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Natural Products and Ion Channel Pharmacology

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Abstract

An accelerated rate of natural-product discovery is critical for the future of ion channel pharmacology. For the full potential of natural products to be realized, an interdisciplinary initiative is required that combines chemical ecology and ion channel physiology. A prime source of future drug leads targeted to ion channels is the vast assortment of compounds that mediate biotic interactions in the marine environment. Many animals have evolved a chemical strategy to change the behavior of their prey, predators or competitors, which appears to require a large set of ion-channel targeted compounds acting in concert. Some of these compounds (e.g. Ziconotide (Prialt)) have already found important biomedical applications. The elucidation of molecular mechanisms mediating biotic interactions should yield a rich stream of potent and selective natural products for the drug pipeline.

Natural product discovery is critical for rapid progress in ion channel pharmacology

An examination of the present state of drug discovery and development reveals a crisis situation in the pharmaceutical industry, with high attrition rates for candidate compounds. We discuss why natural products may be more successful drug leads than small molecule candidates derived from random chemical libraries. In order to provide a sufficient stream of drug leads for the pharmaceutical industry, we present the case that natural products represent a mostly untapped resource, but that a paradigm shift will be required in the discovery strategy. These considerations are particularly important for drugs targeted to ion channels. Input from scientific disciplines that are presently completely disconnected from ion channel pharmacology, such as chemical ecology, organismal biology and molecular phylogenetics will be needed to optimize discovery.

Productivity crisis in the pharmaceutical industry

The lack of productivity in research and development (R&D) is widely cited as the greatest problem facing the pharmaceutical industry today [1,2,3]. The industry has increased R&D spending at an average annual rate of 12.3% since 1970. In aggregate, R&D spending increased over 80 fold between 1970 and 2008 [4]. However, the dramatic increase in R&D spending has not resulted in a commensurate increase in new therapeutic drugs. In fact, the number of new drugs approved annually by the United States Food and Drug Administration (FDA) has declined since the mid-1990s to levels seen in the 1970s and 1980s, when R&D spending was much lower [3]. One metric of R&D productivity in the pharmaceutical

industry is total R&D cost per new molecular entity (NME) approved, where NME is defined as a novel drug not previously approved for marketing in any form or for any other indication. As a consequence of dramatically higher R&D spending and lower NME approvals, the cost per NME is approaching \$2 billion, threatening the viability of the pharmaceutical industry [1,2,3,4]. Furthermore, pharmaceutical companies are facing a “patent cliff,” where they are expected to lose \$140 billion in annual sales between 2007 and 2016 from patent expirations, with few potential blockbuster drugs in the pipeline to replace them [5].

High pre-clinical and clinical attrition rates for candidate compounds

It is perplexing that productivity has declined in the pharmaceutical industry, especially when one considers that this has happened concurrent with revolutionary advances in science. It has become progressively easier to identify and validate drug targets. But it still remains a significant challenge to find compounds with the selectivity, potency and pharmacological properties of a viable therapeutic drug candidate, as demonstrated by the high attrition rate of compounds both in clinical and pre-clinical testing. One study indicated that only 11% of drugs successfully progress from first-in-man to FDA approval (89% attrition rate), while other industry experts have claimed that the attrition rate is actually greater than 90% [2]. Clinical safety and toxicology account for approximately 30% of attrition in the clinic [2], suggesting that in many cases failures in the clinic are due to a lack of selectivity for the drug's target. A lack of selectivity is also a major problem in pre-clinical attrition. Even after extensive *in vitro* testing of a promising compound, when applied to animal models, undesirable off-target effects are often discovered, making a candidate compound unsuitable as a therapeutic drug. Although polypharmacology (one drug targeting multiple proteins in concert) has been proposed as a potentially new paradigm in drug discovery, it is still essential for any drug to hit therapeutically useful targets and to avoid interacting with all other proteins that may produce harmful off-target effects [6]. Such off-target activity has contributed significantly to the high pre-clinical and clinical attrition rate of candidate molecules.

Targeting selectivity of natural products vs. random small molecules

An underappreciated fact that may account for much of the off-target activity of failed compounds is that most drug-discovery projects now begin with combinatorial libraries of random small molecules, rather than natural products: the historical source of pharmaceutical drugs (e.g. penicillin, paclitaxel, opiates, etc.). Over the past two decades, concurrent with the productivity decline in the pharmaceutical industry, the major strategy adopted by the pharmaceutical industry to find starting points for medicinal chemistry has been high-throughput screening of combinatorial libraries. This approach has indeed yielded compounds that hit the molecular targets of interest. However, even after more than two decades of combinatorial chemistry, there is only one *de novo* combinatorial new chemical entity that has been approved by the FDA, and the majority of drugs on the market are still derived from natural sources [7,8,9,10].

While high-throughput screening is a powerful tool for finding compounds from random chemical libraries that interact with the molecular target of interest, one can never screen all

other targets that the compound might also potentially interact with, and most of the compounds in large combinatorial libraries have groups suitable for interacting with sites on proteins that are not unique to a single molecular target. Such molecular scaffolds are not necessarily a good starting point for multiple costly cycles of medicinal-chemistry modifications and functional testing, because untested off-target side effects will likely remain even after medicinal-chemistry optimization.

With respect to targeting selectivity, natural products may have a significant advantage. Over millions of years, nature has explored chemical-diversity space extensively with a vast array of organic/biological scaffolds, and we are probably far from having elucidated a significant fraction of these. It is likely that evolutionary forces select against scaffolds that cause a high degree of non-specific interactions with many biological molecules. In a sense, natural products have been field tested by evolution. Structural scaffolds on which many related compounds are based have been under intense selection, both for targeting selectivity and high potency for many millions of years, and therefore off-target properties have been weeded out. We might think of the evolutionary forces that have shaped natural products as being similar to millions of successive rounds of medicinal-chemistry cycles and clinical trials. In contrast to the evolutionary refinement of natural products for potency and selectivity, even a very large combinatorial library is woefully inadequate at exploring chemical-diversity space. Estimates for the possible number of unique organic molecules of reasonable molecular weight (drug like) that could be produced range from 10^{13} to 10^{180} , while the number of known compounds is on the order of 10^8 [11]. One estimate of the potential number of chemical structures that could be produced with a molecular weight less than 500, stable in oxygen and water at room temp, and composed of only H, C, N, O, P, S, F, Cl and Br is 10^{63} [11,12]. Thus, even a very large combinatorial library (e.g. 10^6 molecules) barely scratches the surface of chemical diversity space, whereas natural products have had eons to explore chemical diversity space to maximize potency and selectivity. Therefore, a natural product found to functionally alter a desirable molecular target for a therapeutic application would be less likely to have off-target complications than a compound from a combinatorial library.

The discussion above is a general one that applies widely to all pharmacological agents, and all molecular targets. However, these considerations are especially important for ion channel pharmacology. Functional ion channel complexes can be grouped into superfamilies that in turn are subdivided into ion-channel families with multiple homologous subunits. In most cases, an individual subunit is then assembled into a tetrameric or pentameric complex; homologs within the same ion channel family can co-assemble to form various heteromeric combinations. This generates an enormous diversity of closely-related ion channel isoforms. The pharmacological agents that are often the most useful are those that can discriminate between the different subtypes in a given ion channel family. Generating the required selectivity to discriminate between closely-related isoforms, together with the necessary potency to make a compound an effective pharmacological agent is a formidable challenge; avoiding other off-target interactions makes this even more difficult. Thus, all of the issues presented above become particularly critical whenever an ion channel is the physiological entity that needs to be targeted.

A concerted discovery strategy for natural products

Although natural products have built-in selectivity advantages, we argue that many of the previous natural-product discovery efforts have been misguided. The bioprospecting approach to natural-products discovery is a chemistry-centric strategy that aims to elucidate the structure of novel bioactive molecules from the natural world [13,14,15]. The historical approach for exploring molecular diversity from plants and animals is to collect a large quantity of tissue (typically 1kg), extract compounds and run tests to determine whether there are any pharmacologically-active components in the extract. A potential problem with this approach is that it only detects compounds that are sufficiently potent, and present at sufficiently high levels, to score on the assay(s) used [13,14]. If one takes a kilo of total animal tissue, many compounds that the animal may have evolved may not be present at sufficiently high concentrations to be detectable.

In contrast to the bioprospecting approach, a focused biology-centric strategy has successfully identified many natural compounds, primarily from venomous animals, that are pharmacologically active on ion channels. The study of venom components was initiated mostly by biologists interested in the physiology of prey capture and defense employed by venomous animals. As a result, it was automatically assumed that the compounds of interest would be synthesized in the venom apparatus of the animals and instead of taking a kilo of a particular snake and extracting all compounds, venom was collected and stored or the venom apparatus was dissected. Thus, the specialized fluid produced or the tissue of the animal that produces the pharmacologically-active compounds serves as the starting material, and not mixed tissue extracts. We suspect that if the standard bioprospecting approach had been taken with spiders or cone snails, and one kilo of total tissue were first extracted, since the venom duct of these animals is only a very small fraction of the total body mass, none of the pharmacologically-active compounds targeted to ion channels would have been detected. Although this seems almost a trivial factor, it is a crucial consideration for the discovery efforts for natural products targeted to ion channels.

The purification of α -bungarotoxin from the venom of the Taiwanese banded krait by Chang and Lee [16] marked the historical inception of ion-channel pharmacology, establishing venom as an important source of pharmacological agents targeted to ion channels. The demonstration that α -bungarotoxin targeted the skeletal muscle subtype of nicotinic receptors was a keystone in molecular neuroscience as well, since the toxin made the purification and definition of the first functional ion channel complex possible, and ultimately led to the characterization of the ligand-gated ion channel superfamily. Since that historic work, a large number of investigators have examined snake venoms for additional components targeted to ion channels.

In more recent decades, the expanding exploration of other animal venoms has led to these becoming sources of novel ion channel targeted pharmacological agents. Cone snails, spiders, scorpions and sea anemones all have provided natural products that have pharmacological effects on different types of ion channels. The range of venom components that target various ion channel subtypes has been steadily expanding, and compounds from venom remain a major resource for future discovery. In fact, recent mass-spectrometry profiling of crude venoms has suggested that the diversity of venom components may have

been substantially underestimated previously; approximately 1000 different masses, representing unique compounds, have been detected in the venom of a single species of spider and cone snail [17,18,19]. With ~700 species of cone snails and 41,000 species of spiders, the potential diversity of venom components in these species alone is enormous. One estimate of the total number of distinct peptides in all venomous animals is 20 million; only a tiny fraction have been discovered and characterized [18].

The pace of venom-peptide discovery historically has been dictated by bioassay-guided purification of venom components, followed by Edman sequencing. Recently cDNA cloning of venom-peptide transcripts [20,21,22,23] and *de novo* sequencing of venom peptides by tandem mass spectrometry is accelerating the pace of discovery substantially [18,24]. In one study employing a novel mass-spectrometry approach, 31 full peptide sequences were obtained from only 7% of the crude venom from a single cone-snail venom duct [25].

Proof-of-principle for natural products targeted to ion channels as drug leads

We have chosen two specific examples of venom peptides to illustrate the more general point that natural products targeted to ion channels can be highly suitable for drug development. The most obvious example is the development of Prialt [26] as an analgesic drug for intractable pain, since this has been formally approved for commercial distribution both in the United States and the European Union. The keystone feature of Prialt as an analgesic is that patients do not develop tolerance, in contrast to opiate drugs such as morphine [27]. Thus, it filled an urgent unmet need. A second example discussed is the sea anemone toxin, ShK and its derivatives, a lead for autoimmune diseases such as multiple sclerosis. This case study also illustrates that natural products targeted to ion channels have important applications outside the nervous system.

In addition to Prialt and Shk, other venom components have been developed as drugs for ion channels and other targets. These include the following: Captopril, a drug that mimics the bradykinin-potentiating peptide from a snake venom (*Bothrops jararaca*) for the treatment of hypertension; Byetta (Exenatide) a synthetic peptide originally found in the saliva of the Gila monster, which targets the glucagon-like peptide receptor for treatment of type 2 diabetes; and TM-601, a modified version of a scorpion-venom (*Leiurus quinquestriatus*) peptide known as chlorotoxin that targets a subtype of small-conductance chloride channel, which is upregulated in glioma cells (a form of brain cancer). TM-601 is currently in clinical trials for the treatment of gliomas. These drugs have been reviewed previously [18]. In addition to Prialt, several conotoxins have reached human clinical trials for the treatment of pain and epilepsy [22].

Prialt: a case study in natural-product selectivity

One success story in utilizing venom components for therapeutic drug discovery is the discovery of a peptide, ω -conotoxin MVIIA (Fig. 1), from the marine cone snail, *Conus magus*. When this peptide was originally purified from venom, it was assayed for biological activity by injection into fish and mice. When injected into the periphery of fish, it caused a

delayed neuromuscular paralysis, which was not evident in mice. However, when it was injected intracranially in mice, it caused shaking [28,29]. These results pointed to a species difference in gene expression that ultimately culminated in a critical insight in natural product drug discovery: although orthologous genes (same gene separated by speciation events in evolution) may be highly conserved structurally across species, they may have a different tissue-specific expression pattern in different species. ω -Conotoxin MVIIA targets very selectively the N-type calcium channel ($\text{Ca}_v2.2$) [28,30,31], which is expressed in the motor neurons of fish (hence the peptide is paralytic to the cone-snail's fish prey) but is primarily expressed in the central nervous system in mammals, and is not the primary Ca channel subtype at the neuromuscular synapse. So, when the peptide is administered to the spinal cord of humans, it produces analgesia, rather than paralysis [26]. This difference in expression pattern allowed the peptide to be developed as a drug for intractable neuropathic pain, which was approved for marketing (as Prialt) by the FDA in 2004 in the United States and in Europe in 2005. One notable aspect of the pre-clinical drug development was that many analogs of the natural peptide were made in the attempt to improve the potency or selectivity, but ultimately the natural peptide, without a single modification, was the molecule that was taken to the clinic [26]. Because Prialt is a peptide, and does not cross the blood-brain barrier, it must be administered intrathecally to be delivered to N-type calcium channels in the spinal cord, which limits its potential market as an analgesic drug. However, before the discovery of Prialt, the N-type calcium channel was not known to mediate pain signaling. Prialt effectively validated the N-type calcium channel as a pain target. Consequently, various pharmaceutical companies are now pursuing the development of small-molecule inhibitors of the N-type calcium channel that may penetrate the blood-brain barrier when administered orally. Notably, other venom peptides that target ion channels in the peripheral nervous system for therapeutic applications should not require intrathecal administration.

In the case of Prialt, an advantageous feature of targeting an ion channel directly was that no tolerance to the drug develops in a clinical setting [27]; in contrast, because morphine and other opiates are agonists of G-protein coupled receptors (GPCRs), the down-regulation that occurs when a GPCR is continuously exposed to agonist limits the efficacy of morphine when pain persists over an extended period of time. This feature of drugs with ion channel targets makes them more attractive for development, particularly for chronic conditions.

ShK peptide: ion-channel drug discovery beyond the nervous system

Since the discovery of ω -conotoxin MVIIA (Prialt) in the 1980s, ion channels have proven to play many important roles outside the nervous system. In the immune system, effector memory T cells (T_{EM}) utilize the voltage-gated potassium channel Kv1.3 in a signaling pathway that results in activation and proliferation in response to antigen presentation. Activation and proliferation of T_{EM} cells is a hallmark of several autoimmune diseases, such as multiple sclerosis, rheumatoid arthritis and psoriasis. Inhibitors of Kv1.3 have a demonstrable antiproliferative effect on T_{EM} cells, while sparing naïve and central memory T cells (T_{CM}) [32,33].

Much effort has gone into discovering and/or engineering highly potent and selective inhibitors of Kv1.3 as potential therapeutic drugs for autoimmune disease. A highly potent peptide inhibitor of Kv1.3, known as ShK (Fig. 1), was discovered from the Caribbean sea anemone *Stichodactyla helianthus* [34] [35]. ShK inhibits proliferation of T_{EM} cells via block of Kv1.3 with a K_d of 10 pM [36]. Although ShK is selective for Kv1 channels, it also has high affinity (K_d, 28 pM) for the neuronal Kv1.1 channel [37], which could be a barrier to clinical development of the natural peptide, although no side effects were observed with ShK treatment in an experimental model of multiple sclerosis [38]. Over the past decade, George Chandy and co-workers have generated 380 analogs of ShK to improve targeting specificity and *in vivo* stability the peptide. This effort has resulted in analogs with greater than 100-fold selectivity over Kv1.1 with improved stability [39]. An analog of ShK is expected to enter human clinical trials this year [40]. While small molecule inhibitors of Kv1.3 are attractive for the treatment of MS, they are presently less potent and less selective than the best ShK analogs [33].

Chemical ecology: a cornucopia for the ion channel pharmacology of the future

In this section, the exciting potential of animals as sources of drug leads is presented. At the present time, most pharmacologically-active compounds that target ion channels from animals have been characterized from venoms. A rationale for why venoms predominate as the source of natural product ligands for ion channels is discussed.

We suggest that any biotic interaction in which one animal uses a chemical strategy to change the behavior of another animal is likely to involve diverse natural products targeted to different ion channels; the reasons why such compounds are seldom discovered except from venoms is given below. A key argument for why a paradigm shift in natural-product discovery will be required is the mechanistic complexity that underlies all effective interactions between animals that are mediated by pharmacologically-active compounds. A specific, relatively well understood example is provided, and it is shown why an individual biotic interaction may yield multiple compounds of interest. Since the millions of species of animals each have their own specific sets of biotic interactions, this leads to an amazing level of chemical and pharmacological complexity that could potentially be identified and characterized from animal biodiversity.

Venomous vs. non-venomous animals as potential sources of ion-channel targeted drugs

Natural products comprise a rich resource for the continuing discovery of pharmacologically-active compounds that target ion channels. As illustrated above, the major group of natural products that dominate the pharmacology of ion channels is peptides/polypeptides. The discovery process has been skewed towards peptides from venoms. Non-peptidic natural products targeted to ion channels remain mostly unexplored, but these should become much more accessible in the coming decades.

The emerging field of chemical ecology (the study of chemicals mediating interactions between living organisms) should eventually elucidate many novel biologically active

compounds. Interactions between animals are only beginning to be elucidated at a mechanistic and molecular level. Notably, it is not just venomous animals that produce chemicals to affect the behavior of their potential predators, prey or competitors. In the marine environment in particular, there is persuasive evidence for animals producing diverse compounds to affect the behavior of the other animals around them [22,41,42]. The critical difference is that venomous animals produce the compounds of interest in a well-defined anatomical structure. For most non-venomous animals, what is not established is where and when and how such compounds are produced.

Thus, we believe it likely that in the marine environment, pharmacologically-active compounds that have evolved to modulate the behavior of another animal are targeted to ion channels, since these are the obvious molecular targets that would strongly affect behavior. Such compounds are mostly unexplored. The key problem with pharmacological discovery from non-venomous animal sources is that because the basic biology remains mostly unknown, the specialized anatomical structure(s) where the interesting chemicals are likely to be produced have generally not been directly investigated. However, compounds that have evolved to affect animal behavior would seem prime candidates as ligands for specific ion channels, as will be discussed in the next section.

This raises yet another fundamental scientific issue: if an animal is not venomous, then the compounds produced to modulate behavior of a different animal must be able to reach the physiological target molecules without the specialized delivery systems that venomous animals have evolved. In many ways, the problem is similar to the problem of getting through the gut barriers or the blood/brain barrier encountered in human pharmacology. What makes these issues critical for the next decades is that it may well be that there is a world of “drugable compounds” already produced by these animals, but because of historical biases that are a consequence of how discovery in the pharmaceutical industry has evolved, these have hardly been accessed. At present however, with rapid advances in molecular biology and biotechnology, if these compounds are being produced by any animal, they should, in principle, become accessible.

Biotic interactions and ion-channel-targeted natural products

One reason for optimism in the future of natural products as drug leads are the early indications that a vast trove of yet undiscovered compounds exist that affect the function of ion channels. In the few systems studied, the underlying biochemistry and pharmacology of biotic interactions has proven to be much more sophisticated than could ever have been predicted *a priori*. We discuss one specific case from cone snails to illustrate this. We suggest that this specific predator/prey interaction will prove typical of the multi-layered mechanisms that underlie an interaction between organisms that has arisen through natural selection. Another way of viewing the overall message of the biology discussed next is that for interactions between organisms to be effective in the real world, a sophisticated molecular strategy is required. Evolution is apparently a stringent taskmaster: for one animal to change the behavior of another using chemical compounds demands that natural products with novel and surprising pharmacology be generated.

The specific example of a biotic interaction that is reasonably well-understood at a biochemical and pharmacological level is between a fish-hunting cone snail predator and its fish prey. All cone snails are venomous and predatory, and a significant number (~100 species) hunt fish as their primary, if not exclusive prey. While there is molecular phylogenetic evidence for ~6 distinct clades of fish-hunting cone snails [43], two of these are particularly well understood, both with respect to how they capture their prey, as well as with regard to their evolutionary history. Although the two clades, comprising ~25 different species, evolved fish-hunting independently, their strategy for capturing fish is convergent at a general physiological level.

These two clades of fish-hunting cone snails are usually regarded as subgenera of *Conus*: *Chelyconus*, comprising fish-hunting species in the new world, and *Pionoconus*, a clade of fish-hunting species dispersed throughout the IndoPacific, from Hawaii and Easter Island, to the East African coast. The biogeography and recent molecular phylogenetic evidence suggests that the evolution of piscivory in *Chelyconus* and *Pionoconus* were significantly separated both in time (by several million years) and space (new world vs. IndoPacific) [21,44,45,46].

Both groups of fish-hunting *Conus* species evolved a harpoon-shaped radular tooth that serves both as a hypodermic needle for venom delivery as well as a harpoon for tethering the envenomated fish. The fish prey, depending on the site of injection and the amount of venom that the snail is able to deliver, struggles briefly, but invariably quickly becomes tetanically paralyzed with very stiff fins, and is reeled in towards the false mouth of the snail (in which pre-digestion takes place). After an initial period of tetanic immobilization, the fish sometimes recovers and will struggle to free itself, but in most cases, the second stage, an irreversible neuromuscular block, occurs [29,47,48,49]. As a result, even when the fish is able to free itself loose from the harpoon because it recovered quickly from the original tetanic immobilization, it invariably becomes paralyzed and characteristically cone snail species in both clades will do a quick search of the neighborhood to find the immobilized fish. Thus, a fish able to break free in an aquarium setting is still devoured.

In all species in these two clades of fish-hunting cone snails, fish envenomation evokes two distinct physiological endpoints. First is the very rapid tetanic immobilization (“excitotoxic shock”); the effect is equivalent to being tasered. Second is a complete block of neuromuscular transmission (“neuromuscular block”). In both clades of fish-hunting cone snails, multiple venom components cause each of these effects (See Table 1). We refer to groups of toxins that act together for a common physiological endpoint as “cabals” [29,47,48,49]. The venom components that cause the very rapid onset of a tetanic immobilization are called the “lightning-strike cabal”. Those venom components that cause a complete block of the neuromuscular transmission are known as the “motor cabal”.

What is striking is that the two clades of fish-hunting cone snails have independently evolved both a lightning-strike *and* a motor cabal. At a gross pharmacological level, the molecular targets of the two cabals overlap, and in many cases are identical. However, what provides clear molecular evidence for convergent evolution is that the gene superfamilies that have been recruited to provide these analogous pharmacological functions are often

divergent between the two clades, but they are homologs for all of the species within each clade. Furthermore, in both cases, a large number of venom components comprise each cabal — e.g., for both sets of fish-hunting cone snails, a combination drug strategy has evolved. The critical result is that *the targets of all of the major venom components that comprise these cabals are ion channels*.

Why natural products will prove to be more diverse and selective than ligands for ion channels developed in the past

Two aspects of natural product discovery are presented: first is the likelihood of a vast diversity of natural products targeted to ion channels yet to be discovered; a second aspect we will discuss is why these natural products are likely to be highly selective and potent. Clearly, if this prediction holds true, there should be no shortage of useful compounds for ion channel pharmacology, and a quantum jump will occur in the number of natural product drug leads available that have ion channel targets.

Biodiversity leads to chemical and pharmacological complexity—For the specific biotic interaction described above, a large number of different ion channel modulating peptides were independently evolved in the two clades of fish-hunting cone snails. Cone snails have generated peptide-toxin diversity by multiple mechanisms. One evolutionary mechanism by which cone-snails have generated toxin diversity is by employing multiple gene superfamilies to encode peptides with different structural scaffolds in their venoms. Conotoxin superfamilies are typically identified by cDNA cloning of transcripts encoding conotoxin precursor peptides. Each superfamily is identifiable by a highly conserved signal sequence (see Table 2) that is cleaved, along with a propeptide sequence, in the production of the mature peptide, which is secreted into the venom duct. In addition to the conserved signal sequence, a conotoxin superfamily typically shares a single arrangement of cysteine residues (see Table 2) that creates a common disulfide-bonding framework, although some conotoxin superfamilies contain two different arrangements of cysteine residues, as described in the following paragraph for the A-superfamily. Within a gene superfamily, even conotoxins that share a common arrangement of cysteine residues may diverge in their targeting selectivity, thus defining different conotoxin families within a superfamily. Hence, the κ M-conotoxin family targets K-channels, while the μ -conotoxin family targets Na-channels, but both belong to the M-superfamily (Table 2). Within a conotoxin family, selectivity for a particular subtype of ion channel appears to have been refined by extensive mutagenesis of the non-cysteine residues such that even closely related species of cone-snails encode peptide sequences that are similar but divergent and unique to a single species. Furthermore, it appears that the different cone-snail species emphasize different types of toxins to different extents and can evolve novel venom components not found in other species within the same clade.

A concrete example of differences in venom components between the two clades, *Chelyconus* and *Pionoconus*, is shown in Figure 2. What is illustrated are peptides of the motor cabal that are competitive antagonists of the nicotinic acetylcholine receptor at the post-synaptic terminus of the neuromuscular junction. The peptides found in two different

species of *Pionoconus* (*Conus magus* and *striatus*), and two different species of *Chelyconus* (*Conus purpurascens* and *ermineus*) are shown in the figure. When peptide sequences within a single clade (either *Pionoconus* or *Chelyconus*) are compared, then the sequence similarity is obvious. The peptides within each clade are different in their amino-acid sequences, but there are many conserved and identical positions, and the pattern of cysteine residues (and disulfide bonding) is strictly conserved. In contrast, the peptide sequences of the *Chelyconus* species are markedly different from the *Pionoconus* peptides; not only are they longer, with an additional disulfide linkage, but there is no apparent sequence homology at all. However, if the entire reading frame that encodes the precursor for both sets of peptides is compared (not shown), the peptides are found to belong to the same gene superfamily. The transcripts of the α -conotoxins of the *Pionoconus* clade and the α A-conotoxins of the *Chelyconus* clade have the same conserved signal sequence, as well as conserved sequence elements in the 3' untranslated region that are diagnostic of the A-superfamily of conopeptides (not shown). The long period of divergence and the independent evolution of piscivory in the two clades clearly resulted in sufficient divergence of the mature peptides to make their shared superfamily origin no longer apparent at the amino acid level.

Some components of the cabals shown in Table I are even more divergent between the two clades. The peptides that are K-channel blockers in *Chelyconus*, have 3 disulfide bonds and are typically 25-30AA in length; these belong to the O-gene superfamily. However, in *Pionoconus*, the analogous K-channel blockers are kunitz domain polypeptides, the conkunitzins. These are larger polypeptides with no structural or genetic relationship to the O-superfamily. On the other hand, some of the peptides, particularly those targeted to voltage-gated Na channels, belong to the same family in the two clades. Specific examples are the μ -conotoxins that are Na-channel blockers, and the δ -conotoxins, which delay inactivation of Na channels; peptides in both clades belong to the M-superfamily and O-superfamily, respectively, and their homology is apparent.

In addition to the biochemical/genetic differences between the two clades discussed above, there are systematic differences in the individual molecular pharmacological strategies. Thus, *Chelyconus* species have a non-competitive antagonist of the nicotinic acetylcholine receptor (the ψ -conopeptides) that is a major component of their venoms. These non-competitive antagonists are not found in *Pionoconus* species. In contrast, *Conus magus*, a species in *Pionoconus*, has multiple toxins targeted to voltage-gated Ca channels: one of these is highly specific for the N-type Ca channel (and this has become the drug Prialt), but other peptides in this venom preferentially target the P/Q Ca channel [28,50], which is also found at the pre-synaptic terminus. Thus, *Conus magus* is more highly sophisticated in targeting voltage-gated Ca channels compared to species in the *Chelyconus* clade. All species in the two clades use multiple venom components to completely block neuromuscular transmission, so the physiological end point is indistinguishable. However, in evolution, there is a choice of which of the specific molecular targets important in neuromuscular transmission will be targeted, and some cone snail species focus more on pre-synaptic targets, while others emphasize different pharmacological sites and different subtypes of the post-synaptic receptor. Every species examined also has at least one peptide that inhibits voltage-gated Na channels in the neuromuscular circuitry.

Even within the same clade, there are interspecies differences, most not well understood. Thus in the *Pionoconus* clade, *Conus magus* has a single antagonist for the nicotinic receptor; however, *Conus striatus* has three different competitive nicotinic antagonists (see Table I), at least two of which are generally highly expressed. The selective pressures that generate three different competitive nicotinic antagonists are unclear, and probably reflect our limited insights into the detailed synaptic physiology of the teleost fish prey of these cone snails.

The overall picture that emerges is that at the gross physiological level, the biotic interaction between the predator and fish prey seems indistinguishable between all of the *Conus* species in the two clades. The physiological end points are the same, tetanic immobilization and neuromuscular block. In marked contrast, if the amino-acid sequences of individual peptides are compared, there is no interspecies overlap; every species has generated its own distinctive complement of venom components — peptides from different species never have identical sequences, even within the same clade. Although there is analogy in the molecular targeting, there is also divergence. Thus, the cone snail species in these two fish-hunting clades represent a large library, highly diverse at the molecular level, of natural products with many different ion channel targets. A peptide in the motor cabal of *Conus magus* has become a drug (Prialt); another peptide in the lightning strike cabal of *Conus purpurascens*, a species in *Chelyconus*, is in pre-clinical development for its cardioprotective properties [51]. The highly selective targeting of these peptides is discussed in the next section.

Prospects for target selectivity—The elucidation of the conopeptide cabals (Table 1) revealed that each individual venom component of the cabal had a high degree of pharmacological specificity. For a predator to capture prey, it might be anticipated that the selective pressure would be to evolve compounds that would be the chemical equivalent of bludgeons, but the analysis of the different cabal components, all of which proved to be targeted to ion channels, revealed instead a degree of target selectivity that was unprecedented. Thus, the ability of some members of the motor cabal such as ω -conotoxin MVIIA, the compound that eventually became Prialt, to discriminate between very closely-related Ca channel subtypes was not anticipated.

The argument has been made is that this high selectivity arises from two factors. One is the need for speed: the faster a chemical compound has to act on a specific molecular target, the more unacceptable it is for that compound to bind to physiologically-irrelevant targets. Thus, if the Ca channel targeted members of the motor cabal were to bind Ca channels present in blood vessels, the transit time from injection to binding and blocking neuromuscular presynaptic Ca channels would be greater, since the peptide would go through cycles of binding and dissociation from Ca channels that were similar structurally, though irrelevant physiologically. This therefore selects for high selectivity, and against binding to physiologically-irrelevant (though structurally similar) targets.

The other factor is intrinsic in the evolution of the combination drug strategy itself; to give a concrete example, the fish-hunting cone snails discussed in the section above have at least two types of pharmacologically-active compounds that target Na channels. One of these are Na channel blockers, which are motor cabal components and the other are peptides that keep

Na channels open by inhibiting inactivation, key components of the lightning-strike cabal. Clearly, if both peptides targeted the same Na channel, their activities would be antagonistic, and the motor cabal component would inhibit the lightning-strike cabal. By selectively targeting the Na channel blockers to one (or a few) Na channel subtypes (e.g. muscle Na channel [52]), and targeting the peptides that keep Na channels open to different subtypes of voltage-gated Na channels, (presumably those found in peripheral axons [53]), both sets of peptides can act unimpeded on their respective targets. Thus, a combination strategy requires higher target discrimination, so individual compounds in a combination do not interfere with each other's functions, nor with other combinations of compounds generated by the animal.

We have postulated above that the “cabal” strategy will prove to be general and widespread in Biology; indeed, evidence for two different venom components that can be postulated to act as members of a cabal have been found in diverse venoms (e.g., the α -toxins that activate Na channels and the K-channel blockers from scorpions). Thus, whenever an animal uses a chemical strategy to affect the behavior of another, it is likely that multiple compounds targeted to ion channels will be generated, and these would be expected to be highly selective. Furthermore, as complex biotic interactions are elucidated at a molecular level, we expect that many of the future ion-channel compounds awaiting discovery will be non-peptidic organic compounds that have evolved to cross membrane barriers (particularly in the marine environment).

Future perspective

We presented an overview of the present state of the field of ion channel pharmacology in the first section of this article. A rationale is given for why natural product discovery is likely to be a more promising approach than combinatorial chemical synthesis combined with high-throughput screening, presently the norm in the pharmaceutical industry. We also presented our view that the traditional approach used for natural product discovery, “bioprospecting,” has likely not detected the great diversity of natural products that target ion channels. Two specific examples of peptides from venoms (cone snail and sea anemone) illustrated that natural products targeted to ion channels do indeed have considerable biomedical potential. We have focused on venoms from the marine environment to help us make the case for the general discovery paradigm that we are proposing. However, it is noteworthy that there is enormous untapped potential for pharmacological discovery both from venomous- (cone snail, sea anemone, scorpion, spider, snake, etc.) [18] and non-venomous animals, encompassing both peptides and non-peptidic organic compounds, across marine and terrestrial habitats.

This overview provided the rationale for why a broad natural product discovery initiative should greatly accelerate progress in ion channel pharmacology. What would seem to be a required basic-science component of this initiative would be better understanding of biotic interactions between animals, particularly when animals use a chemical strategy to change the behavior of their prey, predators or competitors.

Venomous and non-venomous interactions that affect behavior are likely to be especially prominent in the marine environment, and the case is made that the mechanistic basis of

each individual biotic interaction will involve natural products targeted to ion channels with great selectivity. This will require a more incisive knowledge of the biology of the animals producing such compounds, and of the chemical ecology of the marine environment. This should provide the raw material for drug leads targeted to ion channels in the coming decade. However, it will require a paradigm shift, abandoning the predominant strategy for natural product discovery at the present time and instead adapting a new interdisciplinary approach to discovery that incorporates phylogenetics and organismal biology with chemical ecology and medicinal chemistry.

Executive Summary

Natural-product discovery is critical for rapid progress in ion channel pharmacology.

- Low R&D productivity is widely cited as the biggest problem facing the pharmaceutical industry. The R&D cost per new drug has increased to billions of dollars, accompanied by high pre-clinical and clinical attrition rates of candidate compounds, which in many cases can be attributed to lack of targeting selectivity.
- Natural products, which have been refined by evolutionary forces for potency and selectivity, may have distinct advantages in targeting selectivity over random small molecules.
- The bioprospecting approach to natural-products discovery was misguided and has probably failed to identify many of the most potent and selective compounds that mediate biotic interactions.

Proof-of-principle for natural products targeted to ion channels as drug leads.

- Two specific examples illustrate that natural products targeted to ion channels are suitable for drug development: Prialt is an FDA-approved drug that is chemically equivalent to ω -conotoxin MVIIA (peptide). An analog of ShK, a sea anemone peptide, is expected to enter clinical trials this year for multiple sclerosis and other autoimmune diseases.

Chemical ecology: a cornucopia for the ion channel pharmacology of the future.

- The discovery of natural products that target ion channels has been skewed toward venoms in the past.
- Chemical ecology should elucidate many novel compounds that mediate biotic interactions between prey, predators and competitors, especially in the marine environment. Such natural products should have built-in drug-like properties and many will likely target ion channels.
- One example that illustrates the potential diversity of natural products targeted to ion channels from the marine environment is from fish-hunting cone snails. Many distinct venom components act in concert for prey capture or defense in the “lightning strike cabal” and “motor cabal.”

Why natural products will prove to be more diverse and selective than ligands for ion channels developed in the past.

- The fact that biodiversity leads to chemical and pharmacological complexity is illustrated by the divergence of venom components between different clades of fish-hunting cone snails.
- Venom components that act in concert for prey capture or defense have evolved to be highly selective, apparently because 1) this allows a compound to reach its target faster and 2) without interference upon other targets or circuitry perturbed by other venom components.
- We argue that the “cabal” strategy (multiple compounds acting in concert to mediate a biotic interaction) will be much more general and widespread in biology than is currently anticipated, leading to the discovery of many novel, highly selective, ion-channel targeted compounds.

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Defined Key Terms

Bioprospecting	The search for previously unknown bioactive chemical compounds from the natural world
Biotic interactions	All behavioral and chemical exchanges between living organisms in an ecosystem.
Cabal	A term originally used to describe secret societies that seek to overthrow existing authority. We have adopted the term to describe diverse compounds acting <u>in concert</u> to achieve a certain physiological end-point in a biotic interaction, e.g. multiple different venom components used to immobilize prey.
Chemical Ecology	The study of chemicals that mediate interactions between living organisms.
Clade	In biological systematics, a clade represents a branch of related species on the tree of life.

Conotoxin	Small, highly disulfide-bonded peptides from the venom of marine cone snails (genus <i>Conus</i>).
Kunitz domain	A conserved domain in certain small, basic, extracellular proteins containing three disulfide bonds for stability. Examples include mechanistically diverse proteins such as bovine pancreatic trypsin inhibitor and dendrotoxins, which block potassium channels.
Natural product	A chemical compound produced by a living organism for a particular biological function.
Prialt	(ziconotide, SNX-111) is a synthetic peptide that is chemically equivalent to ω -conotoxin MVIIA, a natural peptide from the venom of the marine cone snail, <i>Conus magus</i> . The peptide is a highly potent and selective inhibitor of the voltage-gated calcium channel Ca _v 2.2. It was approved as a drug for intractable neuropathic pain by the FDA in 2004 in the United States, and was approved in Europe in 2005.
ShK	A natural peptide from the sea anemone <i>Stichodactyla helionthus</i> that is a potent inhibitor of the voltage-gated potassium channel K _v 1.3. An analog of ShK is expected to enter clinical trials this year for the treatment of multiple sclerosis and other autoimmune disorders.

Fig 1.

Structures of ω -conotoxin MVIIA (Prialt) [70] and ShK [71]. These are venom peptides from the cone snail *Conus magus* and the sea anemone *Stichodactyla helionthus* that are targeted to the voltage-gated Ca^{++} channel $\text{Ca}_v2.2$ and the K^+ channel $\text{K}_v1.3$ respectively. A critical residue for binding its target is shown for each peptide: Tyr-13 in ω -MVIIA and Lys-22 in ShK. Cys residues and disulfide bonds are shown in yellow. The amino-acid sequence for each peptide is shown below its respective structure.

Pionoconus clade

α -MI	GR C CHPAC C GKNY S C
α -SI	I C CNPAC C GPKY S C
α -SIA	Y C CHPAC C GKNF D C

Chelyconus clade

α A-PIVA	G C CGSYONAA C HO C S C CKD-ROSY C GQ
α A-EIVA	G C CGPYONAA C HO C G C CKVGROOY C DROSGG

Fig 2.

Comparison of analogous venom peptides targeted to the acetylcholine binding site of the neuromuscular nicotinic acetylcholine receptor. Amino-acid sequences are compared from *Conus* species belonging to two clades, *Pionoconus* and *Chelyconus*. α -MI is from *Conus magus* while α -SI and α -SIA are from *Conus striatus*. α A-EIVA and α A-PIVA are from *Conus ermineus* and *Conus purpurascens*, respectively. Several α -conotoxins target the α/δ interface of the neuromuscular nAChR [68], while these α A-conotoxins target the α/δ and α/γ interfaces of the neuromuscular nAChR [59]. Note that α -MI is much more similar in sequence to α -SIA than either peptide is to α -SI; α -MI and α -SIA are likely homologs from different species, while α -SIA, though also a competitive nicotinic antagonist, may have a divergent physiological role *in vivo*. Representative shells from each species are shown.

Table 1
Diverse Ion Channel Targeted Peptides for Prey Capture

Molecular Targets ^a	Examples of Venom Peptides in: ^b	
Motor Cabal:	Pionoconus Clade ^c	Chelyconus Clade ^c
Nicotinic receptor: (ACh site inhibitor)	α-MI [54] α-SI [56], α-SIA [57], α-SII [58]	αA-PIVA [55] αA-EIVA [59]
Nicotinic receptor: (Ion channel blocker)	—	Ψ-PHIE [60]
Na channel blockers	μ-MIIIA [61] μ-SIIIA [63]	μ-PIIIA [62]
Ca channel blockers	ω-MVIIA [29] ω-MVIIC [50] ω-SVIA [58]	—
Lightning Strike Cabal:		
Na channel (inactivation inhibitor)	δ-SVIE [64]	δ-PVIA [65] δ-EVIA [53]
K channel blockers	Conkunitzin-S1 [66] Conkunitzin-M	κ-PVIIA [49]
Glutamate receptor (desensitization inhibitor)	Con-ikotikot-S [67]	—

^aThe diverse ion channel targets of venom peptides involved in prey capture that have been characterized are shown. Examples of peptides that target these sites from two species in the *Pionoconus* clade (*Conus magus* and *Conus striatus*) and two species in the *Chelyconus* clade (*Conus purpurascens* and *Conus ermineus*) are given in the two columns indicated. Several α-conotoxins target the α/δ interface of the neuromuscular nAChR [68], while these αA-conotoxins target the α/δ and α/γ interfaces of the neuromuscular nAChR [59].

^bNames of peptides are abbreviated. The amino acid sequence of each peptide is given in the references provided. An overview of the structures, mechanisms and nomenclature of these peptides is Terlau et al., 2004 [69].

^cPeptides from *Conus magus* have M in their abbreviated name (α-MI, μ-MIIIA, etc.), while *Conus striatus* peptides have S, *Conus purpurascens*, P and *Conus ermineus*, E.

Table 2
Examples of Conotoxin Superfamily Sequences

	M-superfamily
κM-RIIIK	MMSKLGVLLTICLLFPLTALPMDGDQPVDRLAERMQDNISSEQHTFFEKRL PSCCS LNRLCPVPACKRNP CCTG
μ-GIIIA	MMSKLGVLLTICLLFPLTALPMDGDDEPANRPVERMQDNISSEQYPLFEKRRD CCTPPKCKDRQCKPQRCC AGR
μ-SmIIIA	MMSKLGVLLTICLLFPLTALPMDGDQPADQPADRMQDDISSEQYPLFDKRQ KCCTGKKGSCSGKACKNLKCCSGR
	A-Superfamily
α-MII	MMFTVFLLVVLATTVV\$FPSDRASDGRNAAANDKASDVITLALK GCCSNPVCHLEHSNLC GRRR
α-EI	MMFTVFLLVVLATTVG\$FTLDRASDGRDAAANDKASDLIALTARRD PCCYHPTCNMSNPQICG
α-CI	MMFTVFLLVVLTTVV\$FPSDRASDGRDDEAKDERSDMYKSKRNGR CCHPACGKHFCGR

Examples of two conotoxin superfamilies are shown. The sequences shown are the predicted amino acids from cDNA cloning of the transcripts encoding these peptides. The highly conserved signal sequence that is diagnostic of a conotoxin superfamily is shown in shaded text. The underlined text indicates the mature-peptide sequence that is cleaved from the precursor peptide and secreted into the venom duct. Cysteine residues are shown in bold type.