A Two-Pronged Attack on Bioterrorism

Livermore scientists are designing tiny synthetic molecules to detect biological warfare agents and fight cancer.

NEWLY designed molecules that bind to and capture biowarfare agents are on the drawing board at Livermore. The goal is for these molecules to quickly and efficiently detect such deadly pathogens as botulinum toxin, anthrax spores, or smallpox. Using synthetic chemistry, scientists produce these new molecules that bind to unique sites on the surface of the toxin or organism. Their two-pronged, or bidentate, structure is critical. When a small molecule binds to a protein, the attachment is usually weak, and the interaction between the two is short-lived. If, however, two or more small molecules that bind to the protein are linked together, their binding to the same protein may be thousands, even millions, of times stronger. By targeting specific proteins, the synthetic molecules will mimic some of the behavior in our immune system where antibodies recognize molecular foreign entities in our bodies and abnormalities such as cancer cells.

A single detector armed with many of these synthetic targeting molecules could simultaneously recognize an equal number of harmful biological agents that might be used in a terrorist attack. Assays using antibodies, known as immunoassays, are widely used to identify pathogens in the laboratory and form the basis for many biowarfare detection systems fielded to date.

Lawrence Livermore National Laboratory

However, only seven good antibodies are currently available for pathogen detection. Other detectors depend on recognizing the bioagent's DNA. "But some pathogens, such as viruses, require human exposure to only a small number of organisms to be acutely toxic," says Livermore biochemist Rod Balhorn. "With so little DNA present in each virus and given the rapid variation that occurs in the base sequences that make up the DNA, those pathogens are typically very difficult to detect."

Similarly designed targeting molecules could zero in on defective or overactive proteins in our bodies and poison them, just as our natural antibodies do. These antibodylike molecules can lock on to cancer cells or other pathogens and kill them—and only them. By targeting unique sites on other proteins that cause disease—for example, the proteases that cause inflammation in arthritis or enable HIV to function—the synthetic molecules would block the activity of the protein without entering its active site. The active site is a cavity on the surface of a protein that is used by the protein to perform its function. Similar active sites can be present in many proteins, both those that are essential to cell function and others that cause disease.

The pharmaceutical industry has already begun using this approach to develop drugs that function as intended without blocking the activity of healthy cells or proteins. Molecules that target unique sites on the surfaces of specific proteins may soon lead to a new generation of drugs that have minimal side effects.

Balhorn is leading the program at Livermore to design synthetic molecules for bioagent detection and cancer treatment. He and a team of Livermore investigators are collaborating with scientists at Brookhaven and Sandia national laboratories and the University of California at Davis Cancer Center. Together, they are developing the methods needed to produce the first of these synthetic antibodylike molecules.

"Terminology is a little tricky," he notes. "It is tempting to call our new molecules 'synthetic antibodies.' But we are designing small molecules that function like antibodies, not large proteins that are synthetic versions of antibodies. So we use the term 'high-affinity ligands' to describe our molecules."

"Ligand" is a general term used to describe a small molecule that binds to proteins or other large molecules. The higher the affinity a ligand has for a specific protein, the more tightly it binds to it. Research by others has demonstrated that bidentate ligands have a vastly increased affinity for the target protein, anywhere from thousands to millions of times greater. Polyvalent ligands—molecules that bind to multiple sites on the surface of a protein—are observed in many biological interactions that require very tight binding. The seek-and-destroy antibodies of our immune system, which normally operate quite successfully, are one example.

"What we're doing is searching for two molecules that bind to two sites next to each other on the surface of a protein," says Balhorn. "Then our synthetic chemist joins them together using a third molecule, called a linker. The linker must be both flexible and robust, or the new molecule will fall apart. This new synthetic ligand will then behave pretty much like an antibody, binding tightly to the protein."

The new bidentate molecules, called high-affinity ligands (HALs), will have several advantages over naturally occurring antibodies. They can be totally inorganic (nonprotein) and can be synthesized in large quantities using methods to ensure that each batch is structurally and functionally identical. They will also be stable over a long period, making them excellent candidates for long-term deployment in detectors for agents of biological warfare.

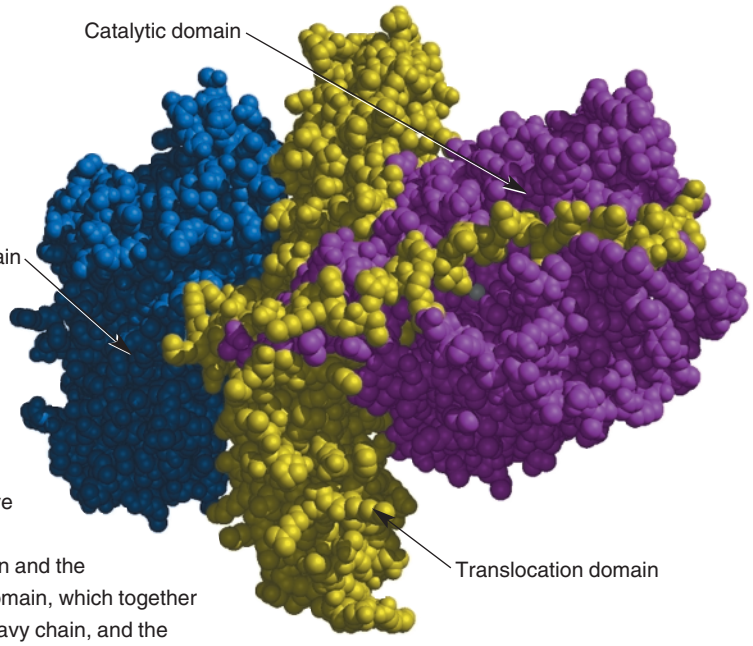
This schematic diagram shows how a linker molecule will connect molecules that bind to two sites on a protein. The goal is to develop a process for designing and producing high-affinity ligands for any structured surface. When two molecules are connected with a linker, they bind with up to a million times higher affinity than does each molecule alone.

The Toxic Targets

As bioagent detectors, HALs can be designed to target protein toxins produced by pathogens as well as any major protein component of pathogenic organisms. For the National Nuclear Security Administration's Chemical and Biological National Security Program, work is under way to develop HALs that bind to the *Clostridium* neurotoxins, which include botulinum and tetanus, the most toxic substances known. The *Clostridium* toxins attack the central nervous system and cause spastic paralysis in the case of tetanus and flaccid paralysis in the case of botulinum.

Balhorn's team is laying the groundwork for future development of HALs to target the *Staphylococcus* enterotoxins, which cause acute intestinal symptoms such as those associated with food poisoning, and ricin, a residue of castor bean processing that causes major intestinal or respiratory complications. The body's response to toxic quantities of either of these substances is swift and often fatal.

Work is also scheduled to begin in the near future on HALs that bind to proteins in the spores of *Bacillus anthracis* (anthrax) and in *Yersinia pestis* (plague). Once these HALs are completed, efforts will focus on the next highest priority agents: smallpox, *Francisella tularensis* (a plaguelike illness), and *Brucella melitensis* (an organism whose infections, often called Mediterranean fever, cause spontaneous abortions). Creating synthetic ligands even for proteins with a known structure is still a research project. Work began in 2000, and Balhorn estimates that high-affinity ligands for these eight bacterial toxins and threat organisms can be delivered in about 2005.



Clostridium neurotoxins have three parts: the targeting domain and the translocation domain, which together make up the heavy chain, and the catalytic domain, which makes up the light chain and is the toxic part of the molecule.

Got Structure?

If the structure of the target protein is known, the team uses that structure to develop a HAL. Work on these molecules is a logical progression from Livermore's protein structure and computational biology effort, with which Balhorn has been involved since its inception. (See *S&TR*, April 1999, pp. 4–9.) Using x-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy, high-resolution structures for many proteins have been determined at laboratories around the world, including Livermore. These include several types of *Clostridium* toxins (botulinum and tetanus) and the *Staphylococcus* enterotoxins.

All toxins in the *Clostridium* family have three parts. The targeting (or binding) domain, which binds to receptor molecules on the nerve cell membrane, and the translocation domain, which makes a pore in the cell through which the toxin passes, together make up what is known as the heavy chain. The light chain, which contains the catalytic domain, is a protease that is injected into the nerve cell and disrupts its functioning.

For the *Clostridium* neurotoxins, the team is developing a HAL to bind to the targeting domain, that fragment of the protein that recognizes and binds to motor neurons. Of these neurotoxins, botulinum is considered a greater threat than tetanus, but tetanus is easier to work with. Fortunately, its targeting domain is sufficiently similar in structure to botulinum's that it serves as a model for botulinum.

In 1998, Livermore's x-ray crystallography group completed a high-resolution structure of the binding domain of the tetanus toxin. Researchers then computationally calculated the molecular surface of the protein to identify sites where binding is likely to occur. "We look

for pockets on the surface of the folded protein, places where another molecule would be able to fit tightly," says computational chemist Felice Lightstone. For the tetanus toxin, Lightstone found two appropriate sites adjacent to one another on the binding domain.

For a HAL to be effective, the sites designated for binding must be on a part of the toxin that is "conserved," meaning that these regions remain essentially identical across all strains of a toxin. When bioagents are being genetically engineered, areas such as these are difficult to modify without altering the toxicity of the agent. Ideally, a high-affinity ligand for tetanus toxin will be able to recognize engineered and other unknown or related *Clostridium* toxins.

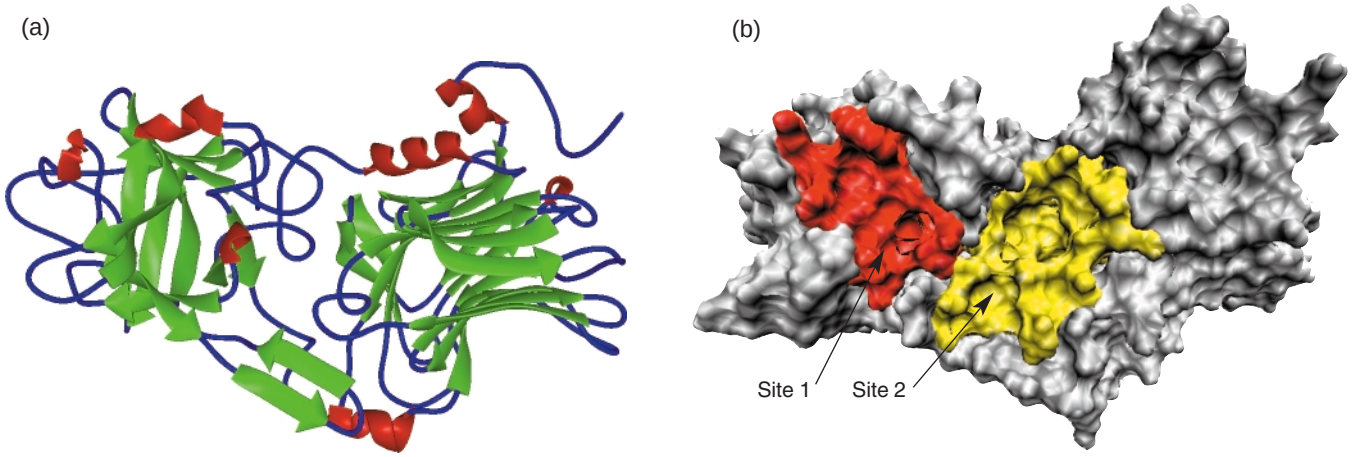
The next step involved selecting compounds that might fit into the two sites. All of the 300,000 compounds in the Available Chemicals Database, a listing of all commercially available compounds, were computationally inserted (docked) into each site. The potential fit and interactions were then assessed. The top 1,000 compounds were run again using a range of structures for each compound representing the different bond orientations and shapes, known as conformations, that each molecule is likely to adopt. In this manner, the top 100 compounds were identified. The calculations for each site took about 3 weeks on a Linux cluster of 40 dual-processor personal computers.

Sandia National Laboratories in Livermore has recently written new programs to expedite this time-consuming process. Each compound is tested in 10 different conformations to see which fits best into the rigid protein. This provides a more realistic test of binding, because many of these small molecules are not rigid and can adopt different conformations. "Computational docking projects typically have success rates of anywhere from 10 to 40 percent," says Lightstone. "Even before we started using our new version of this program, our success rate of identifying molecules that actually bind to the protein was in the 40- to 65-percent range. Now, the likelihood of getting a fit may be even greater."

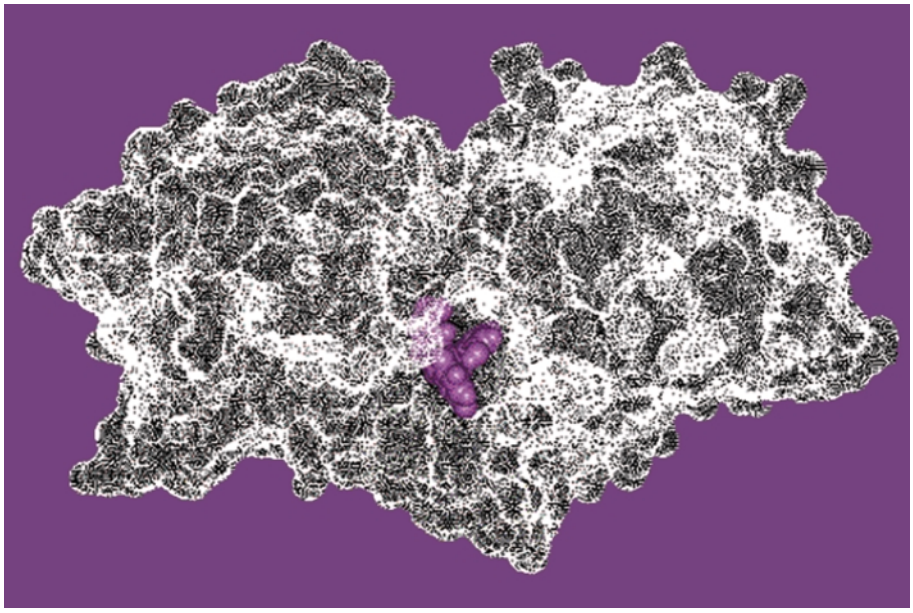
Into the Laboratory

Once possible ligands have been identified computationally, they must be tested in the laboratory to see whether binding actually occurs. Mass spectrometry (MS) and NMR spectroscopy are both effective for testing ligand–protein binding. NMR examines binding in the solution state, while MS looks at binding in the gas phase. MS typically requires much smaller samples, but it cannot handle certain compounds or chemical buffers. NMR can examine mixtures of compounds more easily and determine which combinations bind best in solution. Both techniques can identify where on the target protein binding is occurring.

The initial computational screening process to find new compounds that bind to tetanus neurotoxins resulted in 100 possible ligands that were predicted to bind to one of two sites (site 1 and site 2) on the tetanus neurotoxin's targeting domain. Experiments using electrospray ionization–mass spectrometry (ESI–MS) suggested that



(a) The x-ray crystal structure for the tetanus toxin showing how the amino acid chain is folded and (b) its calculated molecular surface showing sites 1 and 2, predicted binding sites for ligands.



The predicted structure of the tetanus–lavendustin A complex. Lavendustin A is shown in purple binding to site 2.

7 of the first 13 tested compounds bound to the toxin. With ESI–MS, ligand binding is confirmed when a new mass peak appears at the expected mass-to-charge ratio for the ligand–tetanus complex.

The antitumor drug doxorubicin was discovered to be the best fit at site 1. The binding of this ligand to site 1 was later confirmed by x-ray crystallography of doxorubicin–tetanus toxin and doxorubicin–botulinum toxin complexes. For site 2, the same MS method was used to screen 1 of 100 compounds, six of which were observed to bind. The figure above shows one of these ligands, lavendustin A, docked into site 2 in the predicted structure of the tetanus–lavendustin A complex.

The six ligands predicted to bind to site 2 were then screened for binding to the targeting domain using NMR. The six molecules were tested individually, as mixtures of different combinations of the compounds, and in the presence or absence of the known site 1 binder, doxorubicin.

When examined by NMR, small molecules exhibit weak, negative signals referred to as NOEs (nuclear Overhauser effects). Large molecules such as proteins exhibit strong, positive NOEs. When small molecules bind to proteins, the characteristics of the NOE for the large molecule are transferred to the small molecule. Thus, strong NOEs are detected for ligands that bind to the protein.

The NMR screening of mixtures containing the six predicted site 2 ligands confirmed that four bind to tetanus toxin in solution. Using a novel transfer NOE (trNOE) competition assay, researchers have determined that three of these ligands bind in the same site, presumably at site 2. The fourth ligand was determined to bind in a third site distinct from site 1 and site 2.

NMR experiments were also performed to evaluate how possible structural changes induced by the binding of one ligand in site 1 could influence the binding of the second ligand in another site. In these experiments, doxorubicin, which was added first, remained bound to site 1 throughout the additions of all six of the predicted site 2 ligands. The mixture containing doxorubicin and lavendustin A produced the strongest positive trNOE signal in the presence of the tetanus toxin. This experiment confirmed that both lavendustin A and doxorubicin bind simultaneously to the toxin, indicating that each must bind to a different site.

"Unfortunately, this assay cannot define the location of the binding site," says physical chemist Monique Cosman, leader of the NMR group at Livermore. "But since doxorubicin is known to bind to site 1, we know that lavendustin A must bind to a different site, which may be site 2.

By performing these trNOE binding experiments with pairs of molecules that were determined to compete for binding to the same site, Cosman developed a new NMR method for identifying the relative strength of binding of each ligand to a particular site on the protein. MP-biocytin, another molecule that binds to site 2, did so with a relatively lower affinity than lavendustin A. The affinity of the third ligand is similar to that of lavendustin A, but it was not studied further because it is too perishable.

Mass spectrometry was then used to verify where the molecules are binding. Chemist Sharon Shields developed a new method that combines MS with proteolysis, a process in which a protein is digested by enzymes. "This is unique," she notes. "Now we can study solution-phase biological processes using a gas-phase mass spectrometric method."

She first treated the targeting domain of tetanus toxin with proteases that make clips in the amino acid chain either alone or on the tetanus–

doxorubicin complex using various ratios of doxorubicin to the neurotoxin. Then she used matrix-assisted laser desorption ionization and ESI–MS to determine the pattern of enzymatic degradation that had occurred. In the tetanus–doxorubicin combinations, doxorubicin prevented the enzyme from digesting the protein at the binding site by limiting access to the amino acids located in that region.

The figure below shows a map of peptides (amino acid chains) produced by digesting the tetanus–doxorubicin complex compared to the tetanus toxin alone. In this experiment, Shields used the enzyme trypsin. The decreased abundance of peptides indicates the location where binding is occurring. That location contains amino acids 299–304, 351–376, and 394–434. Molecular docking calculations had predicted that doxorubicin would reside near amino acids 356, 358, 359, 407, 409, 419, 427, and 437. These predictions are a close match to MS results. Comparable locational experiments using other enzymes had similar results.

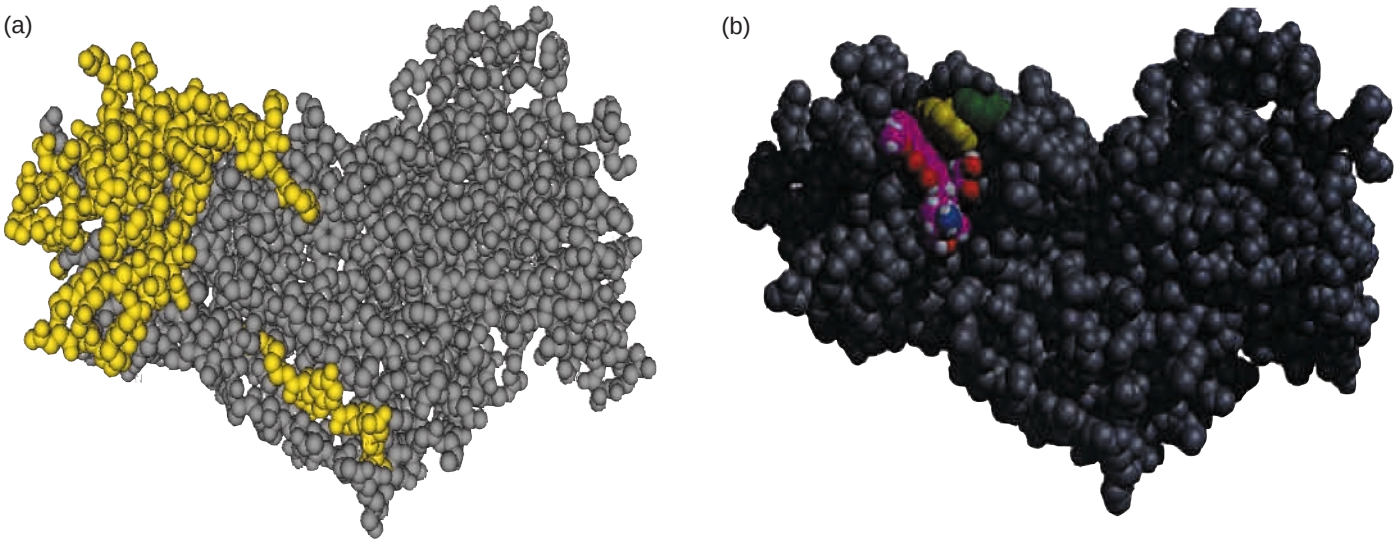
Shields also found that the presence of doxorubicin induces subtle changes in the tetanus toxin's three-dimensional structure, suggesting that the protein may envelope, or wrap around, doxorubicin when it binds. Further experiments are needed to confirm these results.

Creating a New Molecule

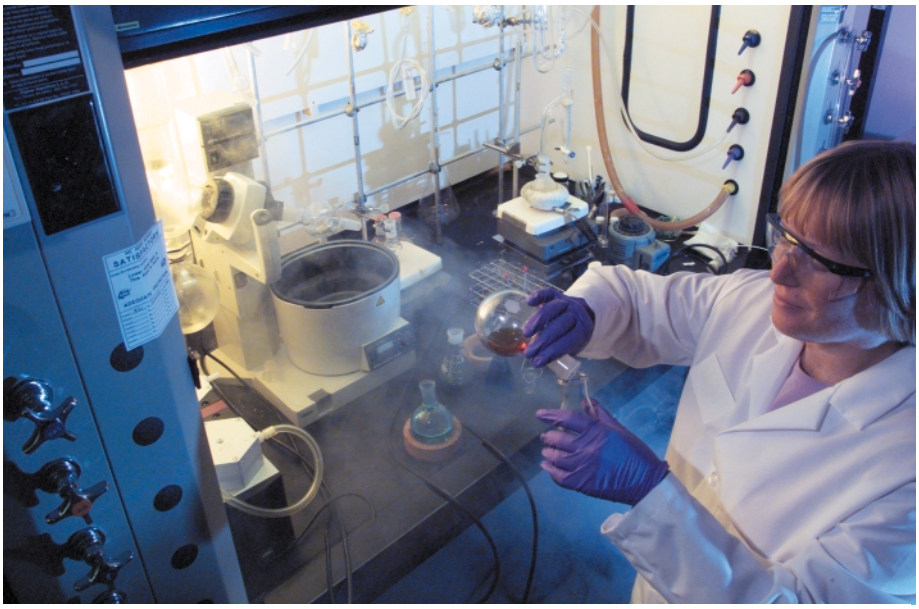
Synthetic chemist Julie Perkins has the job of linking the two molecules that bind to sites 1 and 2 to create a new HAL. This is the critical step. She is experimenting with linkers that will connect doxorubicin and MP-biocytin as well as doxorubicin and lavendustin A. "We know that each of these compounds binds individually to sites 1 and 2, but because they bind weakly, they can also float away," Perkins says. "When the compounds are linked together, they are much more likely to stay bound.

She is starting with the amino acid lysine as a linker. Lysine is an ideal building block because it has three distinct functional groups upon which she can perform synthetic chemistry experiments. Many derivatives of lysine are commercially available as well. The molecules that have been identified to bind into site 1 and site 2 can either be attached directly to lysine, resulting in their close proximity, or with a linker, which increases the distance between them. Increasing the distance between the two compounds with a flexible chain may also help increase the affinity of the ligand for the protein.

"To achieve maximum affinity of the ligand for the protein, we have to find the optimal length and rigidity of the linker," says Perkins, "and that can only be done experimentally." She is experimenting with a flexible glycol chain that can be attached to the lysine to increase the distance separating the two ligands.



(a) This map of peptides (amino acid chains) compares the doxorubicin–tetanus toxin complex to the tetanus toxin alone. Amino acids in yellow represent the peptides that showed a decreased abundance, which indicates that binding is occurring. (b) Computational docking studies predicted that binding would occur at the location shown. The two match quite well.



Synthetic chemist Julie Perkins works to link two molecules, each of which binds to two protein binding sites. The new molecule will bind more strongly and securely to a specific toxin protein than the individual molecules can.

Once she has synthesized each new compound containing the two linked ligands, conventional binding studies will identify the highest affinity and most selective ligand combinations. These studies will determine how tightly the HALs bind and confirm that they selectively bind only to *Clostridium* neurotoxins.

Targeting Cancer

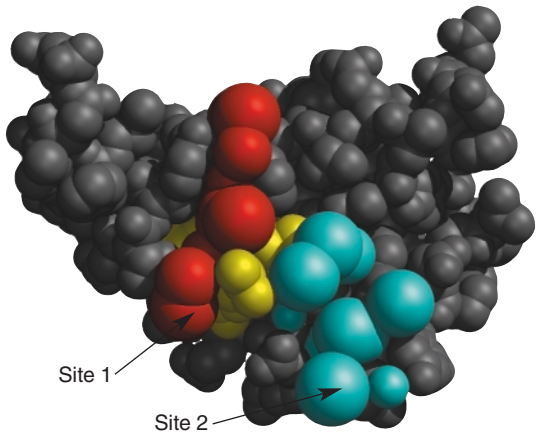
For cancer therapy, the challenge is to synthesize molecules that bind with high affinity to each cancer cell without themselves generating an immune reaction from the body. Targeting molecules therefore must be smaller and more specific and have higher affinities than natural antibodies. They should also not be made of proteins, which elicit an immune response from the body.

The goal is to use these small, exceptionally high-affinity molecules to deliver a lethal radiation dose directly to a tumor. In this case, the HALs would be tagged with radioactive isotopes and introduced into the body. Research all over the world is focused on this new technique, known as isotopically enhanced molecular targeting.

To create new HALs for cancer treatment, Livermore is using the same process developed for producing HALs that bind to toxins and pathogens. The first project will be a HAL for a receptor protein found on the surface of non-Hodgkin's lymphoma, HLA-DR10. The crystal structures of four HLA-DR molecules are known, and unique binding sites on the HLA-DR10 protein have been identified using computer models of the protein generated by computational biochemists Adam Zemla and Daniel Barsky. Computational docking experiments are under way.

The HAL developed for binding HLA-DR10 and targeting human lymphomas will be designed to rapidly pass through the liver and kidney and thus minimize the systemic damage that can occur when antibodies carry radionuclides. "We are striving to convert the meaning of the word 'cancer' from 'fear, pain, suffering, and death' to 'just another treatable disease,'" says Balhorn.

Sites 1 and 2 on the HLA-DR10 molecule (a protein receptor for non-Hodgkin's lymphoma) have been identified.

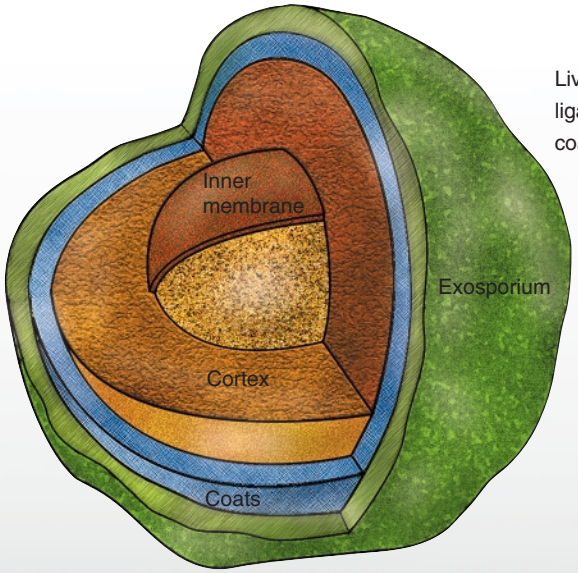


Targets of Unknown Structure

When a target protein's structure is not known, the team will use a different route to design and synthesize HALs. Computers cannot be used to predict the binding of molecules to

sites on these proteins. But NMR and MS processes that are being developed and fine-tuned now for identifying ligands that bind to known protein structures will identify ligands that bind to unknown structures.

Libraries of molecules will be experimentally screened for their ability to bind to the protein using a combination of Cosman's NMR technique and mass spectrometry methods being developed by chemist Lori Zeller. The molecules that bind will be segregated into sets that bind to different sites. Perkins will then synthesize all possible combinations of pairs of these small molecules using a series of different-size linkers. With Livermore's new Fourier transform ion cyclotron resonance mass spectrometer, mixtures of the HALs and protein can be quickly screened to identify the particular combination of ligands and linkers that produce HALs that bind to the protein. This approach should work well for creating detection reagents for pathogens. In collaboration with groups at Porton Down Defense Science and Technology Laboratory in England, Livermore researchers will design the first HAL for a protein with an unknown structure to bind to a protein on the coat of the anthrax spore.



Livermore will design a high-affinity ligand to bind to protein in the spore coat of *Bacillus anthracis* (anthrax).

Measuring Success

The Livermore team will soon produce its first HAL for the *Clostridium* neurotoxins. To know whether this work has been successful—whether the ligand works as designed in a bioagent detector—the team will send its results to the Department of Defense's Critical Reagent Program to be assessed for quality and specificity.

In the war against bioterrorism, the best defense begins with having the best possible data. Work has begun on docking studies to identify binding sites on the light chain of botulinum toxin. In this case, the goal is to synthesize HALs that can distinguish between the different types of *Clostridium* neurotoxins. That kind of fine-tuning is essential for accurate bioagent detection and identification during a crisis.

—Katie Walter

Key Words: antibodies, bioterrorism, botulinum toxin, cancer treatment, *Clostridium* neurotoxins, high-affinity ligands (HALs), mass spectrometry (MS), nuclear magnetic resonance (NMR), protein structure, synthetic chemistry, tetanus.

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