Difficile defense

By Chris Cain, Staff Writer

A team led by **The University of Texas Medical Branch** has identified the nitrosylation of *Clostridium difficile* toxins as a host defense mechanism that reduces cellular damage caused by the pathogen.[1] The researchers are now developing compounds that augment this response as an adjunct therapy to antibiotics.

C. difficile is an opportunistic bacterial pathogen that can cause severe diarrhea and pseudomembranous colitis, and it is a leading cause of hospital-acquired infections worldwide.

The infections are treated with antibiotics including metronidazole, oral Vancocin vancomycin and the recently approved Dificid fidaxomicin. However, these drugs also affect the gut flora that keep *C. difficile* in check. Indeed, *C. difficile* recurs in 15%–30% of treated patients, and the rate increases with subsequent recurrences.

Vancocin, a broad-spectrum tricyclic glycopeptide antibiotic, is marketed by **ViroPharma Inc.** Dificid, a macrocyclic narrow-spectrum antibiotic that is superior to vancomycin at sustaining clinical response through 25 days beyond the end of treatment, was approved in May and is marketed by **Optimer Pharmaceuticals Inc.** and **Cubist Pharmaceuticals Inc.**

To find new ways to both combat *C. difficile* infection and decrease recurrence, Tor Savidge looked at how the immune system naturally combats the pathogen. Savidge is an associate professor of gastroenterology and hepatology at the University of Texas Medical Branch (UTMB).

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—Roger Pomerantz, Merck & Co. Inc.

Earlier work had suggested that nitric oxide (NO) could lower *C. difficile* toxicity in the gut.[2] Nitric oxide is a signaling molecule that is induced by inflammation. It modifies protein function through the S-nitrosylation of cysteine residues.

Thus, Savidge sought to identify specific S-nitrosylated proteins that could explain nitric oxide's protective function. "Our novel method to look at the entire nitrosyl proteome by mass spectrometry led us to explore the specific mechanisms underlying this effect," he said.

In mice, the team exposed the intestines to one of *C. difficile*'s major virulence factors, toxin A (TcdA), then performed a quantitative analysis of S-nitrosylated proteins.

Unexpectedly, they found that TcdA itself had been S-nitrosylated. This was surprising because S-nitrosylation of a foreign protein had never before been identified.

In vitro assays confirmed that both TcdA and the closely related TcdB could be specifically S-nitrosylated by a nitric oxide donor called *S*-nitrosoglutathione (GSNO) that is upregulated by the host after exposure to toxin.

In cultured intestinal cells, the S-nitrosylated toxins induced less cytotoxicity than unmodified toxins. In a mouse model of *C. difficile* infection, addition of exogenous GSNO significantly increased survival compared with addition of vehicle control.

The team included researchers from **Case Western Reserve University**, **Tufts University**, **The Commonwealth Medical College** and the **University of California, Los Angeles**.

Results were published in *Nature Medicine*.

Glenn Tillotson, SVP of medical affairs at Optimer, told *SciBX*, "In patients with severe infection, this nitrosylation mechanism could have a tremendous impact because you can't always give enough antibiotics to take care of the bacteria quickly enough to stop the toxins from causing intestinal damage."

He noted that 1%–2% of *C. difficile*–infected patients eventually require a colectomy, and this new approach could be most beneficial for those patients.

However, he cautioned, "This is very elegant work scientifically, but it is not clear yet whether this can be turned into a therapeutic weapon against *C. difficile* infection."

Roger Pomerantz, SVP and global franchise head of infectious diseases at **Merck & Co. Inc.**, agreed. "This is a very different mechanism than what has been described before. It is a very interesting paper—the science is excellent, well controlled and very persuasive."

He added, "The question for us at Merck is whether Savidge's work presents a druggable mechanism. The key is not only whether we can make a drug—there is good structural data for these toxins—but would it have an effect that is clinically meaningful."

Pomerantz noted that the host typically has many synergistic defenses that it uses to tackle infection, and it is not clear that nitrosylating the toxins would have enough of an effect to make a significant impact on the disease.

Both Tillotson and Pomerantz wanted to see the therapeutic effect investigated in model systems such as hamster and pig models of *C. difficile* infection, which more closely mimic the symptoms of the disease in humans.

Savidge said he plans to test the effect of S-nitrosylation in such models, including a pig model of the disease.

Merck's MK-3415A is an injected formulation of two mAbs against TcdA and TcdB that neutralize toxin function. In a Phase II trial published in *The New England Journal of Medicine* last year, MK-3415A decreased recurrence rates by about fourfold when used as an adjunct therapy to antibiotic treatment.[3]

Phase III testing of MK-3415A as an adjunct therapy to metronidazole, Vancocin or Dificid is expected to start by year end.

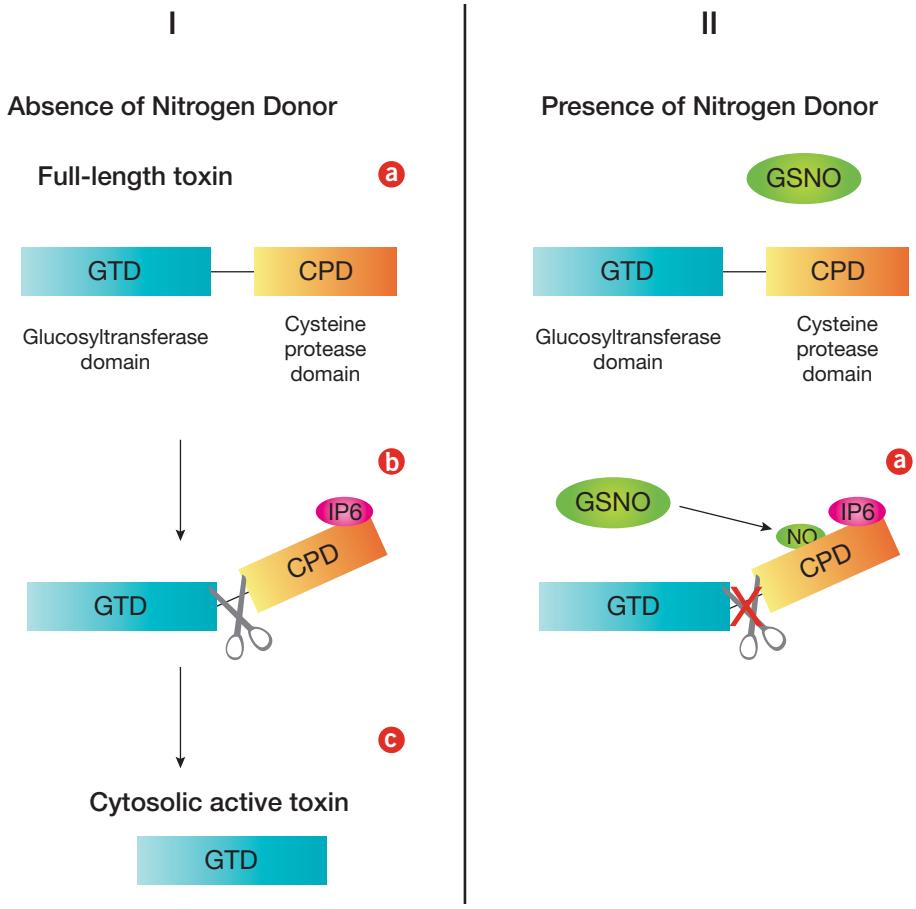

Figure 1. Nitrosylation of *Clostridium difficile* TcdA and TcdB inhibits toxin activation. *C. difficile* secretes two closely related cytotoxins, TcdA and TcdB, which induce cell death by glucosylating intracellular host proteins.

The toxins are secreted in an unprocessed form (**I**[**a**]) that is delivered into intestinal cells. Once inside cells, host inositol hexakisphosphate (IP6) binds to an allosteric site on the toxin and induces conformational changes (**I**[**b**]). This triggers autoproteolysis through the cysteine protease domain (CPD). Proteolysis leads to release of the glucosyltransferase domain (GTD) into the cytosol (**I**[**c**]).

In the presence of the nitric oxide (NO) donor *S*-nitrosoglutathione (GSNO), the unprocessed toxins are nitrosylated at the CPD after binding of host IP6 induces conformational changes (**II**[**a**]) but before proteolysis can occur. This nitrosylation prevents autoproteolysis *in vitro* and decreases toxicity *in vivo*.

The primary endpoint will be a reduction in the proportion of patients who have *C. difficile* recurrence after 12 weeks.

Adding an IP address

Savidge now is pursuing multiple strategies to bring toxin inhibitors into the clinic. His overarching goal is to combine antibiotics with compounds that disrupt intracellular toxin activation, which should decrease the damage caused to the gut by *C. difficile* and thus could reduce the risk of recurrence.

To help ensure that toxin S-nitrosylation produces actual benefit, Savidge is combining GSNO action with an additional mechanism that disrupts the timing of toxin activation. That mechanism comes from inositol hexakisphosphate (IP6).

TcdA and TcdB are secreted by *C. difficile* in a large, unprocessed form that is taken up into host cells through endocytosis. Host-produced IP6 is required to induce autoproteolysis, which releases the toxins within cells.[4,5]

S-Nitrosylation lowers toxin activity by preventing this cleavage (*see* **Figure 1, "Nitrosylation of *Clostridium difficile* TcdA and TcdB inhibits toxin activation"**).

"The toxin has evolved to use host IP6 as a sensor for when it is inside the cell. If you overload the system with IP6, it has some therapeutic effect because it prematurely activates the toxins before they get to their intracellular site of action," said Savidge.

Thus, addition of IP6 would prematurely activate *C. difficile* toxins outside the cell, whereas GSNO would prevent the activation of unprocessed toxin that the pathogen manages to deliver to its desired location of inside a cell.

In a mouse model of *C. difficile* infection, combined treatment with IP6 and GSNO significantly increased survival over either therapy alone.

Savidge is now developing a series of new nitrosyl-IP6 derivatives that will bind allosterically to the toxins with high affinity. This would enable one compound to bind the toxin, induce conformational changes and then inactivate autoproteolysis by S-nitrosylating the toxin.

Aimee Shen, assistant professor of microbiology and molecular biology at **The University of Vermont**, wants to see the approach tested in hypervirulent *C. difficile* strains, which can overexpress mutated versions of the toxins that undergo more rapid conformational changes. This could make it harder for S-nitrosylation to prevent toxin autoproteolysis.

"The hypervirulent strains are the ones causing outbreaks, and their prevalence has dramatically increased over the last decade," she noted.

Optimer's Tillotson added that hypervirulent strains have greater toxin production, intestinal adherence and spore-forming capabilities that together lead to more toxic infections and increased rates of recurrence.

He was concerned this might make it difficult to find an appropriate therapeutic window to prevent the damage caused by secreted *C. difficile* toxins. "This reminds me of attempts to develop drugs to

treat bacterial sepsis—the problem is that by the time you recognize the patient is septic, the train is leaving the station," he said.

Pathogen partners

Although Savidge's nitrosyl-IP6 derivatives are progressing through preclinical development, he also is working with the Institute of Translational Sciences at UTMB to begin a Phase I/II trial of IP6 as an adjunct therapy in patients receiving metronidazole for *C. difficile* infection.

In addition to developing his own compounds, Savidge is in talks to collaborate with **N30 Pharmaceuticals LLC**, a company that is pursuing an alternative approach to increase S-nitrosylation *in vivo*. N30 is developing inhibitors of alcohol dehydrogenase 5 (ADH5; GSNOR), an enzyme that breaks down GSNO.

Preclinical data have suggested that inhibiting GSNOR could help treat inflammatory conditions including asthma and inflammatory bowel disease (IBD) by raising GSNO levels.[6] However, the therapeutic effect of increasing GSNO in *C. difficile* infection was not previously known.

"We evaluated exogenous delivery of GSNO as a therapeutic, including in man. For a variety of reasons, including GSNO's instability, we moved our resources to GSNOR inhibition. Our GSNOR inhibitor program increases bioavailable nitric oxide by inhibiting or slowing the breakdown of endogenous GSNO—we saw this allowed for finer control and superior pharmacology compared to the NO donor approach," said Gary Rosenthal, EVP of research at N30.

N30 is in talks with Savidge to test the company's GSNOR inhibitors in models of *C. difficile* infection. Rosenthal also said the company is investigating chemically linking GSNOR inhibitors to other therapeutics and could consider linking a GSNOR inhibitor to an inositol phosphate.

N30's lead GSNOR inhibitor, N6022, is in Phase I/II trials to treat acute inflammatory asthma.

Shen also is exploring pharmacological modification of bacterial toxin activity. Last year, she published the synthesis and testing of a panel of compounds that are the first series of inhibitors of autoproteolytic TcdB activation.[7]

UTMB has filed two patents covering Savidge's work, and the IP is available for licensing.

Cain, C. *SciBX* 4(34); doi:10.1038/scibx.2011.954
Published online Sept. 1, 2011

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COMPANIES AND INSTITUTIONS MENTIONED

Case Western Reserve University, Cleveland, Ohio
The Commonwealth Medical College, Scranton, Pa.
Cubist Pharmaceuticals Inc. (NASDAQ:CBST), Lexington, Mass.
Merck & Co. Inc. (NYSE:MRK), Whitehouse Station, N.J.
N30 Pharmaceuticals LLC, Boulder, Colo.
Optimer Pharmaceuticals Inc. (NASDAQ:OPTR), San Diego, Calif.
Tufts University, Medford, Mass.
University of California, Los Angeles, Calif.
The University of Texas Medical Branch, Galveston, Texas
The University of Vermont, Burlington, Vt.
ViroPharma Inc. (NASDAQ:VPHM), Exton, Pa.