full-length 12-mer peptides were more effective at rescue of cell viability. A higher off-rate (k_{off}) for the 8-mer peptides determined in kinetic analyses of binding may account for this difference.

PERSPECTIVE

The results of the present study by Bracci and co-workers [11] identifies a peptide inhibitor capable of neutralizing anthrax toxin *in vivo* and provides the basis for the rational design of novel peptide inhibitors of PA–LF binding. Furthermore, evaluation of the ability of MAP3V/A and MAP-YWWLT PPP to antagonize the EF-induced elevation of cAMP in various cell lines showed that these peptides were also effective inhibitors of ET action. Since LT and ET can act synergistically to suppress innate and adaptive immune responses, such peptides may be more effective antidotes for anthrax than exclusive LT inhibitors. Additionally, the use in the current study of peptides in MAP form demonstrates the advantages of this methodology in the identification of biologically active toxin inhibitors of defined chemical structure and with highly reproducible activity. Coupled with the enhanced resistance of MAPs to degradation by peptidases and proteases of biological fluids demonstrated in the study by Bracci and co-workers [11], MAP peptides, in combination with antibiotics, may prove an effective and useful component of post-exposure emergency therapy of inhalational anthrax.

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