Disarming *Clostridium difficile*

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In this issue, Puri et al. (2010) present inhibitors that prevent the autocatalytic activation of the clostridial toxin TcdB in vivo. Their approach is likely to provide guidance for the development of novel drugs targeting virulence factors and thereby rendering bacterial pathogens innocuous.

Nosocomial infections caused by the bacterial pathogen *Clostridium difficile* are increasing worldwide due to the presence of hypervirulent and multiresistant strains. Colonization of *C. difficile* usually occurs in the colon if the natural gut flora is diminished by treatment with antibiotics. An infection can lead to antibiotic-associated diarrhea and sometimes pseudomembranous colitis through the secretion of the large glucosylating toxins TcdA and TcdB, which constitute the primary virulence factors of *C. difficile*. During the last decade, several outbreaks of the hypervirulent Quebec strain "NAP1/027," which produces these toxins at drastically elevated levels, have lead to numerous deaths in hospitals in the United States, Canada, the United Kingdom, the Netherlands, Germany, and Finland (O'Connor et al., 2009).

TcdA and TcdB have a multidomain structure for which an ABCD model (activity, receptor binding, cleavage, delivery) was proposed by Jank and Aktories (2008). The mechanism of TcdB action is believed to be a five-step process. First, the C-terminal receptor-binding domain of TcdB binds to an extracellular carbohydrate-based receptor. This binding triggers off endocytosis that leads to cellular uptake of the toxin. Acidification of the endosome is thought to initiate a conformational rearrangement within the second domain, which comprises a hydrophobic region that penetrates the membrane and presumably exposes the two N-terminal domains to the cytosol. Release of the effector domain, i.e., the N-terminal glucosyltransferase (GT) domain, is achieved by an autocatalytic cleavage mediated by a cysteine protease domain (CPD). The presence of the GT domain in the cytosol leads to the glycosylation of several Rho GTPases that cause alterations of the actin cytoskeleton and ultimately cell death. TcdB arguably has one of the most elegant mechanisms of activation: the autocatalytic cleavage event carried out by the CPD is triggered by the eukaryotic-specific cytosolic small molecule inositol hexakisphosphate ($InsP_6$). Thus, $InsP_6$ serves as a signal that the toxin has arrived at its destination.

In a study published in this issue, Puri et al. (2010) have designed inhibitors and activity-based probes to inhibit this autocatalytic cleavage. Using a gel-based assay that visualizes the protein fragments formed upon autocleavage, they have identified dipeptide and tripeptide acyloxymethyl ketone (AOMK)-based inhibitors that are able to block toxin activation in the presence of $InsP_6$. AOMKs form a covalent intermediate with the nucleophilic active site cysteine residue of the protease that blocks binding of the natural cleavage site. During two rounds of structural optimization, they subsequently synthesized a small focused library of AOMK-inhibitors and assessed their potency with the autocleavage assay, yielding Hpa-SerLeu-AOMK as the most potent inhibitor with an in vitro IC_{50} value of 0.71 μM.

In order to determine the potency of their inhibitors in vivo, Puri et al. (2010) developed a cell-based assay in which fibroblasts were pretreated with an AOMK inhibitor and then exposed to recombinant full-length TcdB. As cell intoxication leads to the disruption of the actin cytoskeleton, changes in cell morphology can be used to measure toxin activation. The authors were able to show that the potencies determined in vitro correlated with the results in vivo with noncytotoxic Hpa-SerLeu-AOMK completely inhibiting toxin function at 100 μM with an observed IC_{50} of 20 μM. Hence, they could validate the CPD as a viable drug target, even though the drug has to compete with an autocatalytic substrate necessitating a high concentration of the inhibitor.

The report also includes a crystal structure of the TcdB-CPD bound to an Ac-GlySerLeu-AOMK inhibitor and $InsP_6$. A highly hydrophobic pocket accompanying the inhibitor leucine side chain and observed back-bone hydrogen bonding for the P2 and P3 residues Ser and Gly, respectively, provide the structural basis of CPD substrate recognition. Binding of $InsP_6$ occurs in a highly basic cage that comprises several arginine and lysine residues and shows a high resemblance to the respective site in *Vibrio cholera* MARTX toxin CPD (Lupardus et al., 2008). Molecular docking based on the crystal structure was used to rationalize the measured potencies of the AOMK library.

In pursuit of a tool that allows for the direct visualization of CPD activity, Puri et al. (2010) turned their inhibitor into the activity-based probe AWP19 by replacing the N-terminal capping group with a spacer and a Cy5 fluorophore. AWP19 was found to fluorescently label holotoxin at probe concentrations below 50 nM. The probe was furthermore used to characterize cathepsin B as a possible off-target of TcdB CPD inhibitors. Due to the short specificity region of only two amino acids, cross-reactivity must be considered and could also account for the cytotoxicity of the Cbz-SerLeu-AOMK inhibitor that only differs in the capping group from the noncytotoxic lead compound Hpa-SerLeu-AOMK. However, this result will hopefully aid the development of more advanced probes that could enable the direct visualization of toxin activation at a single-toxin level,

answering the questions of when and where CPD activation takes place in the cell.

The observation that the CPD alone is more effectively labeled than the autocleavage substrate led the authors to speculate about a conformational regulation of toxin activity. This is based on the assumption that in the full length toxin the natural cleavage site is located close to the active site, reducing its accessibility, e.g., for CPD inhibitors. Hence, a clinically applicable CPD inhibitor will have to cope with the tough task of both competing with an autocatalytic substrate and reacting with a rather unreactive protease. However, Puri et al. (2010) have demonstrated that it is possible to take up this challenge. Their promising approach is likely to provide guidance for the development of a new generation of drugs targeting *C. difficile* virulence factors rather than cellular viability, thereby rendering this important bacterial pathogen innocuous (Clatworthy et al., 2007).

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Reeling in the Catch: Advancing Cleavable Linkers for Proteomics

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In global proteomic applications that focus on the characterization of proteins that have been derivatized using bioorthogonal chemistry, a challenge persists in the release of labeled proteins from supports used for purification. In this issue, Yang and coworkers (2010) present a detailed study of the diazobenzene-cleavable linker system as an effective solution for proteomic studies.

In recent years, critical technical advances have profoundly enhanced the ability to perform global analysis of biological systems, including novel bioorthogonal labeling reactions and mass spectrometry-based proteomic techniques. For the former, the ability to achieve selective labeling of biomolecules within the extremely complex environments of organisms, cells, and cell extracts has opened numerous avenues for the efficient characterization of biological systems. Here, bioorthogonal reactions, including the azide–alkyne cycloadditions, either the copper-catalyzed or copper-free reactions, as well as the Staudinger Ligation, have emerged as vital chemical tools (Sletten and Bertozzi, 2009). In examples of powerful applications, the analogs of sugars (Saxon and Bertozzi, 2000), amino acids (Link et al., 2003; Liu and Schultz, 2010), and lipids (Kho et al., 2004; Hang et al., 2007) bearing diminutive reactive tags that represent only minor structural perturbations (azide or alkyne) have been shown to effectively infiltrate biosynthetic pathways. This has been exploited to achieve the bioorthogonal labeling of resulting cell surface glycoproteins, newly synthesized proteins, and posttranslationally lipidated proteins, respectively. In addition, the strategy of activity-based protein profiling has been advanced for the collective labeling of proteins using small molecule probes that label target proteins (Speers et al., 2003; Cravatt et al., 2008).

Despite these advances, technical challenges persist that complicate such applications, particularly when the goal is to isolate and identify labeled proteins and to characterize the location of the labeling event. For this purpose, bioorthogonal chemistry is typically used to introduce biotin onto labeled proteins, followed by subsequent purification via streptavidin enrichment (Figure 1). However, a problem exists: it is challenging to achieve quantitative release of proteins from avidin supports after purification due to the high affinity of the streptavidin–biotin interaction. As a result, there has been considerable interest in devising cleavable affinity tags to release proteins following the labeling and purification stages. To be effective, this chemistry must survive all aspects of the labeling and separation processes, but must be cleanly released using mild conditions so as not to affect labeled proteins and subsequent mass spectrometry-based detection. In this issue of *Chemistry and Biology*, Yang and coworkers (2010) present a detailed study of a diazobenzene system that is shown to be highly effective for cleavage in sophisticated proteomics applications.

In the article, Yang and coworkers (2010) describe the design, synthesis,