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Conotoxins and their Regulatory Considerations

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Abstract

Venom derived peptides from marine cone snails, conotoxins, have demonstrated unique pharmacological targeting properties that have been pivotal in advancing medical research. The awareness of their true toxic origins and potent pharmacological nature is emphasized by their 'select agent' classification by the US Centers for Disease Control and Prevention. We briefly introduce the biochemical and pharmacological aspects of conotoxins, highlighting current advancements into their biological engineering, and provide details to the present regulations that govern their use in research.

Keywords: Conotoxins; Peptides; Cyclic peptides; Regulation; Select agents

Introduction to Conotoxins

The venom of *Conus* marine snails has evolved as an efficient means of prey incapacitation and as an effective defense mechanism [1]. The deadly effects of this cocktail are a direct result of Conotoxins, small disulfide-rich peptides that are potent antagonists of neuronal receptors and ion channels including nicotinic acetylcholine receptors (nAChRs), voltage-sensitive sodium and calcium channels, N-methyl D-aspartate (NMDA) receptors, and more [2] (Supplementary Table 1). These bioactive peptides express selectivity towards their target receptors and are even able to discriminate between receptor subtypes as demonstrated with α-conotoxin ImI. α-Conotoxin ImI has an Inhibitory Concentration (IC_{50}) of 40.8 nM in the human acetylcholine receptor subtype α3β2 and an IC_{50} greater than 10 μM in the receptor subtype α1β1δε [3]. This property can be utilized to design receptor-modulating ligands with therapeutic applications.

Conotoxins are typically about 10-40 amino acids in length, but exhibit many motifs such as α-helices and β-sheets that are normally found in larger proteins. These structural features are stabilized by disulfide bonds generated from the abundance of cysteine moieties in its primary structure [1,4]. Interestingly, these Cys residues are found in predictable locations within a conotoxin's sequence, giving rise to loops of known amino acid lengths, which influence their bioactivity [4,5]. Conotoxins can contain anywhere from one to five disulfide bonds which typically produce intramolecular connections [6]. However, conotoxins are also capable of forming intermolecular disulfide bridges as seen with conotoxin Vt3.1 [7]. These dimeric conotoxins are worth investigating for unique receptor specificity and or augmented pharmacokinetic properties.

Although disulfide bonds play a major role in determining conotoxin structure activity relationships, assessing how they influence bioactivity of conotoxins should be handled on a case-by-case basis as they stabilize peptide structure in varying degrees. Finn et al. [8] demonstrated that elimination of a single disulfide bond by replacing cysteine with serine residues at position 15 and 26 resulted in a 8000-fold loss of potency for ω-conotoxin GVIA. It was found that elimination of this single bond resulted in the disruption of the peptide's tertiary structure. Contrastingly, Han et al. [9] had demonstrated that deletion of a single disulfide bridge in μ-conotoxin KIIIA, through an alanine substitution did not result in a significant loss of bioactivity (Kd of 0.004 ± 0.004 μM in native toxin versus Kd of 0.008 ± 0.002 μM in C1A, C9A). It was found that elimination of a single disulfide bond did not result in the disruption of the α-helix motif that was necessary for μ-conotoxins KIIIA activity [10].

Permutations of disulfide bond connectivity can also produce conotoxin isomers that may possess enhanced pharmacological properties. The number of disulfide isomers is directly correlated with an increasing number of cysteines within a peptide sequence [11]. A conotoxin with four cysteines can undergo three different disulfide conformations, producing three forms or isomers: the globular, ribbon and bead forms [12]. Notably, when the disulfide bond connectivity of α-conotoxin AuIB was rearranged, the resulting ribbon isomer exhibited greater bioactivity than the globular native toxin [13]. This finding is valuable to conotoxin bioengineering as it expands the repertoire of modifiable conotoxins to include non-native isomers.

Cysteine positioning within the primary structure of α-conotoxins can also impact some aspects of biological activity. Despite their great sequence diversity, the cysteine frameworks of these bioactive peptides are quite predictable. For conotoxins containing four cysteines, their cysteine framework is given by the formula $(X)_{n1}C^{i}C^{ii}(X)_{n2}C^{iii}(X)_{n3}C^{iv}(X)_{n4}$, in which $n1$ can contain anywhere from 1-4 amino acids, $n2$ can contain 3 to 4 amino acids, $n3$ can contain 3-7 amino acids and $n4$ can contain 0-5 amino acids [4]. Interestingly, the number of amino acids in $n2$ and $n3$ of their cysteine framework can have profound consequences in their selectivity toward a specific receptor class. Generally, α-conotoxins with a 3/5 cysteine framework ($n2$=3 and $n3$=5) will target muscle nAChRs [14] while conotoxins with a 4/7 framework ($n2$=4 and $n3$=7) will be specific for neuronal nAChRs [1,14,15]. This has important implications regarding conotoxin classification.

Conotoxins can be systematically ordered into superfamilies and further organized into families (sometimes referred to as classes). Superfamilies are based on disulfide bond framework patterns, while families are founded on their pharmacological target [1,16]. Currently, there are twenty main conotoxin superfamilies (A-, B-, C-, D-, E-, F-, G-, H-, I-, J-, K-, L-, M-, N-, O-, P-, S-, T-, V- and Y- superfamilies [17]). Within these inclusive categories lie their respective families, often denoted as single Greek letters prefix, among which, there are five

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main families of considerable interest in research: α-, ω-, μ-, δ- and κ-families [16]. These categories are rapidly expanding with the discovery of new conotoxins (Supplementary Table 2).

Along with conotoxins, cyclotides are another category of natural products that are garnering immense interest due to their therapeutic potential. Cyclotides are disulfide rich plant peptide characterized by head to tail cyclization and six conserved cysteine residues arranged in a knotted topology [18,19]. Cyclotides have insecticidal properties and function in plants as host-defense agents [20]. In addition to their natural function cyclotides have been reported to possess anti HIV, anticancer and hemolytic activities [18,21]. Like conotoxins, cyclotides contain a high frequency of cysteine residues in their sequence. The presence of disulfide bonds confers considerable stability to these natural products thereby making them worthwhile research tools for structural studies and as scaffolds for stable delivery of drugs [22,23]. The idea of merging the notable pharmacological properties of cyclotides with the specificity of conotoxins has drawn much attention to bioengineering of cyclotide forms of conotoxins [15].

Biological Spectrum of Conotoxins

Phyla selectivity

One of the most important and well known aspects of conotoxins is their ability to selectively target ion channel subtypes [16]. This dynamic receptor-ligand interaction can be utilized to understand subtle differences in the normal physiology of various ion channel subtypes. As such, conotoxins are viewed as valuable resources for phyla selective receptor probes. Phyla selectivity is commonly seen amongst individual conotoxins, representing their native predatory preferences [16]. Many peptides isolated from a piscivorous cone snail such as *Conus magus*, will only display activity in receptor subtypes related to higher order organisms, whereas molluscivorous cone snails tend to produce conotoxins which target mollusk receptor subtypes [24]. This type of selectivity can be greatly utilized in terms of creating safe and effective pesticides. Potential pesticides produced from the venoms of molluscivorous cone snails may provide compounds that could be naturally degraded by microorganisms without producing harmful byproducts or causing environmental damage [25].

κ-Conotoxin PVIIA and δ-conotoxin PVIA isolated from *Conus purpurascens* are conotoxins present in the same venom profile however exhibit two distinct mechanisms of action [26]. These peptides were categorically determined to be part of two individual "cabals" or modes of attack: κ-conotoxin PVIIA on the lightning strike cabal, and δ-conotoxin PVIA on the motor cabal. κ-conotoxin PVIIA inhibits the Shaker potassium channel K_v2.4 and δ-conotoxin PVIA delays the inactivation of sodium channels, allowing for a reinforced strategy of prey paralysis and capture, a synergistic technique akin to what modern drug companies are only now attempting to mimic. These techniques are often seen in *Conus* venoms and occasionally appear to be perplexing and contradictory. This is especially true when considering conotoxins from the motor cabal that inhibit sodium channel conductance and conotoxins from the lighting strike cabal that inhibit sodium channel inactivation. These two seemingly contradictory mechanisms are only beneficial if the afflicting conotoxins are highly selective for receptor subtypes acting in different areas of the cone snail's prey [16].

General and clinical pharmacological activity of conotoxins

A second feature of conotoxins is their nature to exhibit a high amount of biological activity. ω-Conotoxin MVIIA is the only United States Food and Drug Administration (FDA) approved conotoxin for use as a therapeutic to date and is known for being extremely potent, displaying bioactivity in the low nanomolar range (IC_{50} 7.6 nM) [27,28]. This type of activity is not unique to this single peptide however, as many conotoxins appear be exceedingly potent. Examples of nanomolar potency among conotoxins include but are not limited to: the μO-conotoxin MrVIA (IC_{50} = 345 nM) [29,30] and the α-conotoxin MIC (K_i = 248.7 nM) [31].

Clinically ω-conotoxin MVIIA, otherwise known as Ziconotide or Prialt®, has undergone extensive clinical testing [32]. Pharmacokinetics on Intravenous (IV) and Intrathecal (IT) injections has been established. Classical distribution, metabolism, and excretion studies were undertaken before Ziconotide was entered into clinical trial. Ziconotide is the first non-opioid IT treatment for the management of chronic refractory pain and is known by its brand name Prialt® (Jazz Pharmaceuticals). The therapeutic is designed to be delivered intrathecally and should not be administered via any other Route of Administration (RoA). Prialt® is meant to be delivered only under the supervision of a physician using a programmable implanted variable-rate microinfusion device or an external microinfusion device and catheter. Prialt® is available as 1mL, 2mL or 5 mL vials (100 mcg/mL) and each vial is intended for single use only. It should be stored at 2 to 8°C and protected from light. Patients with existing psychotic conditions should not be treated with Prialt® as severe psychotic symptoms and neurological impairment may occur during treatment with Prialt®. Regular monitoring for cognitive impairment, hallucinations and mood swings should be undertaken during the treatment regimen [32,33]. Several publications describing the safety and efficacy of Prialt® have been published and can be studied for more comprehensive information.

Oral bioavailability

A third feature of conotoxins is their poor oral bioavailability. Many peptides and biologic based compounds are degraded easily by the digestive tract due to their susceptibility to peptidases, making them virtually ineffective in terms of oral administration. A second reason for the poor oral bioavailability of conotoxins (along with most peptides) is their large size. According to Lipinski's rule of five compounds over 500 Daltons are typically seen as ineffective orally due to poor membrane and paracellular permeability. These properties have provided a justifiable means for the current "relaxed" regulatory handling of material as ingestion is not seen as an immediate threat to health [34].

The problem with poor bioavailability of conotoxins may soon be solved by current research on N- to C-terminal cyclization strategies, as seen with the cyclotides discussed above. N- to C-terminal cyclization of peptides has shown to produce characteristics of increasing stability and even increased absorption in disulfide rich compounds containing the cysteine knot motif such as Kalata B1 [35]. This technique, when applied to synthetic conotoxins, could potentially provide these peptides with the properties necessary for oral bioavailability. These new N- to C-terminal cyclized conotoxins may then require a re-evaluation of handling and regulation standards.

Synthesis of Conotoxins

Biosynthesis of conotoxin

Conus species approximately have 100 to 200 different venoms at their disposal. Each species of cone snail venom profile is unique and has minimal overlap with venom from other species. Given that there are 100 to 200 unique conopeptide in each species, the 500-700 living *Conus* species have a possible repertoire of 50,000 to >140,000

different conopeptides [36-38]. Conopeptide biosynthesis involves a single open reading frame, transcribed to mRNA and translated into a prepropeptide approximately 80 to 100 amino acids in length [39]. The prepropeptide is cleaved by proteases to give a final mature peptide devoid of the prepro region. The signal sequence from a given gene superfamily is highly conserved. In ω-conotoxin MVIIA and GVIA the 22 amino acid containing pre region has no variation and the 23 amino acid containing pro region has 13% divergence (3 out the 23 amino acid are different). With the exception of conserved cysteine residues the mature peptide is highly variable. In ω-conotoxin MVIIA and GVIA the 16 of 22 non cysteine residues are different resulting in a 73% divergence in the mature toxin region [38]. Conotoxins are also heavily Post-Translationally Modified (PTM). PTM's seen in conotoxin are proteolytic processing of propeptide to mature peptide, disulfide bridge formation, hydroxylation, C-terminal amidation, carboxylation, bromination, epimerization, cyclization, sulfation and O-glycosylation [39]. The hypervariable peptide region in conjugation with PTM's provide great diversity to cone snail venom.

Chemical synthesis of conotoxin

The synthesis of conotoxins is largely undertaken through Solid Phase Peptide Synthesis (SPPS), however conotoxins have also been produced recombinantly [25]. SPPS remains the dominant technique due to its simplicity, automation and versatility. It can be used in concert with other synthetic techniques such as chemically directed oxidation strategies of disulfide bonds and native chemical ligation. Such benefits allow for the investigation of a greater diversity of synthetic isomers, chemical modifications to enhance desirable pharmacokinetic properties, and avoid complications with recombinant expression and folding. Further desirable qualities of SPPS include the ability to incorporate non-native and PTM amino acids with ease, automated combination syntheses to obtain epitope information such as alanine walks, and epitope replacement using stabilized backbone scaffolds [40].

Conotoxins used as structural tools

Conotoxins are currently known to be of great benefit for use as molecular probes due to their ability to selectively interact with receptor subtypes. A prominent example of this is ω-conotoxin MVIIA, which has been cited as a probe in over 2,000 scientific publications [16]. The potential for activity and structural investigation is being fostered by the ligation of visually traceable media such as biotin and fluorophores [41]. Many examples of the usefulness of conotoxins as probes exist in literature and include receptor subtypes in calcium channels, potassium channels, sodium channels, 5-HT_3 receptors, NMDA receptors and more [6,42].

Conopeptide based therapeutics in the pipeline

In January 2013 Kineta® a Seattle based Biotech Company announced that it had acquired the developmental rights to α-conotoxin RgIA (U2902) from the University of Utah research foundation. α-Conotoxin RgIA was isolated by the team of Dr. Olivera and found to selectively block α9α10 nAChRs [43,44]. Kineta® intends to develop α-conotoxin RgIA as a non-narcotic treatment for severe pain. Currently α-conotoxin RgIA is in preclinical stage of development and Kineta® states that early results have been promising. Several conotoxins like ω-conotoxin CVID (Leconotide; Relevare Pharmaceuticals), Conantokin-G (CGX 1007; Cognetix), Contulakin-G (CGX 1160; Cognetix), and Xen2174 (structural analog of χ-conotoxin MrIA; Xenome Pharmaceuticals) have reached various stages of clinical trial testing [45]. So far Prialt® remains the only FDA approved drug derived from conotoxin.

Present Regulations Regarding Conotoxin Usage

USA federal regulations

The Federal Select Agent Program is a collaborative venture between the Division of Select Agents and Toxins of the Center for Disease Control and Prevention (CDC) and the Agricultural Select Agents Program under the Animal and Plant Health Inspection Services. As stated on the select agent website "The Federal Select Agent Program oversees the possession, use and transfer of biological select agents and toxins, which have the potential to pose a severe threat to public, animal or plant health or to animal or plant products". The federal select agent programs contribute to national security by performing tasks such as maintaining a database of select agents, and inspecting entities that use, possess or transfer select agents. In addition the program also develops policies and regulations regarding select agents and ensures that these regulations are being implemented.

The program in accordance to 42 USC 262a and 7 USC 8401 defines select agents and toxins as a "subset of biological agents and toxins that the Departments of Health and Human Services (HHS) and Agriculture (USDA) have determined to have the potential to pose a severe threat to public health and safety, to animal or plant health, or to animal or plant products". A subset of the select agents and toxins has been classified as Tier 1 Select Agents and toxins. Tier 1 consists of biologic agents and toxins that possess the maximum risk of being misused to inflict mass causality and poses a high level of threat to public health and safety; further these agents could also be used to negatively impact the economy and cause damage to essential public infrastructure. Some of the Tier 1 select agents and toxins are Botulism neurotoxin, Ebola virus, Marburg virus, *Bacillus anthracis* (anthrax) and Rinderpest virus. A complete list of select agents and Tier 1 select agents and toxins can be found at National select agent registry or on the select agent list website at http://www.selectagents.gov/ (last updated 4/2/2014).

An important question to answer is how the select agent program determines which agents or toxins to include in the HHS select agent list. The "Public Health Security and Bioterrorism Preparedness and Response Act" (116 Stature 594; Public Law 107-188) considers the impact on human health after exposures, the severity of contagiousness, the possible delivery routes of exposure, the availability and efficacy of drug therapies and immunizations, and special criteria as deemed necessary by the secretary to protect more susceptible or vulnerable demographics (e.g., children, senior citizens, etc.) before including an agent or toxin in the HHS list. The list is reviewed after every two years and republished with the necessary amendments.

The CDC states that based on the available experimental data majority of known conotoxins do not possess sufficient acute toxicity to generate a considerable threat to human health. Furthermore the CDC acknowledges that conotoxins are invaluable research tools and have the potential to be developed into human therapeutics. Nevertheless, the CDC mentions that based on the available data one subclass of conotoxin referred to as "short paralytic alpha conotoxins, illustrated by α-conotoxin GI and α-conotoxin MI, do possess sufficient acute toxicity by multiple routes of exposure, biophysical stability, ease of synthesis, and availability. The conotoxins that remain on the HHS list will be limited to the short, paralytic alpha conotoxins containing the following amino acid sequence $X_1CCX_2PACGX_3X_4X_5X_6CX_7$" (Table 1)." Where X_1 is any amino acid or amino acid deletion, the cysteines are present in a disulfide bond between C1 and C3 and C2 and C4 giving the conotoxin its stable globular form, X_2 is Asparagine or Histidine, P is Proline, A is Alanine, G is Glycine, X_3 is Arginine or Lysine, X_4 is

Asparagine, Histidine, Lysine, Arginine, Tyrosine, Phenylalanine or Tryptophan, X_5 is Tyrosine, Phenylalanine, or Tryptophan, X_6 is Serine, Threonine, Glutamate, Aspartate, Glutamine, or Asparagine, X_7 is any amino acid(s) or amino acid deletion.

This provides the potential to generate thousands of peptide combinations that may possess biological activity, an approach that has already been tested using synthetic peptide combinatorial libraries [46]. Short paralytic α-conotoxins are listed in the HHS select agents and toxin list but they are not considered Tier 1 select agents.

Permissible amounts as defined by the CDC

An "entity", which is defined as "any government agency (Federal, State, or local) academic institution, corporation, company, partnership, society, association, firm, sole proprietorship, or other legal entity" by the CDC select agent registry [47], is allowed to possess up to 100 mg of each of the short, paralytic α-conotoxins [48]. This amount is only applicable to biologically active conotoxins that have been folded in their three-dimensional conformation. Non-functional conotoxins are excluded from this restriction. Conotoxins possess a high amount of Cysteines in their sequence and unless these cysteines are oxidized to form disulfide bonds the conotoxins will not possess biological activity. Thus, the 100 mg restriction does not apply to disulfide reduced conotoxin sequences lacking oxidized cysteines, or resin bound synthetic peptides.

FDA approval process and requirements

The US FDA approval process, especially in consideration of translational research (from a university setting to a clinical one) consists of three important stages that typically have extensive timelines (on average it takes approximately 12 years for the entire process). These stages include: preclinical research done by the submitting entity (commercial or university) and the successive filing of an Investigational New Drug Application or IND, Phase I, II, and III clinical trials, and the New Drug Application (NDA) or Biologic License Application (BLA) [49].

Preclinical data necessary for the filing of an IND includes three broad areas of information. The first area of information is the animal pharmacology and toxicology studies. These studies are often done on mice or other mammals and must demonstrate that the compound is safe for human use. The second area is the manufacturing information. This information must include compound composition, information about the manufacturer itself, compound stability, and demonstrate that the company is able to maintain a high degree of product reproducibility. The final area of information is the clinical protocols and investigator information. This information must include detailed protocols for the clinical study and states what risks may be involved. This final area also determines whether the physician(s) in charge of the study is/are qualified to meet the study's requirements. Lastly, this area dictates that the company must demonstrate "commitments to obtain informed consent from the research subjects, to obtain review of the study by an Institutional Review Board (IRB), and to adhere to the investigational new drug regulations" [50].

Once the IND has been accepted 30 days must pass before the phase I clinical trial may begin to allow the FDA to complete a review on the prospective study [49]. Clinical trials typically consist of three phases: phase I, phase II, and phase III [51]. Phase I trials consists of a small group of patients and is undertaken to evaluate safety, effective dosage range, and identify any side effects. This process may take approximately one year. In phase II trials the drug candidate is given to a larger and diverse population of patients and further reviewed for safety for a longer time period, this typically takes a two-year period to complete. Finally in phase III trials the drug candidate is further reviewed for safety in an even larger group of patients and is compared to the current standard of therapy if one exists, a process that may take additional three years. Occasionally phase IV trials may be conducted post-marketing to gather additional information on various populations; this approach was required for the approval of Prialt®.

Once the trials have concluded and the data has been adequately reviewed, the drug sponsor may file for an NDA or BLA depending on whether the compound is of natural or synthetic origin. The NDA includes "full information on manufacturing specifications, stability and bioavailability data, method of analysis of each of the dosage forms the sponsor intends to market, packaging and labeling for both physician and consumer, and the results of any additional toxicological studies not already submitted in the Investigational New Drug application" [49]. Once this process has been completed the drug may be marketed to the public.

Future direction for conotoxin regulation – Bioengineered conotoxins

Conotoxins and Conopeptides have been the subject of extensive bioengineering and chemical manipulation [52]. Their ease of synthesis has positioned them as primary choice among peptide chemists who manipulate the native peptide to enhance stability and activity. The resulting bioengineered peptide with augmented properties has proved to be an invaluable tool in studies pertaining to structural activity relationships, drug leads and ion channel characterization. The development of bioengineered conotoxins presents special cases and equivocal situations that may require amendments to the current regulations.

Conotoxins contain numerous Post-translational Modifications [17]. These PTMs are responsible for phyla and receptor selectivity. Removing some or all of the PTMs can change receptor selectivity [24]. Through bioengineering, a native conotoxin not active in mammalian receptors can be modified to have activity in mammalian receptors. Peptidomimetics has further advanced the field of conopeptide bioengineering. Allowing researchers to incorporate synthetic residues or combine active regions of various peptides, giving them the ability to create a new peptide with enhanced properties [53]. With the advent of Native Chemical Ligation (NCL) the linking of a peptide's N- terminus to its C-terminus has been successfully performed [54]. Conotoxins, with their numerous cysteine residues, present themselves as a prime target for cyclization. Cyclization is known to enhance the stability of conotoxins. This synthetic strategy has been applied to Vc1.1, α-conotoxins AuIB, RgIA and MII using NCL; the cyclized analogs have been shown increased oral bioavailability and systemic stability [44,54-56]. In addition to the use of synthetic chemistry, cyclization has also been accomplished through advanced molecular biology techniques such as interim mediated ligation, which is a potential route for future recombinantly-produced cyclic conotoxins [57]. Another approach to increase the *in vivo* stability is using cystathione and dicarba linkages in replacement to essential disulfide bonds. This approach has been successfully applied in α-conotoxin ImI [58]. Enhancing oral stability of conotoxins provides a new route of administration and hence requires a reassessment of current conotoxin directives.

Bioengineering of conotoxins has undoubtedly opened new challenges for regulation. A potential new approach to regulating conotoxins is to distinguish native and non-native peptides. Combining all forms of conotoxins in one all-encompassing umbrella is not the way

forward. Distinguishing between various types of conotoxins will allow better classification and undoubtedly lead to enhanced regulation.

Conflict of Interest

Authors state that there is no conflict of interest.

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