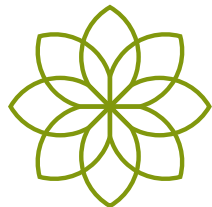
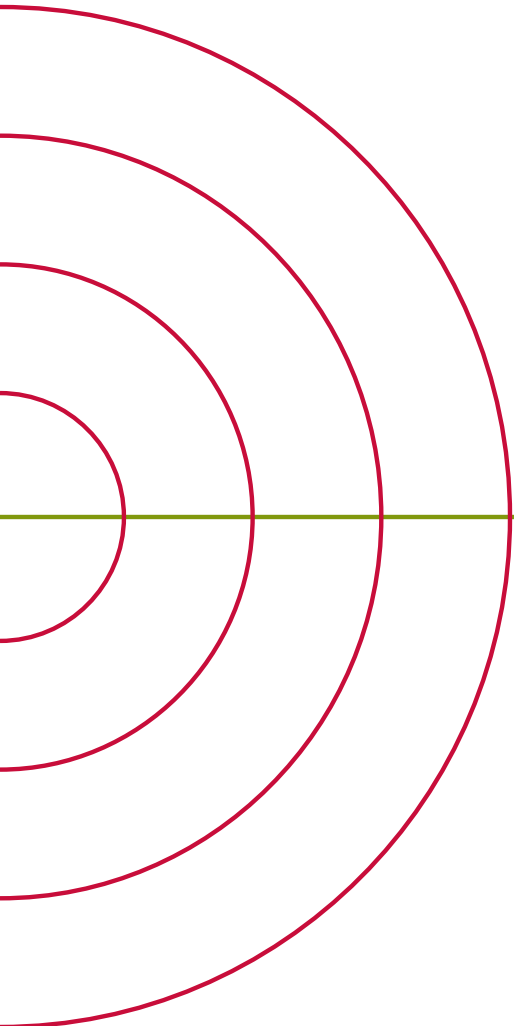




National Center for  
Complementary and  
Integrative Health



# Natural Products and Pain



The Search for Novel  
Nonopioid Analgesics

**February 6, 2019**

Natcher Conference Center  
National Institutes of Health  
Bethesda, Maryland

Workshop Summary

# Opening Remarks

Dr. Helene Langevin, Director of the National Center for Complementary and Integrative Health (NCCIH), welcomed everyone to the workshop and explained that it brings together two of NCCIH's top research priorities—natural products and pain management. She thanked Dr. David Julius, who is chairing the workshop, the National Institutes of Health (NIH) workshop planning committee, and the speakers.

Dr. Emmeline Edwards, Director of the Division of Extramural Research at NCCIH, explained that NCCIH has long had a robust portfolio in natural products research, including efforts to improve technology and methodology for pushing the natural products field forward, but this is the first time the Center's research efforts have focused on natural products for pain and as a tool for addressing the opioid crisis.

# Session One

## Overview: How To Find the Needles in a Haystack?

### **Nature's Pharmacy: How To Find Natural Products for Pain Management**

Dr. Cassandra Quave

Many of the medicines used today were discovered or modeled after compounds found in plants. At least 28,000 plant species have been used in human medicine. Only a few hundred of these have been studied in depth, so there's much more to explore. The science of ethnobotany—Dr. Quave's field—is the study of the relationships between people and plants. It may be considered the science of survival because people can't live without this knowledge. Historically, pharmacology and medicine were botanical arts.

Ethnobotanical information and historic texts can be starting points for the development of drugs of plant origin. The process begins with identifying locations with interesting biological materials for study, including hotspots of biodiversity, such as the Mediterranean basin. The preparation for field research is extensive, involving ethical and community consent, as well as international collaborative research agreements if plants are collected and interviews conducted outside the United States.

The natural products library maintained by Dr. Quave's lab has more than 1,800 botanical and fungal extracts used in traditional medicine to treat inflammatory and infectious diseases. The isolation and identification of active compounds from these extracts is a multistep process, involving several stages of chromatographic separation and analytical chemistry. In some instances, isolation of compounds may cause loss of activity because components of the original extract acted synergistically. Synergy can be assessed by recombining fractions and by synthesizing compounds identified in the extract and testing combinations. The presence of synergy need not prevent drug development. The U.S. Food and Drug Administration (FDA) botanical drug pathway offers options for the development of synergistic compositions if the plant has a history of use for a related indication.

NIH has made an impressive investment in the development of the NIH/National Cancer Institute (NCI) Natural Products Repository, which includes more than 230,000 unique extracts from plants, marine organisms, and microbes.

In summary, there is a huge amount of chemical space to explore in natural product diversity collections. The tools of ethnobotany can be used to identify promising leads, and knowledge from traditional medicine may help researchers narrow their scope as they search for new leads.

**Discussion:** In response to questions, Dr. Quave explained that her laboratory focuses on small molecules, but there are also interesting plant peptides that can be studied. She and her colleagues use knowledge from traditional medicine to determine which part of a plant to use as a starting point. Isolating compounds is the slow step in the discovery pipeline. Better nuclear magnetic resonance (NMR) and mass spectrometry (MS) databases could facilitate research in this field. Platforms exist for growing plant cells in culture and taking research *in vitro*. Recombinant DNA technology could be used to move enzymes into different systems to ramp up production of useful compounds. For the NIH/NCI repository, extractions are done using both aqueous and organic solvents.

## Animal Venoms: Evolving Combinatorial Peptide Libraries for Pain Research

Dr. David Julius

Studying animal venoms is valuable because venomous creatures have evolved chemical ways to defend themselves, such as by inflicting pain. Toxins have evolved to target key sites such as functionally important parts of voltage-gated channels. This provides a way to use evolution not only to identify interesting molecules but also to use them as probes for understanding function. Like other natural products, venoms are chemically complex; some may have multiple active components that show synergy. Many toxins are genetically encoded peptides; this can be an advantage because molecular techniques and gene cloning methods can be used to explore structure-activity relationships, but it is also a disadvantage because peptides pose challenges in terms of bioavailability.

Natural products such as toxins are important not only as sources of pharmaceuticals but also as investigational tools to help identify targets and mechanisms that underlie pain processes.

Obtaining crude venoms is challenging. Commercial sources are limited and academic sources are specialized. A repository for venoms would be valuable. Quantities of venoms can be limiting depending on the species and specific activity. Fortunately, concentrations of venoms in venom sacs are often very high, so only a small volume is needed. Having good phylogenetic and molecular genetic documentation of the animals that make venoms would help with a long-term venom mining strategy and with cloning and analyses of toxin genes.

Two approaches have been used for functional identification. One involves taking a cell line that expresses a target of interest, such as a cloned ion channel, and applying crude venoms to the cultured cells. This kind of target-specific screening has been done for targets already known to play a role in pain sensation. The other approach is target-blind; it involves culturing primary sensory neurons, applying a venom, and looking for an activity. This type of screen puts a spotlight on new targets that may play a role in pain sensation.

Mass spectrometry (MS) is an essential tool for understanding toxin composition. Transcriptome analysis is a powerful tool for studying venoms because it can reveal related toxins and families. Several databases of venom data are available, including NIH-sponsored resources. Producing synthetic toxin can make it possible to have biochemical quantities and generate derivatives, but it is challenging for peptides because of folding problems and posttranslational modifications.

Work with a spider toxin allowed visualization of the structure and function of transient receptor potential (TRP) V1; work with a coral snake toxin provided insights about acid-

sensing ion channel 1 (ASIC1) and opportunities to explore its role in pain sensation; and work with another spider toxin,  $\delta$ -theraphotoxin-Hm1a and b (Hm1a/b), allowed exploration of the role of the previously overlooked voltage-gated sodium ( $\text{Na}_v$ ) 1.1 channel in acute pain and mechanical hypersensitivity.

**Discussion:** Study of the effects of toxins on immune cells and glial cells would be valuable, but Dr. Julius's lab has not focused on this area. Current screening methods do not focus on reactions to toxins in specific prey. However, there are interesting predator-prey relationships where prey have evolved immunity to various toxins. Producing venoms in culture should be possible, but not much work has been done on the functioning of venom sacs. Synergy between peptides in venoms has only been assessed in one instance, but it's well known that synergies can exist.

## Natural Products as Tool Compounds and Therapeutic Leads

Dr. Justin Du Bois

Sodium ion channels have become an extremely interesting targets for next-generation analgesics. In recent years,  $Na_v1.7$  in particular has been recognized as a principal target for pain sensitivity, and other  $Na_v$ s have also been studied. The eukaryotic voltage-gated sodium ion channel is a massive polypeptide (the alpha subunit), coexpressed with auxiliary proteins (beta subunits). Nine different genes encode for alpha subunits, so one of the challenges in research on this channel is finding small molecules that engage one subtype selectively.

Sodium ion channels play critical roles in all of life's processes. Many pathologies related to their malfunction extend beyond pain. Natural products can be used as tool compounds to study the protein structure and function of sodium channels at the biochemical level and to understand the roles of individual subtypes in electrical signaling.

Tetrodotoxin and saxitoxin have played important roles in identifying sodium ion channels. These toxins act as molecular forks, binding to the outer vestibule of the channel and occluding ion permeation through the central core. Saxitoxin is the easier toxin to work with. Toxin conjugates can be created as molecular probes of  $Na_v$  function.

Saxitoxin and tetrodotoxin act as molecular "corks." They bind to the outer vestibule of the channel near the selectivity filter and occlude ion permeation through the central core. Saxitoxin shows selectivity for a subgroup of the nine  $Na_v$ s. Single amino acid changes in the mouth of the channel are responsible for differences in the affinity of the compound. It might be possible to exploit this variation to develop subtype-selective saxitoxins.

Double mutant cycle analysis was used to study a group of saxitoxins created through *de novo* synthesis. The information obtained in this way led to changes in the view of how saxitoxin is bound to the outer vestibule. Results obtained using the newer technique of electron cryomicroscopy (cryo-EM) have confirmed those obtained with double mutant cycle analysis.

Working with a small business partner, SiteOne, Dr. Du Bois's group has designed saxitoxins selective for  $Na_v1.7$ . Some have more than 10,000:1 selectivity. Work with these toxins suggests that the outer vestibule of the channel is a druggable site.

Some toxins that act on sodium ion channels modulate the activity of the channel rather than block it. One of these is batrachotoxin, first isolated from the venom of the Colombian arrow poison frog. This toxin affects multiple biophysical properties of the channel. Molecules like this toxin can be used to increase understanding of the channel's function at the molecular level and to explore pathologies that involve

changes in the activity of channels rather than complete loss of function. It may be possible to learn enough to design molecular “rheostats”—molecules that would fine tune channel gating rather than just block the pore—but this is a very difficult problem in molecular design.

**Discussion:** In response to a question, Dr. Du Bois explained that researchers are looking for ways to image nerve damage associated with pain—a kind of “x-ray for pain.” The concept involves finding ways to visualize upregulation of  $\text{Na}_v\text{s}$ . This works for sciatic nerve injury, but the upregulation of the channel is relatively small, so the signal-to-noise ratio is low. Peptide leads—such as the effects of Hm1a/b—can be used in the design of small molecules that modulate ion channels. Computational modeling can guide this process, but ligand allostery is difficult to understand.



## Session One Panel Discussion

Dr. D. Craig Hopp, moderator of the session, noted that the session had emphasized that natural products are valuable not only as potential therapeutics but also as tools to probe pain biology. He asked the panel what they see as needed resources to facilitate further research in this area. The speakers mentioned repositories for natural product material, especially if samples are well categorized and annotated; having people available who are strong in terms of analysis; better databases for NMR and MS; and screening centers that could perform high-throughput studies, such as whole cell electrophysiology. Assessing synergy is an important methodologic challenge.

Dr. Hopp referred to the tension between targeted assays, which have high throughput but don't allow the identification of new targets, versus agnostic or phenotypic screens, and asked about the balance between these two approaches. Dr. Quave said that she prefers phenotypic screens because many targets haven't been discovered yet.

Dr. Wen Chen pointed out that different types of information—NMR, genetic coding, etc.—may be needed for repositories of different types of substances. The panel noted that NMR is a powerful technique for peptides but not as much of a core component as it is for small molecules. The main challenge for repositories is collecting species and categorizing them. Collecting venoms has additional challenges in terms of milking the venom from the animal. Many relevant small molecules are produced by symbionts, which adds to the complexity of their characterization. Sometimes, the identity of the microorganism that produces the small molecule is not known. Similarly, the roles of plants and endophytic fungi in producing relevant molecules may not be well understood.

Dr. Hopp clarified that NCCIH does not have special collaborations to get natural products to market, although some small business programs are available. Some other NIH institutes and centers (ICs) do have initiatives in which they partner with others (not necessarily small businesses) on research in preparation for an Investigational New Drug (IND) application or even Phase I studies, for example on neurotherapeutics.

The knowledge of traditional healers and community leaders is a valuable resource, but obtaining access to traditional information can be difficult because of challenges in establishing trust. Knowledge that exists today is rapidly disappearing as exposure to Western medicine spreads. Thus, documenting the details of traditional practices is a matter of urgency. This requires trust, formal agreements, and an interdisciplinary effort between bench and field scientists. The Convention on Biological Diversity website and the ethnobotanical literature, both historical texts and current journal articles, are important resources.

In response to a question about the possible role of citizen science in collecting traditional medical knowledge, the panel said that NIH does not have a program of this type, but the idea is a good one.

Many pharmaceutical companies moved away from natural products isolation research in the past 20 years. Pharmaceutical companies may still be interested in natural products if improved methodologies for studying them become available. NCCIH is trying to tackle methods development aggressively so natural products research can keep pace with high-throughput methods. With advances in technology, including identification of targets, much could also be learned by reexamining existing isolates.

Phenotypic screens can play a major role in the discovery of new scaffolds. Rapid dereplication is necessary to avoid repeating investigations of substances that have already been identified. New techniques for studying complex mixtures are very powerful and can generate much knowledge before individual components are analyzed.

NCCIH is launching an effort to develop an NMR database for natural products. Some MS databases already exist.

A member of the audience pointed out that traditional medicines were developed by specific ethnic groups and may not have the same effects in other groups because of genetic differences. The panel pointed out that genetics has also played a role in the study of pain in that studies of rare individuals with mutations that affect pain sensation have pointed to important targets such as  $Na_v1.7$ .

Natural products that interact with RNA or DNA may be valuable as tool compounds, but there are challenges with the delivery of nucleic acid drugs, and it may not be desirable to modify protein synthesis. Nucleic acids as ligands would require different types of extraction procedures than those used to extract peptides or small molecules.

# Session Two

## The Science of Animal-Derived Natural Products

### The Transformative Power of Snail Venom in Pain Therapeutics

Dr. Mandë Holford

People tend to think of venoms with fear, but venoms can also be agents of hope and innovation. Venoms have evolved convergently in many groups of animals, empowering them and transforming physical warfare into biochemical warfare. Research can leverage this power to enhance human lives.

The power of venom comes from three key features: their complex nature, which includes mixtures of compounds that have evolved to hit a specific molecular target; the conservation of physiological targets, often including sodium and calcium ion channels; and their ability to turn cellular communication on and off. Venoms have been used to develop novel drugs: six venom-derived therapies are currently on the market. They treat conditions such as diabetes, high blood pressure, and pain.

Dr. Holford and her colleagues study marine cone snails, which have evolved venoms that enable them to hunt much faster-moving creatures such as fish. The toxins in the venom of cone snails work to shut down the nervous systems of the prey. Snail venoms have potentially valuable effects, including inhibition of pain without the potential for addiction, but they are peptides, and as with other peptides, delivery to target sites is a challenge. The one marketed pain medicine derived from snail venom, ziconotide (Prialt), can't cross the blood-brain barrier and therefore can only be administered intrathecally (by spinal tap). For this reason, although ziconotide has advantages over morphine, it's only used when morphine is not a possibility.

Dr. Holford's group is building a phylogenetic tree to identify terebrid snails that actively produce venom. The tools of transcriptomics and proteomics are then used to find active peptides from the venom. One interesting peptide is Tv1, which elicits avoidance of noxious heat in flies when administered orally or by injection. Its mechanism of action is currently being investigated and may involve the TRPA channel. *Drosophila* is being used for in vivo assays because it is so well studied and because only tiny amounts of the peptide are needed.

The World Economic Forum has recognized venom research as a frontier of science. Although the work is difficult, it is a moonshot for science and requires collaborative efforts to identify the relevant peptides, elucidate their structures, and determine how they exert their effects.

**Discussion:** In response to a question on the role of natural history, Dr. Holford commented that knowledge of the animal's natural ecology facilitates target-driven discovery, and this type of work needs to be supported. A repository of venom peptides would be very valuable, as would more transcriptome and genome work on the animals. At least 15 percent of animals produce venoms. Dr. Holford's group has not yet performed *in vivo* tests in mice. Because of the extensive prior research on cone snail venoms and the existence of an extensive library of conotoxins, prediction of structure based on amino acid sequences is realistic. Computational molecular modeling is also helpful.

One current project in Dr. Holford's lab involves putting a snail peptide inside a virus capsid to facilitate delivery.

Side effects of ziconotide are dose dependent and patient specific and may include headaches and a feeling of instability.

## Discovery of Tarantula Venom-Derived Na<sub>v</sub>1.7 Inhibitory Peptides

Dr. Justin Murray

Dr. Murray discussed work being done at his company, Amgen, on discovery of Na<sub>v</sub>1.7 inhibitory peptides from tarantula venom. Na<sub>v</sub>1.7 is a voltage-gated sodium ion channel that has been shown to play a role in pain sensation in humans. Gain-of-function mutations in the gene that encodes the Na<sub>v</sub>1.7 protein cause spontaneous pain; loss-of-function mutations cause insensitivity to pain.

Challenges in discovery of an Na<sub>v</sub>1.7 antagonist include the need for selectivity (Na<sub>v</sub>1.7 is one of nine highly homologous channels), the need to block action potentials as well as channel current, and the difficulty in getting a drug across the blood–nerve barrier to the peripheral neurons.

Amgen's search for a novel peptide inhibitor of Na<sub>v</sub>1.7 began with a venom screen that pinpointed spider venoms as the most promising. Steps in the screening process included milking the venom from the spiders, high performance liquid chromatography fractionation, electrophysiology testing (using a high-throughput electrophysiology platform, the IonWorks Quattro system [IWQ]), identification of active fractions through MS/MS analysis, refractionation, and identification of an active peptide, GpTx-1. This substance has a potent effect on Na<sub>v</sub>1.7 but is not ideally selective. Analogs were created through an alanine scan to identify the most relevant residues, and saturation mutagenesis was used to create analogs in which these residues were replaced with other amino acids. Each of the more than 130 analogs created in this way was tested for functional activity against Na<sub>v</sub>1.7 and profiled against off-target channels. This process provided insights into the structure of the peptide.

Another Na<sub>v</sub>1.7 inhibitory peptide identified in the venom screen is JzTx-V, produced by the Chinese earth tiger tarantula. It is a novel peptide with an inhibitory cysteine knot (ICK) fold. A more selective analog was created, and its structure and molecular docking to Na<sub>v</sub>1.7 were studied. A key substitution was identified, and additional selectivity optimization provided the lead compound AM-6120, which blocked Na<sub>v</sub>1.7-dependent scratching behavior in mice.

In summary, Na<sub>v</sub>1.7 is a compelling target for pain because of validation from human mutations. Venom screens identified two peptide blockers from venoms, and potency and selectivity enhancements produced AM-6120, which blocked Na<sub>v</sub>1.7-dependent effects *in vivo*.

**Discussion:** In response to a question about how the role of the compound in the tarantula differs from its role in pain, Dr. Murray said that tarantula venom is targeted at insects as prey. Insects are believed to have one sodium channel, which is expressed differently in different species. The venom is likely optimized to target insect sodium channels. A modified version may more effectively target human sodium channels.

Peptides can be synthesized that include both natural and unnatural amino acids to enhance selectivity.

Amgen is just starting to think about interactions of the peptides with lipids as well as proteins. One amino acid points out toward the lipid interface. Making the position of this amino acid as hydrophobic as possible, without preventing folding, seemed helpful but did not translate to the primary assay. It's important to be careful so that the toxin doesn't end up interacting with a membrane other than the desired one.

Issues with folding are addressed through a screen that involves different folding conditions. The goal is to identify an optimal folding condition. Sometimes apparent hits turn out not to be useful because they don't fold appropriately. It may be possible to optimize folding by modulating the charge of the molecule. Within the venom sac, the same peptide may be folded in different ways.

## **Fish Oil and Pain: Resolution of Pain and Inflammation by Fish Oil–Derived Specialized Proresolving Mediators (SPMs)**

Dr. Ru-Rong Ji

The two major components in fish oil are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Both are omega-3 unsaturated fatty acids. EPA has 20 carbons and 5 double bonds, and DHA has 22 carbons and 6 double bonds. Many fish oil products are on the market; they typically contain EPA and DHA in a 1.5:1 ratio. The daily requirement for omega-3 fatty acids is a few hundred milligrams.

Purported benefits of fish oil include effects on bones and joints, mental health, cholesterol levels, eye health, skin health, heart health, blood glucose, energy and endurance, and pain relief, but large clinical trials have had mixed results, and the mechanism by which fish oil might produce beneficial actions is not well understood. The human need for EPA is constant throughout life, and EPA deficiency in adolescence and adulthood correlates with mental issues. Demand for DHA is time dependent; it is higher in the early stage of life to support brain development and in the late stage of life to minimize neurodegeneration.

Dr. Ji's laboratory studies inflammation and pain. Tissue injury in nerve cells can produce proinflammatory mediators that activate TRP channels and sodium channels. Pain medicines may act on either immune modulation (as nonsteroidal anti-inflammatory drugs do) or neuromodulation. An ideal pain drug would act on both. Fish oil-derived SPMs may have targets of both types.

EPA and DHA have weak anti-inflammatory and analgesic effects, acting through receptors such as GPR40 and the long-chain free fatty acid receptor (FFAR1). EPA and DHA may also act indirectly through conversion to SPMs, which have potent anti-inflammatory, proresolution, and analgesic effects. SPMs may be a thousand times more potent than fish oil itself. The SPMs include both resolvins and protectins. The resolvins include an E series derived from EPA and a D series derived from DHA. Neuroprotectin D1 (NPD1), which protects against both inflammation and neurodegeneration, is derived from DHA.

Various SPMs have been shown to have effects in laboratory models of pain. In a persistent inflammatory pain model, complete Freund's adjuvant (CFA)-induced heat hyperalgesia in mice, intrathecal resolvin E1 was much more effective than EPA or DHA, although EPA and DHA were given at doses 1,000 times higher.

Resolvin D2 was potent against TRPV in capsaicin-induced pain, while D2 had a smaller effect and E1 was not effective. D1 and D2 were potent against TRPA1 in the mustard oil model. In both models, large doses of DHA and EPA had mild effects. Resolvin E1 inhibited TRPV signaling in dorsal root ganglion (DRG) and spinal cord, and also counteracted the effects of capsaicin in the spinal cord.

Pretreatment with NPD1 or DHA protects against acute neuropathic pain in mice after nerve trauma (chronic constriction injury). NPD1 is effective at much lower concentrations than DHA. NPD1 and gabapentin were also effective against established neuropathic pain, but DHA was not. Thus, fish oil components may be effective in the early stage of chronic pain but have much less effect in the later stages.

G-protein coupled receptor 37 (GPR37) is a novel receptor for NPD1 in macrophages. NPD1 induces macrophage phagocytosis via GPR37 in zymosan particles. GPR37 regulates the resolution of inflammatory pain. GPR37 knockout mice develop prolonged inflammatory pain. Natural compounds have been screened for GPR37 agonist activity, and several promising substances have been identified. These substances could potentially be used to treat inflammation, pain, infection, sepsis, malaria, and cancer.

In summary, EPA and DHA have direct but weak effects on inflammation and pain, but SPMs have potent effects. In chronic pain conditions, there is failed conversion from fish oil to SPMs. Loss of SPMs may result in transition from acute to chronic pain. Identification of natural compounds as SPM receptor agonists may lead to novel therapeutics.

**Discussion:** Omega-3 fatty acids and their SPM derivatives have both neuronal and immune actions because their receptors are expressed by different cell types. Thus, they may help resolve both inflammation and pain. Dr. Ji explained that it is difficult to identify the specific types of fish used as sources for fish oil in research studies, but all fish oils contain EPA and DHA. Pure DHA is also commercially available. Dr. Langevin suggested that SPMs might actually act on three targets—nerve cells, inflammation in peripheral tissues, and inflammation within the nervous system. Dr. Ji explained that work has been done on neuroinflammation in the spinal cord and the DRG, and SPM receptors exist on microglial cells.



## Session Two Panel Discussion

In response to a question from session moderator Dr. Michael Oshinsky about ways in which NIH can partner with academia or industry to accelerate development of therapeutics from animal-derived natural products, panelists explained that it is important to have funding for discovery research as well as hypothesis-driven research. Discovery research is laborious but important. Having more genomes would facilitate comparative genomic analysis. It would be valuable for NIH to support the creation of repositories and databases.

Improvements in instrumentation would also be valuable. High-throughput electrophysiology platforms could be improved. With regard to fish oil, research priorities include understanding and improving the conversion of EPA and DHA to SPMs, particularly in preventive settings; providing SPMs to be used directly; and identifying more ways to screen small molecules, especially natural compounds.

In response to a question about the accessibility of pharmaceutical companies' natural product libraries, Dr. Murray explained that his company acquired their library through collaboration with another company, but it was eventually made available to everyone. Amgen wants to collaborate with others and can supply specific compounds through various agreements.

In response to a question about failed conversion of EPA and DHA to SPMs, Dr. Ji explained that SPMs are produced during the resolution phase of inflammation, and it is necessary to have the right enzymes available at the right time. In chronic pain, the necessary biosynthetic pathways are not there. Fish oil components may be converted into something else rather than SPMs. It may be possible to find strategies to promote the synthesis of SPMs; acupuncture might have this effect.

SPM receptors are expressed by macrophages and neurons. It may be possible to screen for receptor expression. Loss of GPR37 changes the phenotype of macrophages.

For agents that act on Na<sub>v</sub>1.7, ideas about the most appropriate pain conditions to target have evolved over time. Currently, the greatest interest is in chronic pain. A drug that targets this condition would have the greatest impact. For long-term recovery from chronic pain, it may be helpful to partially inhibit multiple targets involved in the causation of pain to help the system return to its normal state. Attenuating chronic pain is also an important target for venoms.

Barriers in funding discovery research include a lack of funding opportunities specifically designed for this purpose. Reviewers of applications submitted in response to funding opportunities not specifically designed for discovery research may react negatively to applications that are not hypothesis driven. Dr. Oshinsky said that NIH receives few discovery-focused applications, even though it is possible to structure aims around a discovery base. This may reflect the perception that reviewers would

dismiss this type of application as a “fishing expedition.” Dr. Julius suggested that some of the work in this field is at the intersection of the interests of NIH and the National Science Foundation (NSF). There are some joint efforts by NIH and the NSF. Some NIH funding mechanisms, such as the R35, are designed to support a research program over many years, not a specific project, and therefore are less hypothesis driven. Phased, milestone-driven funding mechanisms and NIH’s high-risk, high-reward programs may also be appropriate to support discovery research.

Oral delivery of therapeutics is ideal but difficult to achieve for peptides. Other delivery devices, such as a depot formulation, may be more practical.

# Session Three

## The Science of Plant-Based Natural Products and Supplements

### A Cactuslike Plant, Resiniferatoxin, and Pain Control

Dr. Michael Iadarola

Knowledge of the plant “euphorbium” traces back to a 2000-year-old textbook of pharmacology written by King Juba II of Mauretania. Euphorbium was later mentioned in Greek and Latin medical literature as a treatment for skin and nose irritation. Euphorbias that produce resiniferatoxin (RTX) are found in Morocco and northern Nigeria. RTX is a capsaicin analog but is more potent, with a much more prolonged channel open time.

RTX is highly selective for TRPV1, which is selectively expressed only in subpopulations of DRG nociceptors. Other sensations, such as proprioception, touch, pressure, and pinch, remain intact because the corresponding neurons do not make TRPV1. RTX induces rapid cell death in TRPV1-expressing cells. The neuronal calcium overload RTX produces is permanent when the toxin is administered intrathecally or intraganglionically but reversible over time when it is administered at peripheral sites. RTX interventions can be used for a variety of indications, including corneal neuropathic pain, postincisional pain, complex regional pain, cancer pain, burns, and osteoarthritis.

RTX is effective as a long-duration, nonopioid single-administration treatment for bone cancer pain. It has been used successfully for long-term pain control in dogs with bone cancer. It has also been used successfully for cancer pain in the lower half of the body in a small number of people. For example, intrathecal administration of RTX in a man with bone and pelvic involvement from metastatic cancer reduced his pain ratings and use of oxycodone.

Intra-articular administration of RTX in dogs with osteoarthritis reduced pain severity and interference within 7 days, with a median time to retreatment of 5 months. Preliminary findings from an ongoing clinical trial suggest that RTX can also be helpful for knee osteoarthritis pain in people. In peripheral sites such as the joints, the effect of RTX is reversible.

**Discussion:** RTX has not yet been studied to determine whether it has an effect on joint degradation in osteoarthritis. It's likely that residual pain is present in animals or humans treated with RTX because some cells are less susceptible to RTX than others.

When RTX is injected into dogs intrathecally, it cuts the axon; variation between individuals is due to differences in administration. RTX has been evaluated in a variety of animals. The half-life of RTX is between 4 and 20 minutes; effects after that time are due to changes in the calcium channels induced by RTX rather than the presence of the drug. The portions of the body that receive RTX lose the sensation of thermal pain, but endogenous thermal regulation is not affected. Compounds from a different species of euphorbia are used as a fish stunner in the Mediterranean; these compounds have not been studied with respect to pain.

## Plants, Conolidine, and Pain

Dr. Laura Bohn

Conolidine is derived from *Tabernaemontana divericata*, known as pinwheel jasmine or crepe jasmine, which comes from Southeast Asia and is grown as an ornamental plant in South Florida. The plant has been used in traditional medicine for snake and scorpion poisoning and in Ayurvedic medicine for parasite treatment and dental health. In modern medicine, the roots have been used to treat hypertension, headache, and scabies.

One component of this plant studied previously is apparacine, which is present in very low quantities and was proposed to be an opioid ligand and analgesic. However, its actions in the brain indicate that it is not likely to be a selective compound at opioid receptors. Apparacine showed efficacy in a mouse acetic acid writhing assay, but this assay is crude and does not demonstrate that the compound is an opioid or even an analgesic.

More recently, the related compound conolidine has been studied. The yield of conolidine is so low that it is not practical to extract enough of it for profiling, but the compound has been synthesized. Radioligand binding assays and G protein coupling in cells expressing mu opioid receptors indicate that conolidine is not an opioid. The compound also did not exert an effect in hot plate and tail flick assays where opioids do have an effect.

Conolidine was effective in the nonspecific writhing test and also in more specific pain assays, including both phases of the formalin test in mice, so it appears to be an analgesic. Behavioral screenings in transgenic mice did not provide clues to help identify its mechanism of action. Conolidine was tested in the National Institute of Mental Health's (NIMH) Psychoactive Drug Screening Program, with possible hits examined further in cell-based assays, but no clear effects were demonstrated.

Further efforts to identify the target of conolidine included testing against various G protein-coupled receptors (GPCRs) including orphan GPCRs, a Cerep screen, and a kinase panel screen (the last two performed in collaboration with Pfizer), but no effects were identified. Conolidine has also been tested in a variety of cell lines of neuronal lineage and kinase assays, and no clear positive effects were demonstrated. An effect was seen on N-type calcium channels, but the quantity used was so large that the effect may not have been specific. It's possible to displace conolidine, but *in vitro* studies of the N-methyl-D-aspartate (NMDA) receptor (multiple subunit combinations) revealed no significant effects.

Conolidine has favorable properties: it enters the brain, is orally available, has very little affinity for *p*-glycoprotein, does not block hepatic P450 enzymes, is not cytotoxic, is

soluble to 180  $\mu\text{m}$ , and appears to have a stable shelf life. However, its mechanism of action remains unknown.

**Discussion:** There has not yet been an opportunity for further *in vivo* testing of conolidine. Currently, the supply of the compound has been completely used up. Another synthesis will be necessary. The effect of conolidine on mitochondrial function (which may influence the transition from acute to chronic pain) has not yet been examined. No active metabolite of conolidine has been identified.

## Cannabinoids and Glycinergic Modulation of Pain

Dr. Yan Xu

Cannabis has been used for medical purposes for about 2,000 years. In China 1,800 years ago, it was combined with alcohol as a general anesthetic for surgery.

Components of cannabis include its major psychoactive component, tetrahydrocannabinol (THC), as well as the nonpsychoactive cannabidiol (CBD). Both bind to a specific glycine receptor (GlyR), especially its  $\alpha 3$  subtype, blocking the passage of inflammation from the periphery to the spinal cord and brain. Thus, the perception of pain is stopped. In  $\alpha 3$  knockout mice, the analgesic effects of CBD are greatly reduced. The GlyR is not involved in the psychoactive effect of cannabinoids.

GlyRs are pentameric ligand-gated ion channels (pLGICs). These channels include an extracellular domain (ECD) and a transmembrane domain (TMD). A ligand binds to the ECD and opens the channel. Compounds that modulate the resting or open states may have value as analgesics. THC has been shown to enhance the function of both the  $\alpha 1$  and  $\alpha 3$  GlyRs. NMR, mutagenesis, and functional studies show that THC acts on GlyRs via binding to the S296 site in the TMD. Point mutations have confirmed that S296 is the critical residue for both THC and CBD binding.

Efforts have been made to search for other compounds that act on the same GlyR site as THC. Several FDA-approved drugs and novel compounds have been identified that produce greater potentiation of GlyRs than THC does. One such compound, called compound 8, which is selective for  $\alpha 3$  GlyRs rather than both  $\alpha 1$  and  $\alpha 3$ , has been shown to relieve inflammatory pain *in vivo* and is far more potent than morphine.

Dr. Xu described an alternative approach to drug discovery that involves providing the body with potential targets rather than seeking druglike molecules to match existing receptors. In this approach, modifications are made to the receptor so that it will respond to molecules from natural products or their metabolites. This approach focuses on the root of the problem by engineering a receptor in the peripheral nerves. If the artificial receptor is placed in the DRG, it will block the inflammation from going through the central nervous system. This chemogenetic receptor engineering would involve installing highly chloride-selective channels that will not respond to endogenous neurotransmitters or interfere with endogenous GABAergic and glycinergic synaptic transmission but can be activated by small nontoxic molecules, including food metabolites that would otherwise have no analgesic effects.

An example of structure-based receptor design performed as a proof-of-concept involved taking the *Erwinia* ligand-gated ion channel (ELIC), a bacterial pLGIC, and merging it with a GlyR in such a way that the ECD is from ELIC and the TMD is from the GlyR. The modified channel is highly chloride selective. The ligand binding site was modified to increase its selectivity for propylamine. Behavioral tests have shown profound analgesia from these engineered channels in response to propylamine, which

otherwise would not have analgesic effects. The use of this type of approach could make it possible to take advantage of the pathways of the analgesic effects of cannabis while eliminating psychoactive potential.

**Discussion:** A representative of the American Cannabis Nurses Association mentioned the availability of information on the clinical use of cannabis, including dosing, on the organization's website at <https://cannabisnurses.org>.

Cannabinoids bind primarily to the classical CB1 and CB2 receptors. The analgesic effect of cannabinoids is probably independent of CB1 and CB2, although there may be cross-talk between these receptors and the GlyR.



## Nicotinamide Riboside, a Vitamin B3 Precursor of NAD<sup>+</sup>, and Persistent Pain

Dr. Donna Hammond

Nicotinamide riboside (NR) is a vitamin B isoform that is also a precursor of nicotinamide adenine dinucleotide (NAD<sup>+</sup>). It has been known for decades that NR is present in both bacteria and eukaryotes. In eukaryotes, it enters the salvage pathway for synthesis of NAD<sup>+</sup> through the action of the enzyme NR kinase. NR is found in foods such as milk, whey, and brewer's yeast. It is sold as a dietary supplement.

Supplementing the diets of diabetic mice with NR prevents some complications associated with low NAD<sup>+</sup> levels, including tactile hypersensitivity. This is believed to occur as a result of increases in NAD<sup>+</sup>. NAD<sup>+</sup> is a critical coenzyme in many cellular functions, including axonal transport, cell viability, and bioenergetics. Agents that increase NAD<sup>+</sup> protect against nerve injury and mitochondrial dysfunction both *in vitro* and *in vivo* and may also protect against mitochondrial dysfunction in muscle.

Studies have been conducted in Dr. Hammond's laboratory to see whether NR can alleviate peripheral neuropathy induced by the cancer chemotherapy drug paclitaxel. This side effect is experienced by 60 to 70 percent of patients taking the drug and persists in 30 percent of patients after chemotherapy ends. It is so severe that it can hinder continuation of treatment. There are no evidence-based treatments for this problem.

Experiments were performed to determine whether pretreatment with NR could prevent or treat paclitaxel-induced neuropathy in tumor-naïve female rats. Pretreatment and continuous treatment prevented the tactile hypersensitivity associated with paclitaxel-induced peripheral neuropathy, and behavioral testing showed that animals treated with NR did not have an increase in pain behavior. In rats with mammary tumors, NR blunted tactile hypersensitivity and cold hypersensitivity (another symptom of paclitaxel-induced neuropathy).

There are multiple hypotheses—probably not mutually exclusive—about what is responsible for this neuropathy. Loss of intraepidermal nerve fibers (IENF) may play a role. In tumor-bearing animals, prophylactic NR prevents loss of IENF. Further testing showed that the effects of NR were not due to a pharmacodynamic interaction with paclitaxel and that NR did not prevent the antitumor effects of paclitaxel or potentiate tumor growth.

It is uncertain whether the neuroprotective action of NR is related to increases in NAD<sup>+</sup>, or whether increases in the intermediate nicotinamide mononucleotide (NMN) are responsible. Recent research has brought these two hypotheses closer together by demonstrating a mechanism involving both compounds. Axonal injury leads to a loss of the unstable enzyme nicotinamide nucleotide adenylyltransferase 2 (NMNAT2), which

causes accumulation of NMN. NMN activates sterile alpha and Toll/interleukin receptor motif-containing protein 1 (SARM1), which decreases levels of NAD<sup>+</sup>. It's possible that NR-induced increases in NAD<sup>+</sup> may compensate for SARM1-induced degradation. Knockout animals are currently being created to further investigate the pathway and to address other unanswered questions about NR, and an IND has been approved for an NCI-approved pilot phase II trial of NR in metastatic breast cancer patients.

**Discussion:** NR is available as the dietary supplement Niagen<sup>®</sup>. Its manufacturer has conducted pharmacokinetic and safety studies and funded a study to confirm that NR does not interfere with P450 enzymes. This evidence was used to support the IND. A dose of 1 g/day has been shown to be safe and will be used in the clinical trial. Clinical research has been approved only in patients with metastatic cancer, not in those with potentially curable cancer. Patients could treat themselves with NR outside of a clinical trial, but the cost is prohibitive.

## Session Three Panel Discussion

Dr. Julius announced the National Center for Advancing Translational Sciences (NCATS) A Specialized Platform for Innovative Research Exploration (ASPIRE)—a design challenge for drug discovery innovation in pain and opioid use disorder and overdose. This is in the discovery space, and it offers cash prizes. Further information is available at <https://ncats.nih.gov/aspire/challenges>.

Dr. Chen announced that there is a current Request For Applications (RFA) from NCCIH on the mechanisms of action of minor cannabinoids and terpenes.

Dr. Philip Sanderson, chair of this session, asked the panelists to comment on barriers to further translation. Dr. Iadarola said that in the case of the drug he is studying, an omnibus clearance from the FDA to administer it in a variety of body compartments would accelerate research. In response to a question, he clarified that the drug is still isolated from plants. Dr. Hammond commented that the lack of side effects could hamper development of conolidine because the FDA needs to establish a dose with side effects. She suggested that NIH might want to support further research on conolidine because conolidine relieves pain and is not an opioid. Dr. Bohn pointed out that scale has been a problem in conolidine research because a large supply of the compound has never been available. More NIH support for synthesis of compounds like conolidine would be helpful.

The NIH Blueprint Therapeutics Network provides support for some types of research on drug discovery. Dr. Xu suggested that expansion of this program would be valuable. The National Institute of Neurological Disorders and Stroke (NINDS) supports all phases of research in the drug discovery space.

Dr. Chen suggested that conolidine would be a good candidate for target-blind screening. Dr. Cohn said that ion channel screening has not been performed on conolidine. Pfizer was unable to take conolidine research further because of the lack of a target.

NCATS is developing a screening platform as part of the NIH Helping to End Addiction Long-term (HEAL) Initiative. They are supporting translational therapeutics development, assay development, new target identification, and *in vitro* microphysiological models.

Intra-articular capsaicin has been evaluated for arthritis pain, but there were problems with injection pain as well as variable effects. Capsaicin does not hold the channel open the way RTX does, so it must be given in large doses, and those doses cause a lot of pain. RTX causes less pain on injection, probably because smaller doses are used. Thermoregulatory effects are not a problem for RTX because it is given by local injection. Some compounds that act as antagonists to TRPV1 did not proceed far in development because of adverse effects on thermoregulation as well as lack of efficacy. However, RTX is an agonist, not an antagonist.

Phantom limb pain involves the DRG. When the DRG is anesthetized, the pain disappears. If TRPV1-positive cells are involved, RTX instilled in a para-ganglionic fashion might be helpful. In animal research on phantom pain, operant-type tests are needed for assessment. Department of Veterans Affairs funding might be available for this work.

Pain drug development may need to be divided into two branches—peripheral and central. Peripheral drugs may have the advantage of lesser likelihood of side effects in the brain. Receptor therapies, as discussed by Dr. Xu, could be used in a peripheral nerve to “calm it down.”

More attention needs to be paid to cognitive control over pain; this is not currently being addressed in animal studies. Collaborating with clinical psychologists will be critical going forward.

NIH recently sponsored a workshop on animal models for testing of pain therapeutics, including devices, biologics, and other types of therapies as well as small molecules. One of the conclusions was that behavioral outcomes should only be secondary measures in animal models. In the context of chronic pain, it was concluded that therapeutics would need to penetrate the central nervous system because this is probably where the pain is being maintained. However, not everyone agrees with this concept. Some therapeutics, such as RTX, can be used either peripherally or centrally, and they may have value for some types of chronic pain when administered peripherally. Because of central-peripheral interactions, it may not be possible to completely separate these two types of effects.

In response to a question about extraction of compounds from cannabis, Dr. Xu said that his lab works with pure compounds and does not perform extractions.

# Session Four

## The Science of Microbial-Derived Natural Products

### The Science of Bacteria-Derived Natural Products—Botulinum Toxins

Dr. Philippe Picaut

Botulinum toxin is composed of a 100-kDa heavy chain (HC) and a 50-kDa light chain (LC) joined by a disulfide bond. The HC includes a binding domain and a translocation domain, and the LC is a proteolytic domain, with a zinc endopeptidase responsible for the catalytic activity of the toxin. The mechanism of action of botulinum toxin A on motoneurons involves internalization of the whole toxin followed by cleavage to release the LC, which is the active part of the toxin. The LC targets the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins, leading to relaxation of the muscle.

The idea of using botulinum toxin for medical purposes dates back to 1822. Research on therapeutic use for strabismus began in the 1970s, with approvals and licensing in the 1980s and 1990s. AbobotulinumtoxinA (Dysport®) is now approved in more than 85 countries for 7 indications.

Different botulinum toxins have different SNARE substrates and durations of action. In addition to serotype A, which is already used therapeutically, serotype E and modified toxins may also have potential therapeutic uses.

Purification and gene manipulation of botulinum toxin from clostridial cultures is difficult and complex. The toxin can be manufactured more easily using standard *E. coli* expression systems to generate recombinant toxin (rBoNT). Recombinant expression also enables specific modifications to be incorporated into the toxin and the creation of hybrid toxins. For example, the HC binding domain has been replaced so that the toxin can target new cell types, enabling it to be used against conditions other than neuromuscular diseases.

Important characteristics of botulinum toxin include its targeted efficacy after focal injection, with limited and infrequent spread of the toxin effect; the small amount of protein (picogram quantities) injected; and the 3- to 9-month duration of efficacy (with the duration varying in different tissues). Conditions in which Dysport® has been used include stroke, traumatic brain injury, multiple sclerosis, and cervical dystonia. The drug can be used in pediatric patients for conditions such as spasticity. In cervical dystonia, the drug relieves pain as well as correcting abnormal postures, and an

improvement in pain can be seen without muscle relaxation. Physicians have tried botulinum toxin for a variety of painful conditions, such as postsurgical neuralgia, migraine, trigeminal neuralgia, peripheral nerve injury, postherpetic neuralgia, diabetic neuropathy, chronic low-back pain, myofascial pain, refractory shoulder joint pain, and painful knee arthritis. However, more needs to be learned about its mechanism of action, and rigorous clinical trials need to be conducted for these uses.

Botulinum toxin may relieve pain by causing a block of substance P and calcitonin gene-related peptide (CGRP) within the central nervous system, leading to reduction of central sensitization. It may also cause peripheral decreases of substance P, CGRP, glutamate, and TRPV receptor translocation, leading to a block of peripheral sensitization. Dr. Picaut's company is conducting translational research to clarify the mechanism of action of botulinum toxin against pain, identify the types of pain the drug could be used for, and determine the best conditions for its use in terms of method of administration, timing of administration, and interaction with other analgesics. One line of research involves the use of the toxin as a local anesthetic during postoperative wound healing in pigs. Variables being examined include dosing, timing of administration, depth of injection, and synergy with morphine or a nerve block. Studies are also being performed in acute and chronic pain models in humans. Future areas of research include developing indications and defining outcome measures, synergy with local anesthetics and opioid sparing, and external collaborations to help patients get access to novel treatments.

**Discussion:** Dr. Langevin commented that for postsurgical scar pain, dry needling can be very effective. Dr. Picaut explained that the studies of botulinum toxin injection for this type of pain included saline injection controls but not dry needling controls. Thirty-gauge needles were used.

Some transport of peripherally injected toxin to the central nervous system has been demonstrated in rats. Data have not yet been obtained on humans. Because of the size of the toxin, it cannot cross the blood-brain barrier.

Postoperative pain may involve the muscles as well as the skin. Some research on botulinum toxin for postoperative pain has investigated its use in muscles.

Research is under way on modified toxins that may target specific types of fibers.

Decisions about the indications for botulinum toxin for treating pain will need to await the results of studies currently in progress.

## Bacterial Toxins: Anthrax Toxin as a Molecular Platform To Target Pain

Dr. Isaac Chiu

Dr. Chiu's laboratory focuses on bacteria-neuron interactions, both in pain and in host defense. Sensory neurons and nociceptors interface constantly with the environment, including bacterial pathogens and microbiota. There are direct mechanisms by which neurons can sense bacterial products, and both neurons and bacteria interact with the immune system.

In most instances, pain is one of the leading symptoms of infection. However, infections with the anthrax bacterium, *Bacillus anthracis*, are painless.

The molecular mechanisms by which bacterial toxins and molecules directly act on nociceptor neurons are beginning to be identified. For example, lipopolysaccharides (LPS) from *E. coli* act on TRPA1 and TLR4, flagellin from bacteria interacts with TLR5, the *Staphylococcus aureus* toxin  $\alpha$ -hemolysin can directly form pores in membranes of neurons, and N-formylated peptides from *S. aureus* can interact with the formyl peptide receptor. The painless bacterium *Mycobacterium ulcerans* releases a mycolactone that hyperpolarizes neurons through the angiotensin 2 receptor. Dr. Chiu's group recently found that *Streptococcus pyogenes*, which causes painful skin infections, secretes Streptolysin S, which produces pain and silences immunity. In this instance, the pain benefits the bacterium because the release of CGRP silences neutrophil function, promoting bacterial survival.

Anthrax is caused by the Gram-positive spore-forming bacterium *Bacillus anthracis*. In humans, anthrax can cause severe gastrointestinal, cutaneous, or pulmonary infections. Anthrax skin infections are characterized by black, painless lesions. The molecular mechanism of the painlessness is unknown. The anthrax bacterium secretes edema factor (EF), protective antigen (PA), and lethal factor (LF). PA is the receptor-binding part of the toxin. It delivers either EF or LF into cells, where the toxins induce enzyme activities. Edema toxin (ET) is made up of EF and PA. Lethal toxin (LT) is made up of LF and PA. The main receptor for binding of anthrax toxin is anthrax binding receptor 2 (ANTXR2). Nociceptors are highly enriched in ANTXR2. ANTXR2 is enriched in the DRG but is not present in the spinal cord or brain. Two major research questions follow from these observations: Do native anthrax toxins modulate nociceptor neurons and pain? Can chimeric anthrax toxins be engineered to deliver molecular cargoes, such as botulinum toxin, into nociceptor neurons to silence pain?

In mice, injected anthrax ET induces potent analgesic effects, as shown by behavioral assays. ET also blocks phase 2 of formalin-induced pain, improves neuropathic pain in the spared nerve injury model, and blocks pain in the hot plate and carrageenan models. ET specifically causes cyclic adenosine monophosphate (cAMP) induction in the DRG but not in the spinal cord; this induction may be involved in the mechanism of action.

Botulinum toxin–anthrax toxin chimeras have been created in collaboration with Ipsen Pharmaceuticals. In these chimeras, anthrax toxin serves as a delivery mechanism for the botulinum toxin “cargo.” The concept involves combining the specificity of the anthrax toxin with the ability of botulinum toxin to target neural signaling in nociceptors. In one strategy, the PA domain that binds to the receptor is bound to the catalytic and translocation domains of botulinum neurotoxin. In another, PA and the portion of LF that binds to it are combined with the catalytic domain of botulinum toxin. Initial data show that the chimeras cleave synaptosome associated protein 25 (SNAP-25) and inhibit CGRP release in DRG cultures and can inhibit pain in the formalin model after three injections.

Future research directions include determining whether anthrax toxins or proteins engineered from the toxins can be used as a molecular platform to target chronic pain, elucidating the molecular mechanisms by which bacterial toxins such as ET silence pain, and exploring the engineering of bacterial toxins to target pain as a general potential strategy.

**Discussion:** The rationale for using anthrax toxin as a delivery vehicle for botulinum toxin rather than using native anthrax toxin is that native anthrax toxin is more dangerous and acts on a variety of cell types. Combining the receptor specificity of anthrax toxin with cargoes that are very specific for neurons is safer.

ANTXR2 is enriched in Na<sub>v</sub>1.8-expressing neurons as well as in TRPV1-expressing neurons that are Na<sub>v</sub>1.8 negative. Human DRG has not yet been tested.



## Urinary Bacterial Pain Phenotypes: Mechanisms and Therapeutic Potential

Dr. David Klumpp

Urinary tract infections (UTIs) caused by uropathogenic *E. coli* (UPEC) are symptomatic and are associated with a robust inflammatory response. There's also a condition involving *E. coli*, primarily in elderly people, called asymptomatic bacteriuria (ASB). ASB is characterized by heavy bacterial burdens in the bladder and an inflammatory response, but there is no symptomatic response. Dr. Klumpp and his colleagues have explored the basis for this symptomatic difference.

In animal experiments, a UPEC strain induced acute pelvic allodynia but an ASB strain did not. Thus, mice mimic human responses. No correlation was found between inflammation and pain in mice colonized with these bacteria. Virulence factors, such as FimH, that are found on UPEC are not expressed in ASB strains. However, FimH is not essential for causing pain even though it drives much of the pathogenesis of UPEC. Purified LPS from the UPEC strain induces pain, but LPS from the ASB strain does not. The pain response is dependent on TLR4 but independent of inflammation. LPS drives both pain and inflammation, but the two responses appear to be separate.

Dr. Klumpp's lab also studies chronic pain that may follow acute UTI. Many women with chronic pelvic pain have a history of UTIs. However, it has not been possible to induce chronic pain in mice by repeatedly infecting them with the UPEC strain. In contrast, a mutant strain with a defect in LPS (a lack of O antigen ligase) did induce increasing pain with repeated exposures. The role of O antigen in modulating this response was verified using a bacterial strain that lacks this antigen. All of these bacteria are rapidly cleared from the bladder, so effects are not due to persistent colonization. TLR4 mediates post-UTI chronic pain but not hematopoietic pain.

There are more questions than answers about what mediates this response on the host side, but there's evidence that TRPV1 is involved in the establishment but not the maintenance of this kind of chronic pain. Experiments with DRGs in transgenic reporter mice show that CCR2 is upregulated even by bacterial strains that do not produce pain. In experiments using transgenic mice with DREADD (Designer Receptors Exclusively Activated by Designer Drugs), Na<sub>v</sub>1.8 nociceptors have been shown to mediate established chronic pain. Thus, post-UTI chronic pain appears to involve a three-receptor cascade that includes TLR4, TRPV1, and CCR2.

Further investigations in mice indicated that the LPS from the nonpathogenic ASB strain blocked UPEC pain. So this strain may not be completely neutral and may even have analgesic activity. The ASB strain, administered whole by direct instillation into the bladder, also relieved pain. Screening of a panel of other ASB isolates showed that most had an analgesic effect against acute UTI pain, but the strength of the effect differed among strains. ASB bacteria could also reduce chronic pain over a period of

weeks. O antigen was essential for the analgesic effect. Efforts are being made to interrogate O antigen analgesia genetically by making targeted deletions and testing them for an effect on pain. Some deletions reduce analgesic activity, but results with one mutant suggest that it may also be possible to enhance the analgesic activity through certain deletions.

ASB bacteria have been used clinically for UTIs, based on a hypothesis of “bacterial interference.” Dr. Klumpp’s group has collaborated with the researchers who proposed this, looking at canine UTIs, and they found that some animals had microbiologic cures but others became asymptomatic while still colonized with a pathogenic strain, perhaps indicating that they were benefiting from an analgesic activity of the ASB strain.

**Discussion:** Itch is a major symptom of UTIs. Effects of ASB bacteria on itch have not yet been quantified but could be studied in a histamine model.

In response to a question about whether the analgesic effect was mediated by competition and about the lack of effect of the antibiotic ciprofloxacin on pain in UTIs, Dr. Klumpp said that the antibiotic merely clears out the bacteria but does not affect the pain stimulus, and that the analgesic effect of ASB bacteria does not appear to be due to competition because effects can be seen in chronic pain, where pathogenic bacteria are no longer present. Benefits begin to be seen within a day, so they do not appear to be due to an adaptive response. Whether the effect is mediated by the nervous system, the immune system, or both has not yet been established.

## Session Four Panel Discussion

In response to a question, Dr. Klumpp said that the hypothesis put forth by the group studying bacterial interference is that the ASB strain may outcompete the pathogenic strain. However, benefits are also seen in mice that have chronic pain induced by a prior UTI and that no longer have detectable bacteria in the bladder. Work has been done on the phylogenetics of these ASB *E. coli* strains, and they have been found to be “dumbed down” pathogens that still have some virulence genes but do not express them. They may reflect a developmental shift in the normal urinary microbiome, where there is a low-level diverse microbiome throughout most of life, with a shift to a protective function of ASB strain colonization late in life. ASB bacteria may be a potential treatment for human interstitial cystitis. It may be possible to evaluate their use for this condition in cats, which develop a similar condition. Difficulties in obtaining a Good Manufacturing Practices (GMP)-grade *E. coli* at a reasonable price are a barrier to clinical trials.

Chronic itch may be very comparable to chronic pain. Staphylococcal delta toxin may play a role in itch. Dr. Chiu said that his lab is working on whether *S. aureus* contributes to itch, for example in atopic dermatitis. Ninety-five percent of lesions in this condition have staph. Delta toxins as well as staphylococcal enterotoxin may induce an inflammatory response that contributes to itch, or itch fibers may respond directly to these toxins. It would be interesting to know whether the skin microbiome regulates innervation.

Inflammation and pain are not always correlated in the bladder. Inflammatory markers may be present without pain symptoms. In the skin, inflammation typically manifests as itch.

In subcutaneous infections, pain correlates with increases in bacteria but not with tissue swelling. This suggests that the source of the pain is the bacteria themselves. Inflammation and pain appear to be coupled in some situations but not others. In urology, there are multiple examples of inflammation without pain.

In response to a question from Dr. Chen about gaps in knowledge, Dr. Picaut said that one of the most important priorities for botulinum toxin is learning more about its mechanism of action. Additional basic research is needed and will help in pinpointing clinical indications where patients can benefit.

Studying fungi and viruses may yield important knowledge; not all microbial research needs to focus on bacteria. Viruses, such as those involved in cutaneous viral infections that cause itch or herpes viruses that cause pain, may have evolved to exploit the nervous system.

It would be helpful to have a repository of bacterial products, including bacterial strains themselves or supernatants or extracts from bacteria. BEI Resources has some bacterial toxins, and it might be possible to deposit strains there.

Bacterial toxins may be highly potent analgesics but also have significant potential for side effects. Using probiotics may be one way to avoid toxicity. There has not yet been a comprehensive screen to determine how many bacterial toxins have analgesic properties. However, it is known that analgesic properties are not limited to Gram-negative bacteria. A range of staph strains that cause acute or chronic pain or are benign or even analgesic has been recognized.

Figuring out the path forward for research on bacterial toxins and pain is difficult. TLR4 appears to play a fundamental role in some types of pain, but its role in analgesia is uncertain. Bacterial products seem to be intertwined with human physiology in a way that plant and animal products are not. Transcriptional data and functional assays can be combined to identify relevant receptors. The microbiota needs to be considered as a community, so there will be some bacteria that are analgesic and others that are proalgesic. Identifying the relevant organisms and their molecular mechanisms could lead to new strategies to treat pain. Anecdotally, some patients with visceral pain think that the microbiota is important and that they feel an impact when they try to make adjustments to it.

The anthrax chimeras are expected to be more sensory specific than botulinum toxin itself, so it is hoped that side effects such as paralysis can be avoided. Maximum tolerated dose (MTD) studies of the chimeras will be performed. Anthrax toxin used in this way need not be unsafe, especially when combined with botulinum toxin, the safety of which is well established. Nevertheless, the safety profile of all molecules that may be used clinically needs to be characterized, and moving from animal models to humans can be difficult.

The microbiota in the gut have an impact on the brain. Signals from the periphery can affect the central nervous system through this mechanism. Some types of pain, such as visceral pain in irritable bowel syndrome, are almost certainly affected by the microbiota. There may be multiple mechanisms by which the microbiota signal the brain, and some are sex dependent.

Some toxin conjugates have been taken to clinical trials, with varied results.

# Concluding Session

## Discussion and Future Directions

Points made in the general discussion included the following:

- One of the major themes of the day is the potential value of repositories. However, the mechanics and funding of such resources would need to be worked out.
- Bacteria may exert their effects not just by producing small molecules but also by exerting an influence on the colony of microorganisms that is present. Such actions, in combination with the physiology of the individual, could suppress pain.
- The experience of pain may not be based on interactions with a single receptor. Lackluster results with some pain therapeutics may reflect the complexity of pain.
- Collaborations across multiple disciplines, including those between chemists and biologists, are key to the development of better pain therapies, especially those derived from natural products. A lack of access to expertise in multiple fields is often a stumbling block for research. NIH can work to promote collaborations.
- Identifying new leads is an important priority for research on natural products and pain. Exploratory searching is part of this process. However, until a target and an assay are identified, progress will be limited (as in the case of conolidine). Collaboration can be important in getting from a compound of interest to a potential mechanism of action.
- NCCIH isn't the only agency funding research on natural products and pain. NINDS, the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), and others are also involved. The research community needs to be aware that funds are available for this type of work.
- NIH used to have International Cooperative Biodiversity Groups (ICBG) grants that supported the exploration and discovery of novel compounds and natural extracts with potential for development as therapeutic agents. That kind of mechanism could be very useful because it includes exploration, some of the necessary policy work, and sharing with the community. Some countries are closing their borders to drug discovery research because they have not profited from past successful efforts.

- Currently, field expeditions are funded by industry, discretionary funds, or private foundations. NIH grant reviewers are reluctant to fund this type of research. Support for natural history collections is also lacking. There is a need to recapture the spirit of exploration, and it needs to happen soon because of the high rate of loss of biodiversity. Work funded by foundations will not fill the gap in discovery research because these organizations have narrow interests and may not necessarily share their discoveries with the rest of the research community.
- There is a need to explore the cell biology of natural products by growing them in culture and biobanking them.
- Molecules may not have a single target. Many are polypharmacologic. Therefore, studying a single target in a model system may not be sufficient. New technologies are needed to facilitate investigation of all the activities of a molecule in neuronal cells. The Brain Research Through Advancing Innovative Neurotechnologies (BRAIN) initiative is working toward this goal, and for pain specifically, NCATS is sponsoring work of this type. In vitro models of human circuitry are being developed.
- Requirements for nondisclosure can be a barrier to progress at the clinical level.
- As illustrated by several of the presentations today, a natural product may be a starting point for the development of a therapeutic through engineering, rather than a therapeutic itself. This is true for products of plants, animals, and microorganisms. This type of engineering and development work easily fits into the structure of traditional NIH funding mechanisms.
- Some discovery research has been based on traditional medical uses of plants. Questions about whether a particular folk medicine is efficacious or acts as a placebo can only be answered by collecting specimens and investigating them. Traditional remedies from plants are prepared and dosed in very specific ways, and there is some evidence behind these approaches, although it may not meet the standards of today's science. Validation of a traditional therapy can be valuable even if the mechanism of action is not fully understood. Some folk medicines may act on the host rather than the causative agent of a disease. Existing collections of organisms or folk remedies from other countries may be a source of therapeutic agents, but obtaining funding to study them is difficult.
- Because pain is a normal and necessary protective response, there may be situations where blocking pain could be damaging. For example, patients might overuse a damaged joint if the pain from that joint is blocked. The acute warning signal of pain needs to be preserved even when pathological or chronic pain is blocked.

- Studying the toxicologies of mixtures is challenging. The mixture needs to be carefully defined. It's easier to define the risks and manufacturing constraints of a single substance, but the use of mixtures should not be ruled out.
- It's uncertain whether it's best to investigate the actions of a natural product first and isolate the active components later or whether identification of the active components should occur before investigations of mechanisms. It may be best to pursue both approaches. Synergy may exist in a natural product, but it should not be considered an excuse for not studying the mechanisms of individual components of a mixture. It's important to recognize that when traditional therapeutics are assessed in rigorous clinical trials, almost all of them fail. This may occur because the science behind them is not well enough understood to allow the design of a suitable trial in appropriate patients.
- Until the last century, all drugs were natural mixtures. The FDA has a mechanism for approving such mixtures, but few drugs have been approved in this way. Many complex mixtures are marketed as consumer products rather than drugs. NCCIH is interested in supporting basic research on how natural products affect pain pathways. Some of the substances studied may eventually be marketed as drugs, but this is not the agency's focus.
- Lower potency compounds are not good starting points for medicinal chemistry because they tend to yield only additional lower potency compounds.
- The approach for target-specific screening for pain treatments is reasonably well established, although more public-private partnerships would be helpful. However, target-blind screening is not as well established. There may not be enough assays to probe all the possibilities. A library of assays could be useful.
- Mechanisms of action are key. Without knowing how things work, research can't improve on them. Nevertheless, phenotypic screens are important because they act as a funnel. Using them, research can start out broadly and then quickly narrow down the actions of a substance.
- Advances in omics tools have improved methods of natural products discovery and helped to make this work cost effective.
- The use of artemisinin in a form similar to a tea illustrates both the potential and the difficulty in using complex natural products as therapeutics. There are anecdotal reports that the tea-like extract is more effective than pure artemisinin, perhaps because of the presence of structural analogs in the mixture. However, it is difficult to determine whether a particular batch of a particular preparation is effective. Development of an authenticated, standardized complex product may be a solution to this problem.

- Structural modeling usually comes after chemistry, although it may not always be presented in that way in the literature. This method may explain activities after the fact rather than predicting them.