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Review paper

Spider venom: Reprogramming nature's peptide arsenal for future therapeutics

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Abstract

Background and purpose: Spider venom peptides are chemically diverse scaffolds with high potency, selectivity and structural stability, but native toxins often require optimization for therapeutic use. This review examines strategies for reprogramming spider venom peptides to improve selectivity, stability, safety and delivery. **Experimental approach:** Recent literature on venomics, sequence redesign, motif grafting, lipidation, terminal modification, peptidomimetic tuning, ligation and synthesis and delivery engineering was examined. **Key results:** Inhibitor cystine knot peptides provide robust scaffolds for ion-channel modulation, while linear peptides, enzymes and acylpolyamines broaden chemical space. Transcriptomics, proteomics, computational mining and machine learning accelerate discovery and functional prioritization. Rational substitutions, hybridization, lipidation, terminal editing, synthesis and nanocarrier engineering can improve pharmacological and developability profiles, although trade-offs remain. **Conclusion:** Spider venom research is moving from toxin discovery toward developability-focused reprogramming. Future progress requires integrated structural biology, medicinal chemistry, scalable synthesis and delivery science to address short half-life, off-target activity, manufacturability and venom scarcity.

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Keywords

Venomics; inhibitor cystine knot; rational sequence design; drug delivery; ion-channel modulation

Introduction

Pharmacology regarding the complex biomolecular cocktail used by spiders is primarily associated with their defence mechanisms and ecological role as predators [1-3]. Venom's success depends on its ability to disrupt crucial physiological processes in prey after injection with the chelicerae, thereby reducing the prey's escape capabilities and incapacitating predators [3]. Spider venom toxins encompass peptides, proteins, enzymes, polyamines, salts and small organic molecules [4]. However, the majority of identified spider venom toxins are disulfide-rich peptides, which constitute the primary toxic component in many spider species [5]. These short, cysteine-rich peptides typically function as neurotoxins, affecting ion channels and thus contributing to the central neurochemical immobilization observed in spider envenomations [6]. Spider toxins predominantly target ion channels, with voltage-gated sodium (NaV) and calcium (CaV) channels being particularly well-

represented in current databases [7]. This specialization is evident in insecticidal toxins that modulate insect NaV and CaV channels, such as PPTX-04, which binds to a distinct NaV receptor site and ω-Atypitoxin-Cs1a, a potent insect CaV inhibitor [8]. Furthermore, certain toxins exhibit differential activity across various invertebrate and vertebrate channels, supporting the idea that spider venoms have evolved diversified pharmacological mechanisms for efficient predation and defence, rather than a single universal mechanism [9].

Peptides derived from spider venom are valuable probes in molecular research and promising drug candidates due to their critical role in regulating cell excitability, secretion, sensation and contraction [10]. Spider toxins demonstrate high affinity and specificity in modulating ion channels, as well as GPCRs, proteinases and membrane-associated targets [11]. This broad targeting is significant because ion channels and GPCRs represent major drug target classes in biomedicine [12]. In spider venoms, peptides are a primary focus for translation due to their high target recognition, small, engineered structure and defined disulfide scaffolds [13]. The inhibitor cystine knot scaffold is particularly attractive due to its extraordinary conformational and proteolytic stability, combined with potent activity at therapeutically relevant targets [14]. This stability and selectivity underscore the importance of spider venom peptides in research areas such as pain, stroke and channelopathy, including peptides active on NaV1.7-, NaV1.8-, CaV2.2-, TRPA1- and ASIC1a-channels [15]. Several examples already illustrate how native spider peptides illuminate disease-relevant biology: Hi1a inhibits ASIC1a and demonstrates neuroprotective and cardioprotective potential in ischemic injury, while PcTx1- and Hc3a-like peptides reveal how small sequence changes alter ASIC1a conformational pharmacology [16]. Similarly, NaV-active tarantula toxins continue to define subtype-selective pharmacophores, as seen with Lv1a's preference for NaV1.6 and Tl1a's modulation of NaV1.8 alongside off-target activity at NaV1.5 and KV2.1 [9]. Not all promising spider venom components are classic knottins; linear peptides, venom enzymes and acylpolyamines also expand the pharmacological space available for therapeutic design [17]. Spider and other venom-derived peptides can also act through membrane disruption or cell penetration, which is especially relevant to antimicrobial, anticancer and drug delivery applications [18].

Modern venomics has significantly expanded access to spider peptide diversity by integrating transcriptomics, proteomics and functional assays [11]. Species-level studies now routinely uncover large toxin repertoires, including 98 nonredundant toxins in *Lycosa coelestis*, 190 toxin-like peptides in *Heteropoda pingtungensis* and 102 toxin-like peptides in Pandercetes [19]. Protein-level and electrophysiological validation remain essential because sequence discovery alone does not reveal pharmacological function, and many abundant venom peptides still lack target assignment [20]. Existing screening studies highlight this gap, with some fractions or newly synthesized peptides showing activity on NaV1.7, CaV, Kv4, hKv1.5, or Cav1.2, but comprehensive coverage in broader selectivity maps remains incomplete [21]. Improvements in computational methods are beginning to alleviate this annotation problem by using sequence or structural features to predict ionic channel class, subtype preferences and venom pharmacophores [22]. High-throughput and display efforts have also now expanded beyond traditional ion channels to include peptide ligands targeting GPCRs and other membrane targets, thereby broadening the range of possible therapeutic targets found within venom scaffolds [23].

Despite a positive trend in biodiscovery, most reviews have focused on the diversity, coverage, or therapeutic potential of native toxins, rather than on the engineering approaches used to transform them into therapeutics [24]. The current definition of a therapeutic peptide is too narrow, as it must encompass not only potency but also half-life, protease resistance, tissue exposure, selectivity, manufacturability and safety [25]. Challenges in peptide drug research can be addressed through various approaches, with structural modifications proving particularly successful. These include chemical modification, peptide conjugation, cyclization, noncanonical substitution and delivery engineering [26]. Similarly, the havedy of

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venom peptides has advanced significantly through methods such as heterologous expression, total synthesis, ligation chemistry and rational peptide design. These techniques have facilitated access to protease toxins with limited availability and enabled the creation of engineered variants [27]. For instance, studies across various venom systems demonstrate that native peptides are often not optimized for mammalian targets, requiring improved resistance to disulfide scrambling, reduced toxicity, or better biodistribution profiles to become viable therapeutics [13]. The concept of using natural venom templates as starting points rather than final structures has been successfully applied in non-spider systems to generate numerous less toxic analogs that retain anti-infective properties [28]. This same logic can be extended to subtype selectivity, as evidenced by recent work characterizing NaV1.7 and NaV1.8 pharmacophores within spider scaffolds and the availability of recombinant or synthetic peptides such as Phα1β, Tl1a and Hi1a [14].

Evolutionary and chemical foundations of spider venom

Spider venoms exhibit remarkable chemical diversity yet possess an unusual evolutionary trajectory. Over 90% of spiders primarily direct their toxic arsenal toward disulfide-rich peptide scaffolds, particularly ICK-like knottins. These are complemented by other linear peptides, enzymes and low-molecular-weight components that fulfil crucial ecological and translational roles.

Evolution of venom scaffold

While spider venoms are chemically complex mixtures comprising peptides, proteins, enzymes, polyamines, salts and various low-molecular-weight compounds, their evolutionary architecture is predominantly shaped by disulfide-rich peptides rather than a multitude of unrelated scaffold families. A comprehensive comparative phylogenetic study identified 78 novel spider toxin superfamilies, providing evidence of deep common ancestry among many spider toxin families characterized by high concentrations of disulfide bridges. This study traced the shared ancestry to a primordial cystine-knot-like precursor, termed "Adi Shakti," which emerged approximately 375 million years ago in the common ancestor of Araneomorphae and Mygalomorphae. This pattern distinguishes spiders from many other venomous animals. In snakes, venoms are assembled from multiple phylogenetically unrelated toxin families, whereas nearly all spider lineages predominantly rely on a single broad molecular solution: disulfide-rich peptide toxins. Shaikh and Sunagar [5] further observed that disulfide-rich peptides constitute approximately three-quarters of spider venom and dominate the venoms of nearly all spiders, despite significant divergence in prey ecology and lineage history. At a finer scale, scaffold diversification appears to have primarily proceeded through gene duplication, mutation and selection acting on a structurally robust cysteine-rich core. The expansion of ICK neurotoxins can be extreme within a single species, with one funnel-web spider reportedly containing as many as 13 categorized ICK superfamilies. Genomic research in *Hadronyche cerberea* provides mechanistic insight, showing that all ICK toxin genes share a conserved three-exon architecture and that transposable elements may have facilitated repeated gene expansion [29]. A schematic representation of the inhibitory cystine knot (ICK) motif is presented in Figure 1.

Evolution has also generated more complex architectures from the ancestral cystine-knot module. Bivalent spider toxins, containing tandem ICK domains, appear to have arisen through domain duplication, followed by lineage-specific expansion and recurrent domain loss. This indicates that the spider venom's "single scaffold" strategy does not imply structural monotony; instead, the knot scaffold functions as an evolvable platform that can be elaborated into single-domain, double-domain and unusually cysteine-rich variants with altered target range or avidity [30]. Recent functional studies also suggest that some pharmacophores predate major spider lineage splits. The Old-World tarantula *Lampropelma violaceopes* contains a peptide that preferentially interacts with Nav1.6, with activity similar to that found in New World

tarantulas. This supports the hypothesis that at least some of the sodium-channel-targeting venom motifs emerged before the modern geographic divergence of these two tarantula groups [9].

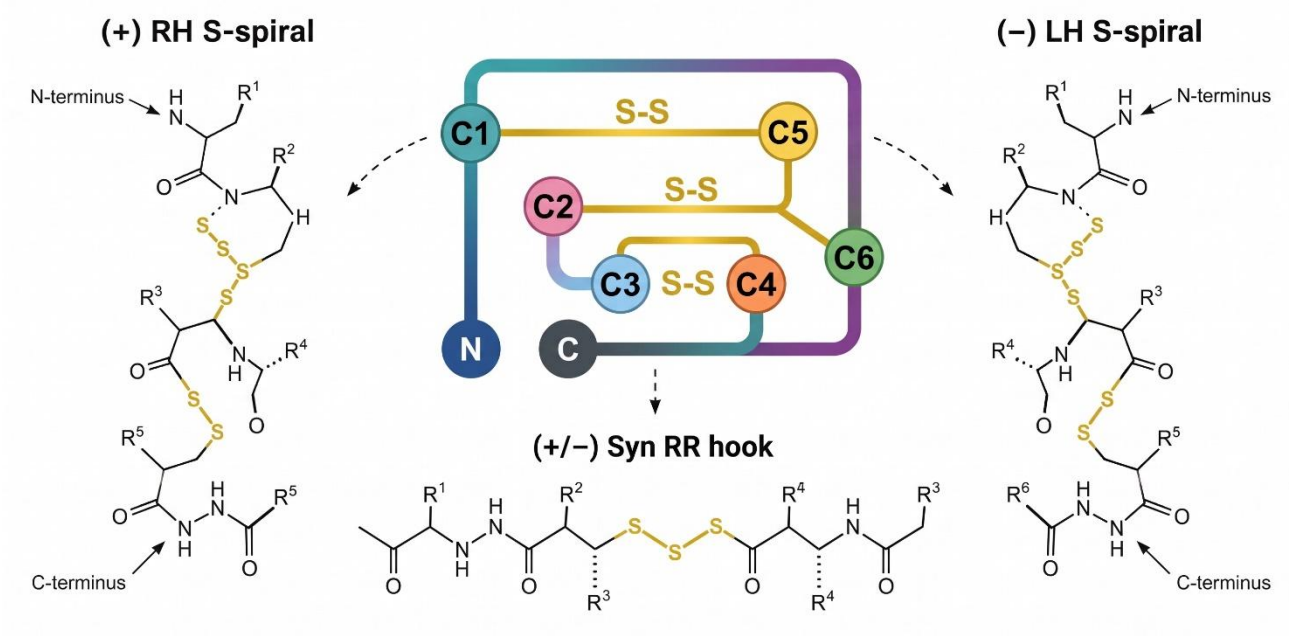


Figure 1. Schematic representation of the inhibitory cystine knot (ICK) motif, illustrating the characteristic disulfide-bond topology formed by six conserved cysteine residues (C_I-C_VI). The central knot architecture highlights the three disulfide bridges that stabilize the compact peptide scaffold, while the surrounding molecular representations depict the characteristic right-handed (RH) spiral, left-handed (LH) spiral and syn RR hook conformations associated with the ICK fold. This highly conserved structural framework confers exceptional thermal, chemical and proteolytic stability, making ICK peptides a prominent scaffold in spider venom toxins and an attractive template for therapeutic peptide engineering

Major chemical classes

Spider venom chemistry is not exclusively dominated by neurotoxins. While disulfide-rich neurotoxins have been the most extensively characterized to date and are considered a primary functional motif in many venoms, predominantly interacting with ion channels such as NaV, KV, CaV, ASIC, TRP and P2X receptors [1], as cited in Table 1, the field of venomics has recently expanded its understanding of other significant venom components. A substantial repository of cysteine-rich toxins has been amassed across various spider species, including 38 disulfide-rich neurotoxins in *Macrothele yani*, 98 peptide toxins in *Lycosa coelestis* and 190 non-redundant toxin-like peptides in *Heteropoda pingtungensis* [19]. However, linear peptides are increasingly recognized as crucial venom constituents, moving beyond their historical perception as rare curiosities. For instance, transcriptomic analysis of *Cupiennius salei* revealed four precursor families responsible for expressing 179 linear cationic peptides, indicating a significant investment in this peptide class by some spiders [31]. Similarly, in *Lycosa poonaensis*, transcriptomics identified 15 contigs encoding 17 linear peptides. All tested representatives exhibited antibacterial activity and adopted amphiphilic α-helical conformations. Mass spectrometry of the same species further detected 120 non-disulfide-bridged peptides among 401 venom components, underscoring the potential numerical significance of linear peptides [32].

Linear peptides also demonstrate functional diversity. Some serve as antimicrobial protectants, potentially inhibiting microbial growth during prey capture or ingestion, while others contribute directly or synergistically to insecticidal or cytolytic activities. Nevertheless, their phylogenetic distribution appears narrower than that of cysteine-rich toxins, having primarily been identified in certain araneomorph families, particularly within the RTA clade [31]. Enzymes represent a clear example of a historically overlooked venom

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class. Modern venomics reveals that spider venoms contain numerous high-molecular-weight proteins with putative or validated enzymatic activity, despite decades of research focusing on short ICK peptides.

Table 1. Major spider venom component classes and their translational relevance

Class	Structural features	Main targets or roles	Translational relevance	Key constraints	Ref.
ICK peptides	Disulfide-rich knot architecture with strong thermal, chemical and proteolytic stability	Frequently modulate ion channels, especially NaV subtypes	Best-developed spider venom scaffold for drug discovery and analgesic engineering	Selectivity, off-target activity and delivery still limit translation	[33]
Linear peptides / AMPs	Non-disulfide or short cationic membrane-active peptides	Antimicrobial, cytolytic and sometimes anti-inflammatory activities	Expand chemical space beyond classic neurotoxins and support anti-infective design	Cytotoxicity and stability can be limiting	[34]
Acylpolyamines	Chemically modular small venom components	Selectively target ionotropic glutamate and nicotinic receptors	Useful templates for synthetic analogues, probes and transport concepts	Comparatively underdeveloped translationally	
Enzymes and proteins	Larger venom proteins including hyaluronidases, phospholipases and proteases	Auxiliary venom functions and possible biomedical or industrial applications	Underexplored class becoming more accessible through recombinant methods	Scarcity and difficult experimental access	[4]
CPP-like toxins	Cell-penetrating peptide-like frameworks	Bioactive agents and cargo-delivery modules	Potential intracellular delivery and tissue-directed transport platforms	Functional validation remains limited	
Bradykinin-like / kinin-family peptides	Short peptides resembling endogenous peptide hormones	Smooth muscle contraction, capillary permeability, ACE-related effects	Illustrate non-ion-channel pharmacology in spider venoms	Sparse translational development so far	[35]

Comparative analysis of validated venom enzymes indicates their roles in toxicity, prey pre-digestion, venom preservation, spreading and venom component activation. Functional profiling of 10 spider venoms identified enzymes from 6 classes and highlighted the presence of significant hyaluronidase activity, disproving the notion that enzymes are merely "heavy residue" in venom and confirming their active role in envenomation [1]. An extremely high enzyme content is also observed in the venoms of some medically relevant taxa, such as *Sicarius thomisoides* venom, which is dominated by phospholipase D, astacin-like metalloproteinases and serine proteases [36].

Other distinct chemical classes include spider acylpolyamines. These compounds are chemically and pharmacologically distinct from peptide neurotoxins, belonging to a venom fraction that includes monoamines and low-molecular-weight polyamine toxins. Their primary functions involve inhibiting ionotropic glutamate receptors, particularly AMPA, NMDA and kainate receptors, as well as nicotinic acetylcholine receptors. The ease of scaffold modification and chemical tractability of acylpolyamines have already inspired the synthesis of analogues with nanomolar potency and receptor selectivity, in addition to fluorescent and photolabile probe derivatives for receptor mapping and ligand design [17].

Structural determinants of activity

For toxins with abundant disulfide bonds, activity is intrinsically linked to the stability of the protein fold. The most extensively studied spider venom scaffold, the Inhibitor Cystine Knot (ICK), possesses a rigid three-dimensional structure that confers significant resistance to thermal stress, proteolysis and harsh chemical environments [2]. Experimental evaluation of the tarantula peptide GTx1-15 further demonstrates this remarkable stability: the peptide remained undegraded in rat and human plasma *in vitro* at 37 °C for 24 h,

exhibited detectability in circulating plasma over several hours, and displayed high heat stability with no measurable cytotoxicity or immunogenicity in the tested system [37]. This inherent robustness likely contributes to the appeal of spider knottins as a bioengineering tool. Their constrained folds surprisingly tolerate substantial functional diversification while maintaining a stable core configuration, facilitating both natural evolution and synthetic redesign [38]. Even minor modifications to the ICK framework can preserve or enhance function. For instance, Tb0-IT2 features an additional five disulfide bonds within an adapted ICK fold, which is believed to enhance its conformational stability. Similarly, Phα1β incorporates an ICK N-terminal domain and a disulfide-stapled C-terminal helix, representing another example where scaffold embellishment uniquely alters membrane interactions and the control of gating-modifiers by Phα1β pharmacophores [14]. Schematic ribbon representations of the principal molecular classes found in spider venoms are presented in Figure 2.

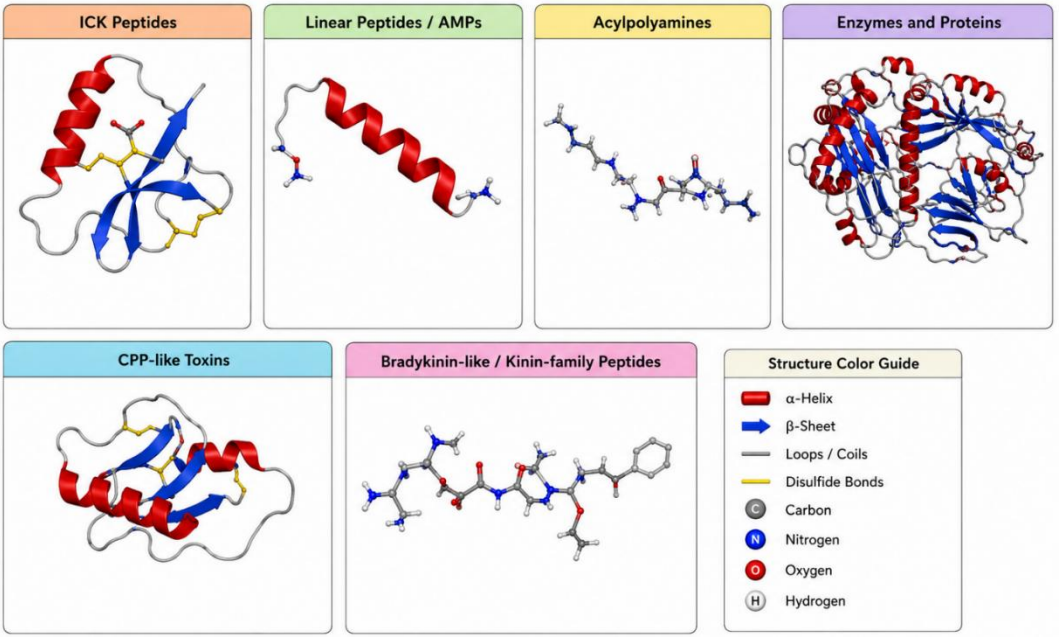


Figure 2. Schematic ribbon representations of the principal molecular classes found in spider venoms, including inhibitor cystine knot (ICK) peptides, linear antimicrobial peptides (AMPs), acylpolyamines, enzymes and proteins, cell-penetrating peptide (CPP)-like toxins and bradykinin-like (kinin-family) peptides

Linear spider peptides operate under different structural principles. Their activity typically necessitates an inducible amphipathic α-helical structure, a positive charge and specific hydrophobic patterning, which enable interactions with negatively charged microbial and/or cellular membranes [39]. While these peptides generally bind to membranes with amphipathically oriented helices, the angle and depth of membrane insertion correlate with their potency. Potent lytic amphiphiles induce strong α-helix orientations at shallow depths, whereas selectively antibacterial peptides exhibit deeper insertion but less intense α-helix orientations [40]. For *L*. *poonaensis*, peptide activity was dictated by net charge, basic/acidic residue sequence and hydrophobicity. In one linear peptide, introducing a single acidic residue on the hydrophilic face reduced antibacterial activity, attributed to disruption of the amphipathic helix's function [32]. Subsequent engineering research has demonstrated the tunability of these membrane-active principles. A significant enhancement in membrane interaction, bacterial selectivity and an overall reduction in eukaryotic toxicity were observed for the LyeTx I-b analog, concomitant with increased amphipathicity [41]. Similarly, stability-improved variants of the amphipathic spider peptides LVTX-8 retained or enhanced anti-cancer activity while reducing haemolytic activity, illustrating that a framework active (and potent) on cell membranes can be "reprogrammed" for therapeutic applications [42]. In summary, the evolutionary and chemical underpinnings of spider venom are characterized by a long-standing reliance on a disulfide-rich scaffold economy,

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complemented by secondary classes such as linear peptides, enzymes and acylpolyamines. This combination of ancient scaffold conservation and chemical-functional diversification positions spider venom as a robust platform for future therapeutic reprogramming.

Venomics and discovery platforms

Current spider venom discovery platforms operate on a well-established methodological consensus. While venom-gland transcriptomics and cDNA libraries have significantly expanded the known peptide diversity, transcript-only catalogues can inflate component counts. Consequently, protein-level confirmation is increasingly recognized as the evidentiary standard. Computational mining has further broadened access to understudied taxa and venoms with low yields; however, functional annotation continues to lag far behind sequence discovery, particularly outside the dominant ion-channel toxin families [1].

Transcriptomics, cDNA libraries and proteo-transcriptomics

Venom-gland sequencing revolutionized spider venomics because many spiders produce insufficient venom for classical activity-guided purification alone, thereby enabling transcript discovery from just a few glands or limited biological material [19]. High-throughput sequencing further advanced this by facilitating de novo venom-gland transcriptomes without prior genomic references, which is especially valuable for rare, non-model, or newly sampled spider species [43]. Species-level studies demonstrate the substantial expansion of apparent toxin diversity achieved through these methods (Table 2). For instance, in *Heteropoda pingtungensis*, a combined venom-gland cDNA library and RNA-seq strategy identified 190 non-redundant toxin-like peptides grouped into 13 families, while RP-HPLC and MALDI-TOF confirmed 110 peptides physically present in the venom [19] In *Lycosa coelestis*, a cDNA library combined with transcriptomics identified 98 non-redundant toxins across 11 families and 147 peptides detected in venom by RP-HPLC and MALDI-TOF [19]. Similarly, in *Pandercetes sp*., cDNA-library analysis yielded 102 non-redundant toxin-like peptides across 11 families, and mass spectrometry detected 154 peptides spanning 1 to 8 kDa [44].

Table 2. Venomics platforms expanding spider toxin discovery

Study system	Discovery approach	Quantitative yield	Contribution	Key caveat	Ref.
Heteropoda pingtungensis	cDNA library plus RNA-seq	991 ESTs; 531 toxin-like; 190 nonredundant toxin-like peptides in 13 families	Two novel NaV1.7 antagonists and many unknown scaffolds	Targets for most toxins remained unpredictable because of low homology	[19]
Lycosa coelestis	cDNA library plus transcriptomics and peptidomics	1131 ESTs; 98 nonredundant toxins in 11 families; 147 peptides detected in venom	Built a foundation for new calcium-channel-active toxin discovery	Functional characterization still lagged sequence discovery	[45]
Four-species comparative venomics	LC-MS/MS plus venom-gland transcriptomics	284 cysteine-rich peptides characterized, including 111 ICK toxins	Showed broad cross-family conservation of ICK toxins	Comparative evolutionary interpretation remains limited by few cross-species studies	[46]
Macrothele washanensis	Transcriptomics, proteomics, bioinformatics	147,492,716 reads; 69 putative toxins in 25 superfamilies; 43 toxin proteins identified	Expanded enzyme and peptide diversity in a newly described species	Biomedical function remains largely prospective	[47]
ArachnoServer 3.0 / bioinformatics infrastructure	Curated database plus automated transcript detection pipeline	Estimates >10 million unique spider venom peptides overall	Standardizes annotation, discovery and mass-based matching	Automated discovery remains difficult because of sequence heterogeneity and false positives	[48]

Transcriptomics has been equally crucial for underappreciated peptide classes. In *Cupiennius salei*, 454 and Illumina venom-gland sequencing revealed four precursor families encoding 179 linear peptides, significantly exceeding the 26 linear peptides previously recovered by classical proteomics and Edman

sequencing [31]. The same study found that linear peptides accounted for 24 % of annotated contigs, surpassing disulfide-containing neurotoxins at 15 %, demonstrating that transcriptomics can reveal venom architectures overlooked by a neurotoxin-centric view. In *Lycosa poonaensis*, transcriptomics identified 15 contigs encoding 17 linear peptides, all of whose representative synthesized peptides exhibited antibacterial activity and amphipathic α-helical structure [32]. Proteo-transcriptomics adds a necessary orthogonal layer by distinguishing expressed transcripts from actual secreted venom components and resolving mature peptide processing. This is now evident across various taxa, including spider studies such as *Macrothele yani*, where integrated genome, venom-gland transcriptomics and venom proteomics identified 194 venom toxins, including 38 disulfide-rich neurotoxins and 12 ICK peptides [49]. Comparable multi-omics logic has also been emphasized in broader venom research, where proteomics confirms translation, secretion and post-translational processing that transcriptomes alone cannot establish [50].

It is important to acknowledge that transcriptomes can underestimate diversity at the core. Modern reviews explicitly state that overestimations of venom complexity can arise from interpretations of assembly artifacts and confidence levels of transcript predictions. They emphasize that the most conservative venom composition predictions should only include components that have been isolated, sequenced and characterized *via* mass spectrometry [1]. This is particularly true for spiders, where normalized cDNA libraries and deep sequencing can identify rare transcripts in great abundance, but most of these low-copy candidates are not definitively known to exist until confirmed by proteomics [19].

Computational mining and AI

Modern computational approaches have enabled venom discovery to advance beyond traditional biochemical methods, which primarily focus on isolating venom components from crude venom. Contemporary workflows frequently integrate next-generation sequencing (NGS) and de novo assembly, homology searches and predictions of signal peptides, cleavage sites, structure and function to identify mature venom peptides from transcriptomes. This has already facilitated the discovery of novel compounds in understudied taxa and limited samples, a particularly valuable capability for spider systems. A representative pipeline, applied to *Argiope bruennichi*, involved extensive analysis of venom-gland transcript homology (comparing venom and molecular library sequences), extraction of mature regions, in silico prediction of molecular activity and subsequent application of AI tools for functional prioritization and experimental validation of two selected molecules as AAPs. In another instance, with *Pardosa astrigera*, a BLAST-based transcriptome screening followed by functional and structural prediction identified Lycotoxin-Pa2a, which subsequent testing showed to possess both antibacterial and anti-inflammatory properties [51]. These studies demonstrate that computation is no longer merely a post hoc annotation tool but an integral part of candidate selection. More advanced pipelines now incorporate structure prediction, toxicity filtering, docking and developability triage prior to synthesis. For example, in a 2025 anticoagulant peptide study, translated venom-gland sequences were filtered based on secretion signals, peptide length, AlphaFold2 structure prediction, and predicted toxicity and haemolysis, then docked against activated protein C. Biochemical validation subsequently identified GC38 as an antithrombotic lead [2]. Similar design principles are evident in antimicrobial engineering studies, where machine learning (ML)-assisted property prediction guided the redesign of spider-derived templates into higher-performing hybrid or multifunctional peptides. An overview of machine learning-driven peptide discovery and optimization workflows is presented in Figure 3.

Neural network screening has already achieved library-scale application for spider linear peptides. Mironov and colleagues assembled a library of approximately 2000 known spider venom linear peptides, fragmented them into 75,235 sequences of 9-22 residues, and utilized pre-trained neural networks to predict

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anti-MRSA activity prior to synthesis and mechanistic validation [52]. Conceptually, this represents a critical paradigm shift from "finding a native toxin" to "mining a native peptide space for functional fragments." The broader field of venom informatics is moving in the same direction. Currently, AI is recognized for its utility in prediction, modelling, interaction, ranking and generative redesign, as highlighted in recent reviews [53].

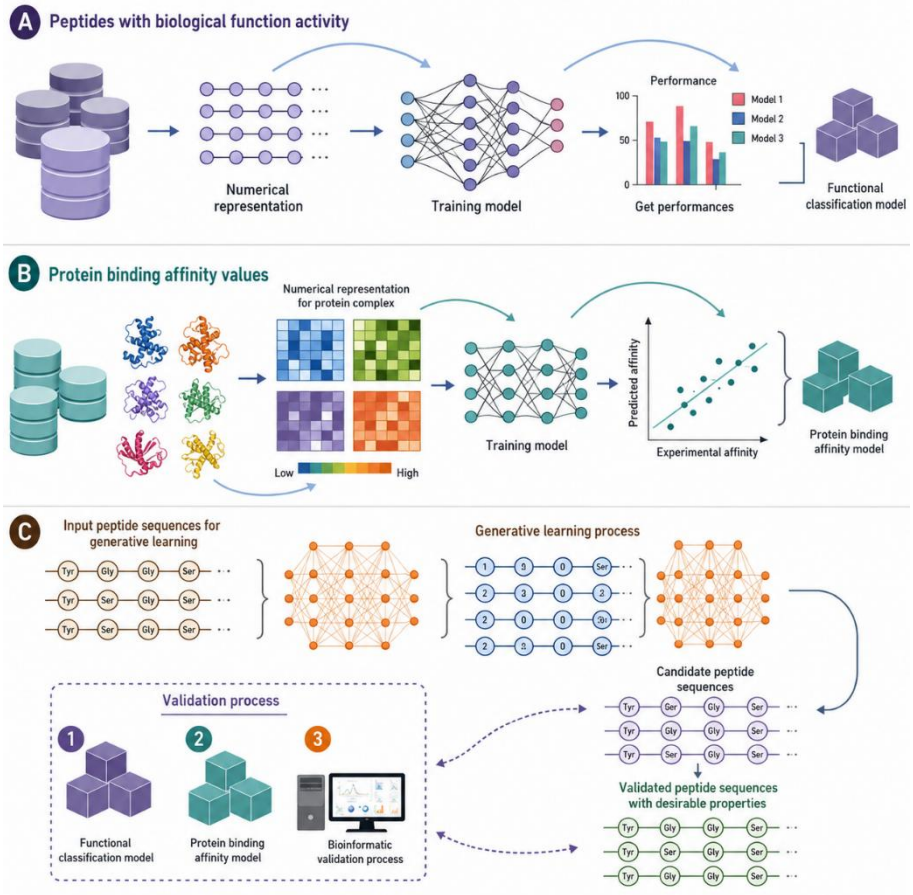


Figure 3. Overview of machine learning-driven peptide discovery and optimization workflows. (A-1) Pipeline for functional peptide classification, functional peptide datasets were converted to numerical sets, trained as predictive models, tested and deployed for functional peptide classification. (B) Protein–peptide binding affinity prediction workflow: encoding of protein–peptide complexes, development of machine learning models, validation by regression analysis and prediction. (C) Generative learning framework for peptide design, in which peptide sequences are used to train deep generative models that produce candidate peptides, followed by functional classification, binding affinity prediction and bioinformatics-based validation to identify peptides with desirable biological properties. This schematic highlights the integration of predictive and generative artificial intelligence approaches for accelerated peptide engineering and rational therapeutic peptide discovery. Adapted from Ref. [53]. under the terms of the Creative Commons Attribution (CC BY 4.0) license

A machine learning classifier has also been developed to predict the most probable ion-channel targets of cysteine-rich venom peptides for a given taxon, offering a method for functional annotation without extensive electrophysiology testing [22]. At the discovery-platform level, phage display and sequencing-assisted molecular display systems are beginning to complement transcriptomics by directly screening venom-inspired scaffold libraries against therapeutic targets [23].

Discovery-to-function bottlenecks

The primary bottleneck in venom research has shifted from sequence acquisition to functional interpretation. For instance, in *Heteropoda pingtungensis*, most identified toxins had unpredictable biological targets due to a lack of homology to toxins with known functions [19]. Similarly, in *Pandercetes sp.*, the biological functions of major toxins remained largely unknown even after cDNA-library and mass-spectrometric characterization [44]. A broader machine-learning study further indicates that most venom peptides still lack

characterized ion-channel targets or mechanisms of action [22]. This bottleneck is particularly pronounced outside dominant neurotoxin families. While disulfide-rich ion-channel toxins benefit from better homology anchors, extensive electrophysiology literature and clearer translational targets, linear peptides, enzymes, short modified peptides and high-molecular-weight venom proteins remain patchily annotated [54]. Spider venom enzymes exemplify this issue: recent syntheses of proteo-transcriptomic datasets reveal that many enzymes, though documented in publications, are underrepresented in public databases, directly impeding annotation and biodiscovery efforts. A secondary bottleneck lies in technical validation. While transcript-based discovery can nominate numerous candidates, confirming mature processing and post-translational modifications (PTMs) often proves challenging. Conformational states frequently cannot be determined, and true secretion into venom can only be confirmed through proteomics or direct biochemical analysis [55]. This is crucial because activity may depend on the exact structural state; for example, Pn3a converts to a different conformer with altered activity, meaning a transcript or even a peptide mass alone does not reliably indicate the correct functional state [56]. A third hurdle involves translating *in vitro* hits into therapeutic leads for human use. The venom peptides reviewed here share common limitations, including proteolysis, short half-lives, delivery issues and off-target toxicity. These challenges necessitate sequence engineering, cyclization and/or conjugation to address issues identified since their initial discovery [57]. This is illustrated by cases such as LVTX-8, where analog design enhanced proteolytic stability and reduced haemolysis and LyeTx I-b, where rational structural modification improved natural selectivity. The ability to rapidly discover candidate spider venom peptides, despite challenges in proving their functional activity, highlights a significant disparity in the research landscape.

Reprogramming strategies for spider venom peptides

Rational sequence redesign

Rational sequence redesign of spider venom peptides offers a viable and increasingly successful approach to generating drug-like leads with enhanced potency, subtype selectivity, safety and metabolic stability. A review of recent literature reveals common themes in redesign strategies, including specific substitutions of channel-contacting residues, engineering of membrane-interacting hydrophobic and electrostatic surfaces, terminal modifications, truncation, hybridization and conformational constraint. NaV-targeting inhibitor cystine knot peptides are particularly noteworthy, supported by strong mechanistic evidence. Single or dual substitutions can induce dramatic changes in topology, subtype preference and safety-relevant activity, as exemplified by designs related to Huwentoxin-IV (HwTx-IV), ProTx-II, GpTx-1 and analogues of Ssp1a [58]. Figure 4 illustrates the sequence alignment, disulfide-bond connectivity and molecular masses (MM) of wild-type Huwentoxin-IV (HwTx-IV) and its 19 engineered analogues. Concurrently, peptides with membrane-disruptive activity, such as LVTX-8, LyeTx-I, latarcin-derived analogues and newly developed transcriptome-activity-based leads, demonstrate that redesign can decrease haemolytic activity and improve proteolytic resistance while retaining or strengthening antimicrobial activity [59].

The premise of rational redesign is that native spider toxins are evolutionarily optimized for ecological fitness rather than therapeutic performance. Consequently, their native state can present both advantages and disadvantages, including poor subtype selectivity, toxic effects on host cells, rapid proteolysis, or, in some cases, difficulties in delivery mechanisms [60]. Recent advancements have shown that these limitations can be overcome without abandoning the native scaffold, as the pharmacological properties of venom peptides are often dictated by a small number of exposed residues rather than the entire molecular structure [61]. General redesign principles have emerged, with most strategies for NaV-modulating spider peptides falling into three categories: those designed to directly interact with channel residues, those used to tune residues controlling peptide membrane partitioning and those used to alter the overall surface charge/hydrophobicity profile to

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rebalance affinity, selectivity and tolerability [62]. This framework aligns with structural studies of NaSpTx family I toxins, which indicate that hydrophobic residues mediate membrane engagement prior to channel binding, while residues in loop 1, loop 4 and the C-terminus interact with the domain II S3-S4 region of NaV1.7 [61].

Peptides	Sequences	MM (Da)
	1 ... 35	
HwTx-IV WT	ECLEIFKACNPSNDQCCKSSKLVCSRKTRWCKYQI*	4107.20
HwTx-IV $E^{1}G$	GCLEIFKACNPSNDQCCKSSKLVCSRKTRWCKYQI*	4031.93
HwTx-IV $E^{1}G$	ECLGIFKACNPSNDQCCKSSKLVCSRKTRWCKYQI*	4031.93
HwTx-IV $E^{1}K$	ECLKIFKACNPSNDQCCKSSKLVCSRKTRWCKYQI*	4103.01
HwTx-IV $E^{1}R$	ECLRIFKACNPSNDQCCKSSKLVCSRKTRWCKYQI*	4131.01
HwTx-IV $K^{18}A$	ECLEIFKACNPSNDQCCASSKLVCSRKTRWCKYQI*	4046.89
HwTx-IV $K^{32}N$	ECLEIFKACNPSNDQCCKSSKLVCSRKTRWCNYQI*	4089.89
HwTx-IV $R^{26}A$	ECLEIFKACNPSNDQCCKSSKLVCSAKTRWCKYQI*	4018.89
HwTx-IV $R^{26}Q$	ECLEIFKACNPSNDQCCKSSKLVCSQKTRWCKYQI*	4075.91
HwTx-IV $T^{28}W$	ECLEIFKACNPSNDQCCKSSKLVCSRKWRWCKYQI*	4188.98
HwTx-IV $Y^{33}W$	ECLEIFKACNPSNDQCCKSSKLVCSRKTRWCKWQI*	4126.97
HwTx-IV $E^{1}G/E^{1}G$	GCLGIFKACNPSNDQCCKSSKLVCSRKTRWCKYQI*	3959.91
HwTx-IV $E^{1}R/K^{32}N$	ECLRIFKACNPSNDQCCKSSKLVCSRKTRWCNYQI*	4116.95
HwTx-IV $E^{1}K/R^{26}Q$	ECLKIFKACNPSNDQCCKSSKLVCSQKTRWCKYQI*	4074.97
HwTx-IV $E^{1}G/E^{1}G/K^{18}A$	GCLGIFKACNPSNDQCCASSKLVCSRKTRWCKYQI*	3902.85
HwTx-IV $E^{1}K/R^{26}A/Y^{33}W$	ECLKIFKACNPSNDQCCKSSKLVCSAKTRWCKWQI*	4040.97
HwTx-IV C_{ter}COOH	ECLEIFKACNPSNDQCCKSSKLVCSRKTRWCKYQI-COOH	4104.93
HwTx-IV K^{36}/C_{ter}COOH	ECLEIFKACNPSNDQCCKSSKLVCSRKTRWCKYQIK-COOH	4233.03
HwTx-IV $E^{1}K/C_{ter}$COOH	ECLKIFKACNPSNDQCCKSSKLVCSRKTRWCKYQI-COOH	4103.99
HwTx-IV $E^{1}G/C_{ter}$COOH	GCLEIFKACNPSNDQCCKSSKLVCSRKTRWCKYQI-COOH	4032.91

Figure 4. Sequence alignment, disulfide-bond connectivity and molecular masses (MM) of wild-type Huwentoxin-IV (HwTx-IV) and its 19 engineered analogues. Amino acid substitutions introduced in the peptide analogues are highlighted in red, whereas the wild-type (WT) sequence is shown in black. MM correspond to the correctly folded and oxidized peptides following disulfide bond formation. The asterisk (*) denotes C-terminal amidation. Adapted from Ref. [62] under the terms of the Creative Commons Attribution 4.0 International License (CC BY 4.0)

Accordingly, rational redesign is most successful when it integrates both target-interface chemistry and membrane-partition behaviour, moving beyond a simplistic lock-and-key binding model [9]. A second broad principle is the increasing reliance on structure-guided and computation-assisted sequence redesign. Docking, molecular dynamics, Rosetta-based design, transcriptomics-enabled lead discovery and AI-assisted screening are now routinely employed to nominate mutations before synthesis and electrophysiological or microbiological testing. In spider peptide discovery and repurposing, in silico workflows have also expanded access to underexplored venom repertoires and enabled function-oriented redesign through truncation, substitution, or hybridization [51].

The primary design objectives for venom-peptide programs are now relatively clear: increase target potency, sharpen subtype or organismal selectivity, lower haemolysis or mast-cell liability, and improve resistance to proteolysis or other physicochemical failure modes [57]. In ion-channel programs, selectivity over closely related mammalian NaV subtypes is crucial because modest off-target activity at NaV1.4, NaV1.5, or NaV1.6 can translate into skeletal, cardiac, or neuromuscular toxicity [63]. Several sequence-level levers recur in literature. Charge tuning is one of the most common, as cationicity can strengthen microbial membrane targeting in linear antimicrobial peptides; however, excessive positive charge can also increase haemolysis or nonselective membrane disruption [52]. Hydrophobicity tuning is equally important; in channel-targeting ICK peptides, it alters membrane partitioning and toxin docking, whereas in linear peptides, it can determine the balance between bacterial killing and host-cell lysis [40]. Amphipathicity is also a third important handle because, as indicated by antimicrobial spider peptides, redesigning to a more structured amphipathic helix can drive greater selectivity for bacteria while simultaneously lowering damage to eukaryotic membranes [41]. Another high-value lever is terminal chemistry. To adjust charge, reduce exopeptidase sensitivity, and fine-tune activity-to-toxicity ratios, charge-changing modifications such as C-terminal amidation, N-terminal acetylation, hydrazide formation, and pyroglutamate protection are

performed [42]. These changes, however, can also increase stability in antimicrobial conditions, albeit with a cost of haemolytic risk. Hence, when considering stability-enhancing chemistry, it is important to consider not only the antimicrobial activity but also the mammalian cell membrane risk [57].

HwTx-IV is the most recent example of the therapeutic potential achievable through rational substitutions. The peptide is highly selective for the validated pain channel NaV1.7 but could be somewhat hampered in its clinical utility due to its actions on NaV1.6, which could be associated with neuromuscular adverse effects [62]. López and colleagues found that the native human NaV1.7/NaV1.6 selectivity ratio was readily tuned in either direction by targeted mutagenesis, demonstrating that even when channel binding motifs are similar, family selectivity for different subunits is highly sequence dependent. Their structure–activity analysis uncovered three appealing paths for redesign: mutations of channel-binding residues, replacement of lipid-binding residues, and indirect modulation of surface charge/hydrophobicity. Certain control points, such as E1, E4, K18, R26 and Y33, held a privileged position in modulating the relative activity of NaV1.7 and NaV1.6, respectively. Importantly, not every "improvement" was beneficial to the desired profile; E4K was more potent at hNaV1.6, while also losing selectivity, whereas the E4K/R26A/Y33W triple mutation favoured NaV1.7 with a corresponding decrease in safety-relevant off-target activity.

The findings from HwTx-IV are not isolated. A similar principle is evident in the rational design of other NaSpTxs derived from various spider venom scaffolds, where minimal judicious substitutions can dissociate potency from off-target activity. Ssp1a, a double mutant, was generated by combining two single-point mutations. One mutant exhibited enhanced potency across all three subtypes (hNaV1.2, hNaV1.3 and hNaV1.7), while the other demonstrated increased selectivity against a wild subtype. These improvements were attributed to alterations in electrostatic, hydrophobic, π–π and loop-mediated contacts [63]. The results for Tl1a indicate that introducing additional charges within the loop 4 region inhibits NaV1.8, providing direct evidence that perturbing the electrostatic interactions between the domain IV S3-S4 extracellular loop and the loop 4 region leads to a loss of function [15]. Furthermore, ProTx-II programs illustrate that refinement extends beyond achieving selective reprogramming; developability liabilities can also be addressed through redesign. Medicinal chemistry has produced ProTx-II analogues with superior *in vivo* properties, including enhanced ion channel selectivity and reduced non-mechanistic safety concerns like mast-cell degranulation, which is a concern for systemic administration [64]. A computational design initiative identified ProTx-II-based peptides with single-digit nanomolar hNaV1.7 potency and >1000-fold selectivity, demonstrating efficacy in various rodent pain models following intrathecal (spinal) administration [65]. These studies suggest that rational redesign can now harness large natural toxins, which are ideally suited for disrupting ion channels but often lack the optimal pharmacological profile for drug development. Collectively, these studies underscore that rational redesign can transform naturally potent, yet pharmacologically less desirable, tarantula toxins into precision ion channel ligands [58].

Hybridization and motif grafting in spider venom peptides

Hybridization involves linking a peptide scaffold or pharmacophore derived from spider venom with another functional component to impart additional biological properties. A similar approach, called motif grafting, defines a specific functional sequence, such as the amino-terminal Cu(II)- and Ni(II)-binding (ATCUN) motif, and grafts it onto an existing peptide backbone [66]. These methods are particularly useful for linear, amphipathic spider venom peptides that lack disulfide bridges, as their sequences can be extended and fused to generate novel functions. Consequently, these hybrids can engage in membrane interactions within catalytic or intracellular mechanisms, thereby expanding antimicrobial activity beyond membrane interaction to include oxidative stress and nucleic acid damage [67]. When combined with conventional antibiotics, complementary

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mechanisms can also enhance the activity of antimicrobial peptides (AMPs) [68]. More broadly, molecular hybridization combines advantageous properties of various molecular entities and is emerging as a tool for creating multifunctional drugs [69]. These constructs can be further optimized for activity, selectivity and physicochemical properties through computational design and medicinal chemistry strategies [70].

One representative hybrid, KFH-Pa1a, features an ATCUN motif N-terminally introduced into Lycotoxin-Pa1a. The parent peptide was identified from the venom gland transcriptome of *Pardosa astrigera*, and the hybrid demonstrated improved antibacterial activity against various microorganisms, including multidrug-resistant (MDR) *Pseudomonas aeruginosa*. Beyond its membrane-disruptive activity, KFH-Pa1a also promoted intracellular reactive oxygen species (ROS) production and bound to bacterial DNA, leading to complementary intracellular mechanisms of bacterial killing [66]. Furthermore, the hybrid exhibited synergism with conventional antibiotics, suggesting the potential to combine membrane permeabilization effects with cytoplasmic effects to enhance the efficacy of antibacterial drugs.

The ATCUN motif, which coordinates Cu(II) and Ni(II) in the N-terminal environment, is implicated in redox reactions that generate reactive oxygen species (ROS) and induce oxidative damage to biomolecules [71]. Incorporating this motif into a cationic, amphipathic spider peptide could therefore couple efficient membrane interaction with intracellular metal-dependent activity [17]. The activity of the metal is highly dependent on its binding environment. For example, replacing histidine with heterocyclic analogues and substituting β-alanine in peptides have been shown to increase ROS production and oxidative DNA cleavage by disrupting metal coordination and redox cycling [72]. The present findings demonstrate that not only can the venom scaffold be modified to optimize future hybrids of spider venom and ATCUNs, but systematic changes can also be made to the grafted metal-binding motif [73]. However, the generation of ROS presents a potential risk, as excessive or non-specific oxidative activity can negatively impact host-cell compatibility [2]. Therefore, future designs must carefully consider antimicrobial activity, serum stability, haemolysis and cytotoxicity to maintain selectivity.

Hybridization of venom peptides is not limited to ATCUN constructs. Generally, hybrid antimicrobial peptides (AMPs) can be formed by combining peptide sequences from different antimicrobial agents to integrate diverse antimicrobial activities and mechanisms of action [9]. Notably, rationally designed derivatives of latarcin-3a have exhibited both antibacterial and antitumor activity with low haemolytic activity under tested conditions, underscoring the functional plasticity of linear spider venom scaffolds [59]. Similarly, transcriptome-based designs have yielded cytocompatible antimicrobial and anti-inflammatory agents such as Ak-N' and Ak-N'm. While not examples of motif grafting, these illustrate the broader potential for multifunctional peptide engineering and functional redesign of spider venom scaffolds. Another hybridization mechanism involves the non-covalent combination of venom peptides with conventional antibiotics at the therapeutic-system level. For instance, LyeTx I mnΔK, a shorter derivative of a spider toxin, increased the susceptibility of MRSA to conventional antibiotics and demonstrated therapeutic efficacy in a mouse wound model [74]. These findings, along with previously reported additive and synergistic effects between other spider-derived AMPs and antibiotics, suggest that venom-derived peptides warrant further investigation as components in antimicrobial combination therapies [68].

Lipidation, terminal modification and peptidomimetic tuning of spider venom peptides

Lipidation

Lipidation represents a direct strategy for reprogramming membrane-active venom peptides. This modification enhances hydrophobic interactions with lipid bilayers, can prolong *in vivo* persistence, and may facilitate albumin association or self-assembly. However, excessive hydrophobicity carries the risk of

increased nonspecific membrane damage. Consequently, general peptide literature often frames lipidation as a potency-selectivity trade-off rather than a universally beneficial modification [75]. A pertinent spider-specific example is LVTX-9, a linear amphipathic α-helical peptide derived from *Lycosa vittata*. Its native cytotoxicity against B16-F10 melanoma cells was modest, with IC_{50} values of 59.2 ± 19.8 µM in serum and 76.3 ± 12.7 µM in serum-free medium. To enhance this activity, N-terminal fatty-acyl analogues, ranging from LVTX-9-C12 to LVTX-9-C20, were synthesized. Among these, the C18 fatty-acyl analogue demonstrated optimal chain length. Specifically, LVTX-9-C18 exhibited a significant optimization of approximately 12.9-fold in serum and 19.3-fold in serum-free media compared to the parent peptide, highlighting fatty-acid tuning as a powerful optimization pathway for this scaffold. It was proposed that this enhanced activity was not due to receptor-specific targeting but rather to stronger, directly induced membrane destruction. Various assays, including SEM, trypan blue uptake, lactate dehydrogenase release and haemolysis, confirmed that the mechanism of action of LVTX-9-C18 on melanoma cells primarily involves plasma membrane damage, consistent with this hypothesis [76]. Many linear amphipathic spider peptides adopt α-helical conformations in membranes, binding to the membrane surface, inducing membrane defects, promoting lipid microdomain clustering, or forming pores [77]. Similarly, the membrane-centric properties of Lycosin-I align with its selective efficacy against leukaemia cells, which appears to stem from its high and selective affinity for negatively charged phosphatidylserine and rapid membrane lysis at higher concentrations [78]. The lipid-dependent conformational transition, selective antileukemic activity, chemical composition and morphology of Lycosin-I are depicted in Figure 5.

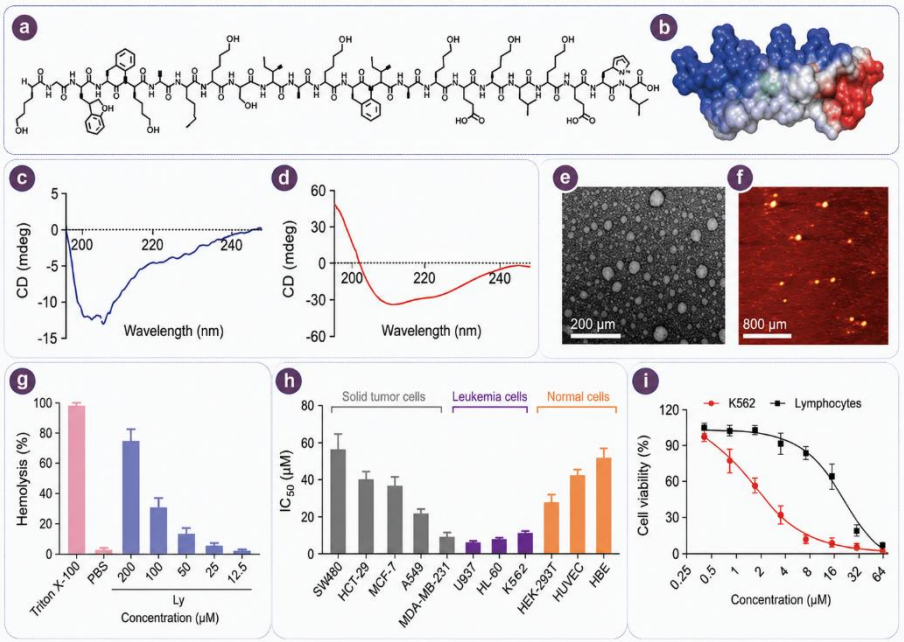


Figure 5. Lipid-dependent conformational transition and selective antileukemic activity of Lycosin-I. (a) Chemical structure of the spider venom peptide Lycosin-I. (b) Three-dimensional structural model of Lycosin-I generated using PyMOL, illustrating its predicted molecular conformation. (c,d) Circular dichroism (CD) spectra of Lycosin-I (50 µM) recorded in phosphate-buffered saline (PBS, pH 7.4) (c) and in POPC/POPS (3:1) lipid vesicles (d), demonstrating membrane-induced secondary structural changes. (e,f) Representative transmission electron microscopy (TEM) (e) and atomic force microscopy (AFM) (f) images showing the self-assembled nanostructures formed by Lycosin-I (100 µM). (g) Hemolytic activity of Lycosin-I against human erythrocytes at different peptide concentrations, indicating concentration-dependent membrane disruption. (h) Half-maximal inhibitory concentration (IC_{50}) values of Lycosin-I against solid tumour cell lines (SW480, HCT-29, MCF-7, A549 and MDA-MB-231), leukaemia cell lines (U937, HL-60 and K562) and non-cancerous cell lines (HEK293T, HUVEC and HBE), as determined by the CCK-8 cell viability assay. (i) Cytotoxic effect of Lycosin-I on K562 leukaemia cells and normal human lymphocytes revealed a dose-dependent toxicity with greater sensitivity to the leukaemia cells. All data are represented as mean ± standard error of the mean (SEM), *n* = 6 (six independent replicates) Adapted from Ref. [78]. under the terms of the Creative Commons Attribution 4.0 International License (CC BY 4.0)

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The broader literature on lipidation provides context for the success of C18 acylation. Both direct and chain-appendant (linker/spacer) fatty-acid derivatization can modify receptor engagement, confer protection against proteases and alter partitioning into biological membranes, with both the length and linkage geometry being critical factors [79]. However, excessively long or incorrectly positioned lipid tails can have undesirable effects, such as enhanced haemolysis and decreased specificity for target cells [80]. Recent studies on the antimicrobial activity of AMPs further suggest that the site of lipidation can be as important as the lipid itself. For instance, the presence of an acylated side chain in an antifungal peptide can maintain or enhance antifungal activity with fewer cytotoxic side effects compared to classic N-terminal acylation [81]. Therefore, a fundamental design consideration for future analogues of LVTX-type spider venom peptides is that N-terminal conjugation, as used for LVTX-9, may not always be the optimal strategy.

Terminal modification

N- and C-terminal capping are common strategies to enhance enzyme resistance to exopeptidases, fine-tune net charge, stabilize secondary structures and alter membrane selectivity [82]. A notable example from 2023 is the optimization of LVTX-8, an amphipathic 25-residue α-helical cell-penetrating peptide (CPP) from the spider *Lycosa vittata*. While native LVTX-8 showed promise as an anticancer precursor, its protease sensitivity indicated vulnerability to a short half-life and poor durability. A subsequent study constructed 10 analogs, with analog 8 being particularly significant. This modified peptide demonstrated increased proteolytic stability, reduced haemolysis and superior efficacy in inhibiting cancer cell growth over a longer duration compared to the native peptide. The study also revealed that LVTX-8's pharmaceutical profile, specifically its mode of cell death, was altered without eliminating the parent mechanism, which involved membrane disruption and mitochondrial targeting with decreased mitochondrial membrane potential [42]. This pattern of venom-peptide engineering extends beyond spiders to other organisms. For instance, a combination of N-terminal acetylation and C-terminal hydrazidation of melittin yielded LJ-5, a derivative exhibiting enhanced proteolytic stability, more persistent anti-cancer activity, increased anti-migration activity and reduced haemolytic activity compared to native melittin [83]. These parallel supports the concept of a universal optimization principle for linear membrane-lytic cytotoxic peptides, indicating that the LVTX-8 (LMCP) case is not isolated. Furthermore, terminal modifications influence activity by altering charge state and structural rigidity. C-terminal amination, frequently observed in antimicrobial peptides, often increases the net positive charge and can enhance antibacterial activity, though it may also increase haemolysis [80]. Other studies have shown that amidation promotes higher helicity and rigidity, which facilitates membrane insertion [84]. Other studies have shown a higher helicity and rigidity in the case of amidation, which would promote insertion into membranes [84]. Consistent with this, the parent peptide LyeTx I-b, engineered through acetylation and increased amphipathicity, exhibited greater membrane interaction and higher vesicle disruption capability while being less toxic to eukaryotic cells [41]. Subtle changes at the C-terminus can even affect subtype selectivity in disulfide-rich spider toxins; for example, replacing C-terminal amidation with a carboxylated motif in Huwentoxin-IV analogues resulted in decreased NaV1.6 potency but increased NaV1.7 selectivity [62]. Collectively, these investigations demonstrate that terminal editing is crucial not only for stability but also for fine-tuning membrane partitioning, toxicity and target selectivity.

Peptidomimetic tuning

Beyond cap-dependent tuning, more significant scaffold modifications are employed to enhance the drug-like properties of compounds while preserving the pharmacophore of the native toxin. Therapeutic peptides often face challenges in peripheral bioavailability due to slow absorption, rapid clearance, conformational flexibility and proteolytic susceptibility. These issues can be addressed through strategies such as backbone editing, incorporation of non-natural residues, cyclization, peptide formation and related mimetic approaches [85].

Recent reviews have clearly defined this field with a translational focus. The core concept of peptidomimetics is to improve stability, binding affinity, specificity and bioavailability while maintaining biologically active recognition motifs [86]. These and other methods, including backbone manipulation, the use of non-canonical amino acids, macrocyclization and side-chain stabilization, are now standard strategies for transforming labile peptides into promising pharmaceutical leads [79]. Non-canonical amino acids are particularly important because they can stabilize secondary structure, modulate function and enhance degradation resistance [7]. While perhaps less advanced than lipidation or terminal capping, full peptidomimetic conversion is a well-established technique in the literature for the rational design of charge, hydrophobicity, amphipathicity and membrane selectivity. Optimization of C-terminal amidation, moderate positive charge and hydrophobic/aromatic N-terminal anchors were performed for fragments of linear spider peptides selected against MRSA using a neural network. This analysis revealed that these parameters were responsible for the peptides' high antimicrobial activity and low haemolytic activity [52]. In one instance, in silico redesign of a spider toxin using the ATCUN motif resulted in a hybrid peptide with enhanced activity against multidrug-resistant *Pseudomonas aeruginosa*, alongside co-mechanisms involving membrane disruption and reactive oxygen species production. Rationally designed derivatives of the latarcin-3a molecule also yielded multi-functional agents possessing antibacterial and antitumor activities without exhibiting hemolytic activity at the assayed concentrations [59]. An important insight from these studies is that success is not simply achieved by maximizing hydrophobicity or increasing charge. Instead, it requires a multidimensional approach to optimize the balance between helicity, amphipathicity, lipid preference, protease resistance and host-cell sparing [80]. This is particularly relevant for peptides designed to inhibit spiders, as some of their therapeutic action is linked to cell membrane engagement. This connection makes unforeseen toxicity a recurring threat when there is a risk of exceeding the optimum range for physicochemical tuning [73].

Design principles and limitations

The general design principles consistently observed across the corpus can be organized into several key rules. First, membrane-active spider peptides benefit from modifications that strengthen beneficial lipid-molecule interactions while avoiding indiscriminate lytic activity. Latarcins exemplify this, showcasing the diverse membrane interaction modes of closely related spider peptides, ranging from subtle (*e.g.* Adducin-like) to highly aggressive (*e.g.* batrachotoxinin-like), including partial haemolysis [40]. Second, for protease-labile parent scaffolds (*e.g.* LVTX-8), terminal protection offers a significant advantage, as demonstrated for all generically related peptide therapeutics [87]. Third, while hydrophobic augmentation enhances potency and membrane insertion, it presents a double-edged sword, as high levels of hydrophobicity tend to increase haemolysis and decrease selectivity [80]. It is crucial to acknowledge important limitations. The majority of modification studies in this corpus are preclinical, relying primarily on cell assays, model membranes, or initial animal studies for evidence rather than clinical translation [57]. While published data confirm higher *in vitro* potency, *in vivo* stability and hemocompatibility for LVTX-9 and LVTX-8, their pharmacokinetics, immunogenicity, formulation considerations and therapeutic window in humans remain unknown [42]. Generally, peptide modification approaches involve trade-offs, where enhancing one desired property often comes at the expense of another. Therefore, the term "optimization" must be understood in a context-sensitive manner [76].

Ligation, synthesis and scalable chemistry

The chemistry of spider peptides has advanced significantly, now permitting access to previously unattainable architectures through total synthesis, native chemical ligation and enzyme-assisted ligation, as exemplified by the double-knot ASIC1a inhibitor Hi1a and other constructed double-knotted peptides. Duggan and coworkers demonstrated that Hi1a can be prepared by total chemical synthesis with native chemical ligation and folded into a bioactive, ASIC1a inhibitory form that functions similarly to its recom-

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binant counterpart [16]. Concurrently, a route to double-knotted constructs and related bivalent toxins was developed *via* the head-to-tail fusion of pre-folded, disulfide-rich peptide domains, without reduction or scrambling [27]. More broadly, these advancements reflect a transformative period in peptide chemistry. Native chemical ligation has emerged as a primary bond-forming reaction, enabling the formation of native amide bonds under mild aqueous conditions. This has provided unprecedented access to large and otherwise inaccessible protein and peptide targets [88]. However, synthetic scaffolds derived from venom peptides pose particular challenges for traditional stepwise synthesis due to increased hydrophobicity, conformational heterogeneity, difficult oxidative folding and the requirement for multiple cysteines [89].

Hi1a access

Hi1a, a bivalent peptide derived from spider venom, holds significant therapeutic promise for acute ischemic conditions. However, research and development have been historically hampered by challenges in producing sufficient quantities of the material [56]. A critical advancement in addressing this supply issue was the successful total synthesis of Hi1a. This breakthrough allowed for the chemical synthesis and *in vitro* folding of the molecule, yielding an active product comparable to recombinantly produced Hi1a *via* native chemical ligation (NCL). This not only demonstrated that a large double-knot spider toxin could be fragmented into ligatable pieces and refolded into its native structure but also underscored the crucial role of the native structure in its toxicity. From a practical perspective, this opens avenues for sequence editing, analogue generation and structure-activity relationship studies across the full spectrum of the original venom [90]. The importance of Hi1a synthesis is further amplified by the versatility of NCL chemistry. While Cys-arginine and Cys-Cys junctions remain prevalent in classical NCL, modern workflows have evolved to incorporate desulfurization, auxiliary-mediated ligation, and the use of alternative thiol- or selenol-containing amino acids. These advancements enable the reconstruction of native junctions post-ligation [91] , which is particularly beneficial for venom peptides where the most synthetically favourable cleavage site may not align with a natural cysteine position [87]. Furthermore, Figure 6 illustrates the modular synthesis of bioactive Hi1a through peptide synthesis, oxidative folding and site-selective ligation of two correctly folded ICK domains.

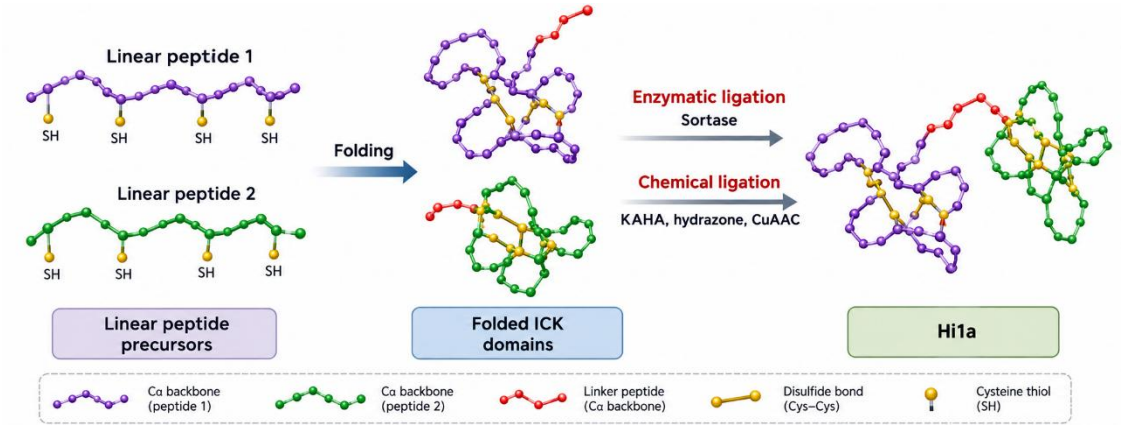


Figure 6. Use of two-domain inhibitor Hi1a in an oxidative folding and site-selective peptide ligation modular synthesis. For the generation of correctly oxidized inhibitor cystine knot (ICK) domains with the native disulfide bond connectivity, the precursors are independently refolded in a linear sequence with cysteine residues. Using these, the folded domains are then ligated (either enzymatically mediated/"sortase" or chemo selectively mediated "catalytic" sort of ligations) to assemble the full-length Hi1a construct that will be delivered to the cell surface. The schematic shows the workflow to efficiently synthesize the corresponding bioactive heterodomain peptide with preservation of the structural integrity of both the ICK domains: peptide synthesis, oxidative folding, site-specific domain coupling [92]

Double-knotted constructs

Given that venom peptides often feature two distinct domains, each characterized by six cysteines and three disulfide bonds, the synthetic pursuit of more complex peptide architectures, particularly "double-

knotted" constructs, has intensified. For several years, naturally occurring double-knotted peptides like Hi1a and DkTx have been successfully isolated from venom and produced recombinantly. However, the synthetic production of these peptides has proven challenging, typically limited to engineered conjugates. A promising strategy involves the independent folding of each knot, followed by their ligation without unfolding. Tran and co-workers investigated various post-folding ligation methods, including sortase A5°, butelase 1, OaAEP1 variants, peptiligase and the mild chemical KAHA reaction, all designed to preserve native disulfide connectivity in both domains. Correctly folded substrates were ligated with varying yields without disulfide reduction or shuffling. Sortase A5° demonstrated the highest efficiency, achieving 60% conversion within 15 minutes. This post-folding ligation strategy is chemically significant as it circumvents a major bottleneck in double-knot synthesis: the low probability of achieving the correct global fold after attempting to oxidize a fully assembled 12-cysteine construct. Instead, each domain can be independently optimized for synthesis and folding before being joined through a native amide linkage. This approach has already been successfully applied in bivalent sodium-channel inhibitors, where the ligation of a spider gating-modifier toxin to a pore-blocking conotoxin yielded a double-knotted construct with enhanced NaV1.7 potency, achieving an IC50 of 58 nM with a 9-glycine linker [56]. Enzymatic ligation offers several advantages over simple dimerization, notably expanding the design space beyond dimer formation. This flexibility, encompassing topological control, the generation of semisynthetic products, and the combination of synthetic and recombinant segments, is increasingly utilized in nature-inspired ligation platforms and is crucial for venom-peptide reprogramming [90]. AEP-based systems are particularly relevant due to their established ability to support cyclization and orthogonal ligation of disulfide-rich peptides, including cystine-knot frameworks up to 34 residues, making them practical maturation tools in recombinant workflows [93].

Enabling chemistry

Recent methodological advancements suggest that future improvements in spider-venom peptide synthesis will likely stem less from the invention of entirely new scaffold-specific reactions and more from enhancing the robustness of ligation around challenging sequences. Several newer NCL variants directly address known bottlenecks such as poor thioester reactivity, sterically hindered junctions, peptide aggregation and the compatibility of one-pot reactions with desulfurization or refolding [94]. For instance, azole-activated peptide acyl pyrazoles eliminate the need for thioesters and thiol additives and can be integrated with metal-free desulfurization or oxidative folding in a single pot, exemplified by the synthesis of the cyclotide kalata B1 [95]. VTT-promoted thioacid ligation offers additive-free, rapid NCL through in situ activation of peptide thioacids and is compatible with one-pot ligation-desulfurization sequences [96]. TSAL ester-induced ligation provides another convergent option from peptide hydrazides, maintaining effectiveness even at sterically hindered junctions, and can be combined with other ligation methods in multistep assembly [97]. Catalyst and junction engineering are also gaining importance. Alkylselenol catalysis accelerates NCL and enables the synthesis of the eight-cysteine snake cardiotoxin A5 without orthogonal cysteine protection, demonstrating how rate enhancement can simplify the assembly of cysteine-dense toxins [98]. Auxiliary-mediated systems now facilitate access to sterically encumbered sites, including Leu-Val and even tyrosine ligation, while proline ligation has been improved through mercaptoproline and selenoproline editing combined with imidazole-promoted acyl transfer [91].

Folding and scale

For knotted venom peptides, bond formation represents only half of the challenge; oxidative folding remains a significant rate- and yield-limiting step. Rapid, regioselective disulfide-installation strategies now offer a complementary solution by enabling the one-pot formation of two or three disulfides in high yield, including therapeutically relevant disulfide-rich peptides and proteins [89]. This is particularly relevant for spider toxins,

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where successful ligation must be followed by predictable access to the native cystine-knot topology. Related advancements are beginning to alleviate the folding bottleneck by rerouting synthetic pathways. A plug-and-play cyclotide grafting strategy utilizes a pre-formed acyclic cyclotide-like scaffold to bypass the challenging oxidative folding of epitope-grafted constructs, even in cases involving additional disulfides that are difficult to achieve by conventional methods [99]. Bracelet cyclotides, historically considered particularly difficult synthetic targets, have now been obtained in reasonable yield, demonstrating that even recalcitrant cystine-knot subclasses can become chemically tractable with improved workflows [100]. While scalability is improving, it remains uneven across different platforms. Automated solid-phase peptide synthesis (SPPS) coupled with programmable downstream chemistry now supports the synthesis of challenging peptides with crude purities exceeding 79%, integrates transformations such as CuAAC and intramolecular NCL, and can deliver multi-milligram products through uninterrupted workflows [88]. General peptide-manufacturing reviews also identify microwave-assisted SPPS, advanced resins, parallel automation and liquid-phase peptide synthesis (LPPS) as key current drivers for scale-up [101]. Nevertheless, peptide desulfurization remains a practical industrial bottleneck, making gram-scale, phosphine-only photo-desulfurization a notable advancement for improving the manufacturability of NCL-derived routes [102].

Delivery engineering of spider-venom peptides

A central tenet of delivery engineering for spider-venom peptides is that formulation should no longer be viewed solely as an optimization tool, but as a prerequisite for determining 'usable exposure,' 'tissue selectivity,' and 'route flexibility,' all critical components for the success of these potent venom peptides. Liposomes, nanocapsules and peptide modifications that facilitate membrane targeting increasingly demonstrate enhanced capabilities to stabilize peptides, penetrate membranes, control their delivery and accumulate at target sites, as evidenced in the aforementioned literature, much of which is currently limited to preclinical systems [103]. For spider toxins, direct evidence now supports their utility as therapeutic agents (*e.g.* Liposomal Shell technology, as previously described) or as agricultural agents (*e.g*. PLGA Nanocapsules, as previously described): the oral insecticidal activity of the spider toxin ω-hexatoxin-Hv1a/Hvn1b was augmented upon encapsulation in PLGA nanocapsules and the ability of the spider peptide LyeTx III to degrade cellular tumour proteins was enhanced by liposomal encapsulation [104]. These case studies are best considered within the broader peptide-delivery literature, where lipid-based carriers protect against degradation, modify pharmacokinetics, and increase peptide residence time through active and physicochemical targeting strategies enabled by surface engineering [105].

Liposomal encapsulation

Liposomal encapsulation holds particular promise for peptides derived from spider venom. Liposomes can shield peptides from degradation and clearance, control delivery and direct activity to targeted tissues [106]. Membrane-active peptides with therapeutic applications often suffer from a low duration of action, a broad spectrum of off-target effects and a lack of tumour or tissue selectivity. This makes them of immediate interest for venom therapeutic peptides [107]. A well-known example is the short peptide LyeTx III, found in the venom of *Lycosa erythrognatha*. A study demonstrated that liposome-encapsulated LyeTx III significantly reduced cell viability in cultured tumour cells (HN13) from 98 % with the free peptide to 38 %. This same study suggested that encapsulation not only enhances activity but also acts as a pharmacological enabler, potentially allowing for a longer *in vivo* half-life and future targeting of specific tumours [104]. This indicates that a well-designed delivery system can transform a broadly membrane-active bioactive venom peptide into a more selective antitumor lead. This perspective aligns with a broader understanding within the liposome literature. Liposomes are among the best-established vesicular drug delivery systems, with a precedent of regulatory approval. They

are biocompatible, protect cargo, enable controlled release and reduce systemic toxicity [108]. Different liposome types, varying in lipid composition or surface modifications, are depicted in Figure 7.

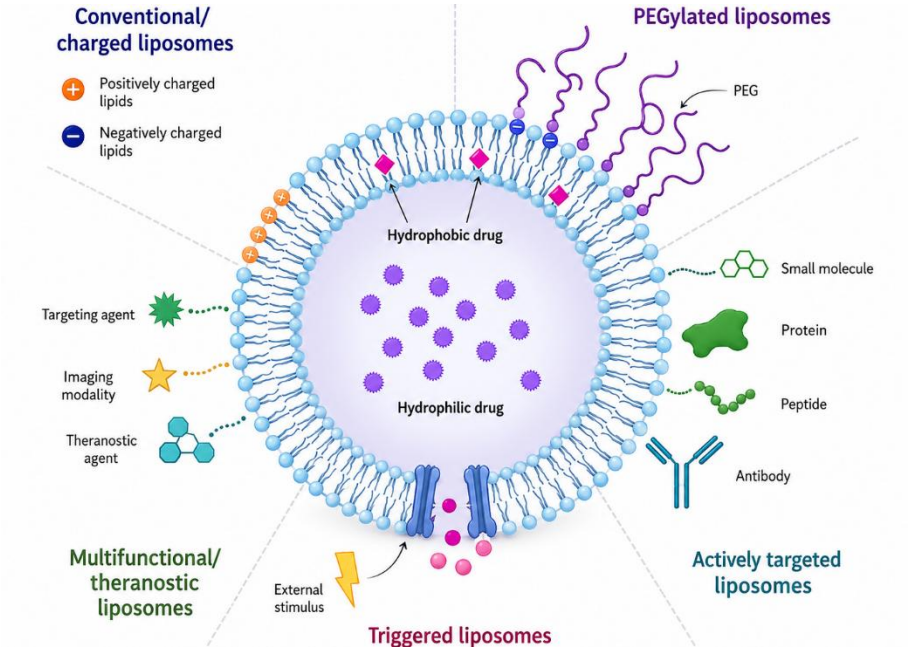


Figure 7. Different liposome types differing in their lipid composition or surface modifications. Adapted from Ref [108] under the terms of the Creative Commons Attribution 4.0 International (CC BY 4.0) License

For peptides and proteins, liposomes can encapsulate hydrophilic cargo in their aqueous core and modulate circulation, biodistribution and uptake through size, charge, lipid composition and PEGylation [109]. Another critical consideration is the design of nano-liposomal surfaces that avoid undesirable interactions with plasma, thereby enhancing blood persistence. This is particularly relevant for venom peptides, which are otherwise rapidly cleared or interact with non-target membranes [110]. Furthermore, liposomal performance is highly dependent on the architectural design of the formulations, not solely on the presence of the liposome and its contents. Limitations of classical first-generation liposomal formulations include a short circulation half-life, physical instability, low payload retention, uncontrolled drug release and a lack of targeting ability. These limitations can be overcome by polymer-assisted liposome engineering. Natural biopolymers (*e.g.* chitosan, alginate, dextran, gelatine and caseins) can enhance the stability of lipid membranes against physicochemical stress and improve the retention of loaded bioactive during storage or gastrointestinal transit [111]. Therefore, when reviewing LyeTx III, its efficacy should not be attributed as a universal liposome effect, but rather as an example of a tuneable platform whose pharmacology is programmed by its composition, surface chemistry and release kinetics.

Nanocapsules and oral/topical bioactivity

A significant challenge for insecticidal spider peptides is their limited bioavailability following oral or cuticular exposure. Nanocapsules offer a promising solution to this problem. Given their mechanisms of action, insecticidal spider toxins are typically larger and less membrane-permeable than conventional small-molecule insecticides, leading to slower and less efficient action when administered *via* routes other than injection. Wei et al. directly demonstrated that nanocapsule engineering can enhance the functional delivery of a spider toxin. The nanocapsules developed in their study were spherical, with an average diameter of 197.70 nm, an encapsulation efficiency of 75.82 % and a zeta potential of -32.90 mV. Penetration experiments revealed that these nanocapsules primarily enhanced the translocation of the peptide through the gut of *Spodoptera litura* and into the haemolymph. This mechanistically superior compound resulted in a corresponding biological activity, with the oral toxicity of the nanocapsule formulation significantly exceeding

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that of the free toxin [112]. This exemplifies the successful application of delivery engineering to overcome a route of administration challenge, rather than modifying the toxin's pharmacodynamics. The broader literature on oral peptide processing supports this observation. Key barriers to successful oral peptide formulations include enzymatic degradation, peptide impermeability across membranes, low epithelial permeability in the intestine and mucus entrapment of peptides. Lipid-based nanocarriers can circumvent these barriers by protecting peptides from proteases, facilitating mucosal adhesion or permeation (depending on surface design), promoting endocytosis and, in some cases, enhancing lymphatic permeation [113]. Core-shell nanocapsules offer particular flexibility, as they can both protect the payload and influence its release behaviour, as well as modulate interactions between the shell and the administration route [114]. This rationale extends to topical/transdermal applications in agriculture, mirroring its applicability to oral delivery. Advanced nanoparticulate delivery systems are currently being developed to deliver peptides to tissues *via* oral, topical, dermal and transdermal routes, offering improved stability, controlled release and extended residence time at the barrier tissue [115]. Model peptides have been used to demonstrate that the transdermal transport of these compounds can be substantially enhanced by formulation chemistry, such as using lipid-based ionic-liquid nano dispersions, where delivery was enhanced up to 65-fold compared to an aqueous solution injected into the same skin [116]. Although this study did not involve spider peptides, it presents a highly relevant precedent for the translational application of venom peptides, particularly given the numerous topical and plasma applications that are likely to encounter similar permeability challenges.

Carrier fusions and peptide-assisted targeting

Carrier fusion strategies introduce an additional layer of engineering, enhancing not only peptide protection but also cell entry and cytosolic delivery. A persistent limitation of conventional nanocarriers is their frequent internalization *via* endosomal pathways, which often leads to payload degradation or failure to reach the cytosol [117]. Peptide-enabled fusion and penetration systems are being developed to overcome this bottleneck. Fusogenic coiled-coil peptides offer a compelling model, where complementary lipopeptides displayed on liposomes and cell membranes can drive direct membrane fusion. This mechanism bypasses inefficient endosomal uptake and improves cytosolic cargo delivery [118]. Dimeric coiled-coil variants have further enhanced liposomal delivery and antitumor efficacy, with membrane fusion identified as the predominant uptake route [117]. For venom peptides, this approach is significant because the same surface-functionalization logic could enable more selective delivery of spider toxins to tumour cells or excitable tissues, thereby minimizing nonspecific membrane lysis. Other peptide-decoration strategies demonstrate similar translational potential. Cell-penetrating and targeting peptides can increase nanocarrier uptake, tissue selectivity and intracellular access, although issues of specificity and safety validation remain significant limitations [107]. For instance, cyclic CPP-modified liposomes increased the oral bioavailability and preserved *in vivo* efficacy of the vancomycin derivative FU002, demonstrating that peptide-decorated liposomes can transform a poorly absorbed, peptide-like agent into an orally active formulation (Figure 8) [119].

Likewise, receptor-directed or penetrative peptide decoration improves uptake and antitumor performance in liposomal systems designed for oncology [120]. For insecticidal peptides, carrier fusion is broadly understood as any engineered association between peptide cargo and a transport-promoting vehicle. Rhamnolipid liposomes improved the bacterial delivery of the otherwise membrane-impermeant peptide ParELC3 and reduced the concentration required for growth inhibition relative to the free peptide [121]. This supports the general principle that carrier design can salvage potent but delivery-limited peptides by enabling barrier crossing and intracellular or intrabody access. The necessary translational application for an insecticidal toxin isolated from a spider (and plant) will involve its surface application to an insect, with the goal of ensuring its survival in the presence of water, adherence to insect surface tissues and efficient passage through epithelial and gut tissue.

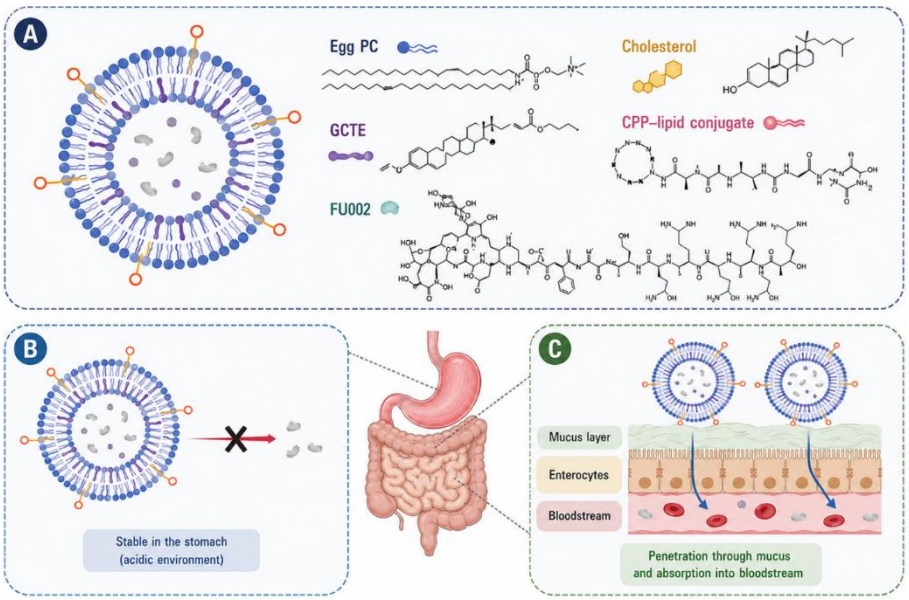


Figure 8. Schematic representation of the composition of CPP-GCTE liposomes that encapsulate FU002 and are delivered orally. The nanoliposomal formulation includes egg phosphatidylcholine (Egg PC), cholesterol, cell-penetrating peptide (CPP)-lipids and the theranostic agent encapsulated in the nanoliposome FU002. (B)When administered orally, GCTE has been shown to protect membrane integrity under the acidic conditions of the stomach, reducing premature drug leakage and providing greater liposome stability than conventional liposomes.(C) In the intestine, surface-functionalized CPPs can penetrate the mucus layer and cross enterocytes *via* trans-epithelial transport, leading to effective systemic absorption of FU002 into the bloodstream and improving the oral bioavailability of FU002

Design principles and limitations

Several general design principles emerge from the literature, as detailed in Table 3.

Table 3. Engineering strategies used to reprogram spider venom peptides

Strategy	Design goal	Example from corpus	Observed gain	Remaining limitation	Ref.
Rational residue substitution	Improve subtype selectivity and reduce off-target toxicity	mGpTx1-SA reduced rKv4.2/4.3 inhibition while maintaining hNav1.7 activity	Improved safety profile with retained analgesia	Requires dense SAR and electrophysiology across isoforms	[122]
Loop and pharmacophore engineering	Increase potency or reshape channel-state preference	Tap1a-OPT variants increased NaV potency; Tap1a-OPT2 avoided NaV1.4 off-target activity seen with OPT1	Enhanced *in vivo* antinociception *via* slowed off-rate and improved channel contacts	Broader human isoform selectivity still needs validation	[123]
Terminal modification and conjugation	Improve stability, half-life and therapeutic window	N-acetyl/C-hydrazide LVTX-8 analogue 825 and MTX-GFLG-LVTX-8 conjugate 827 improved proteolytic stability, durable anticancer activity and lowered haemolysis	Directly addresses the short-half-life problem	Effects remain mostly *in vitro* for this class	[42]
Nanocarrier or nanoparticle coupling	Boost targeting, uptake and reduce systemic toxicity	Lycosin-I-functionalized gold nanoparticles and nanorods selectively entered cancer cells, accumulated in tumours and enabled effective photothermal killing *in vitro* and *in vivo*	Strong proof of concept for delivery engineering	Nanocarrier biocompatibility, scale-up and regulation remain obstacles	[124]
Proteolytic stabilization	Enable persistence or new routes of administration	R9Q stabilization made U1-AGTX-Ta1b orally active and commercially suitable as a bioinsecticide	Demonstrates that single-site stabilization can transform deployability	Therapeutic extrapolation to human drugs remains unproven	[125]

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First, particle size, charge, lipid composition and membrane rigidity are likely to profoundly affect pharmacokinetics, uptake pathways and release behaviour [83]. Second, for oral peptide delivery, typical peptides must possess multiple advantageous properties, including enzymatic protection, mucus navigation, epithelial permeation and controlled release [87]. Third, peptide-functionalized nanocarriers are not yet fully translationally mature due to limited long-term pharmacokinetic, immunogenicity and toxicity data [108]. Furthermore, the literature clearly indicates that encapsulation is not always advantageous. For oral applications, liposomes often exhibit insufficient intrinsic protection, necessitating coating with polymers or co-formulation with excipients such as bile salts [113]. Some nanocarriers improve loading without enhancing efficacy; for instance, cubosome encapsulation of ultra-short antimicrobial peptides failed to augment antimicrobial activity when the peptides already permeated efficiently independently [126]. This serves as a crucial caution for spider-venom research: delivery systems should be selected to address specific bottlenecks, such as instability, off-target toxicity, or epithelial impermeability, rather than being incorporated by default. Scalability and standardization present additional challenges. Liposome manufacturing and standardization are more complex than some competing lipid systems. Broader reviews on peptide delivery also highlight persistent issues in reproducibility, quality control, high production costs and variable batch stability [127].

Therapeutic application domains of spider venom peptides

The current literature highlights three primary therapeutic domains for spider venom peptides: analgesia/ion-channel modulation, which is the most developed; anti-infectives, which are experiencing rapid expansion; and oncology, which shows promise but remains largely preclinical. Additionally, cardiovascular/ /coagulation applications are emerging, albeit from a smaller evidence base.

Analgesia and ion-channel disorders

Pain management represents the most advanced therapeutic application for spider venom peptides due to the natural evolution of spider toxins as high-affinity modulators of excitable membranes, particularly voltage-gated sodium channels and related proton-gated ion channels [4]. Within this area, NaV1.7 has been a dominant target, strongly validated by human genetics in nociception. Engineered spider peptides have provided some of the most selective biologic leads reported for this channel [62]. Over the past decade, progress has led to engineered family-3 scaffold peptides like JNJ-63955918 and AM-6120, which act as potent and highly selective closed-state NaV1.7 blockers, demonstrating convincing *in vivo* efficacy at tolerated doses. Despite this progress, NaV1.7 translation remains incomplete. No spider-peptide analgesic has yet reached approval and long-term systemic use is constrained by unresolved pharmacokinetic, tissue-distribution, target-engagement and safety concerns [58]. Consequently, selectivity engineering is a central translational challenge in the NaV1.7 field. Native peptides such as ProTx-II and HwTx-IV are potent, but their therapeutic development is limited by off-target activity on other sodium-channel isoforms, notably NaV1.6. Structure-guided analogue design has shown this problem to be tractable: synthetic HwTx-IV analogues can significantly alter the human NaV1.7/NaV1.6 selectivity ratio, with some variants achieving improved NaV1.7 bias and better safety profiles [62]. Recent structural work on NaV-modulating spider toxins is also clarifying how gating-modifier peptides engage voltage-sensing domains, which should further accelerate the rational optimization of isoform and state selectivity [128].

Interest has also expanded to NaV1.8, another nociceptor-enriched channel highly relevant to inflammatory and neuropathic pain [129]. Tl1a from *Thrixopelma longicolli* inhibits NaV1.8 with nanomolar potency but also retains activity at NaV1.5, KV2.1 and NaV1.7, illustrating both the promise and the selectivity challenges of early NaV1.8 ligands. This understanding is becoming clearer and more mechanistic: a hydrophobic loop was identified through mutational analysis as an important determinant of Tl1a activity at NaV1. Complementing

these pharmacological findings, cryo-EM structures of human NaV1.8 linked to the Protoxin-I peptide from the South American tarantula reveal that the toxin binds to a site on NaV1.8 near the VSDII S3-S4 linker, preventing its removal and blocking S4II movement upon channel activation. This provides a structural foundation for designing new-generation NaV1.8 inhibitors. A second mature analgesic axis involves acid-sensing ion channels, particularly ASIC1a [130]. These are prevalent in pain pathways and implicated in ischemia-associated injury. Potent inhibitors of ASIC1a, such as the spider peptides PcTx1 and Hi1a, have emerged as well-established chemical probes for understanding ASIC action and pathophysiology [131]. A broader pharmacological literature indicates that ASIC is a target for peptide toxins that affect channel gating in a complex, pH-dependent manner, fully consistent with strong *in vivo* analgesic activity, further supporting their relevance in pain management. The PcTx1-like single-knot peptide Hc3a specifically modulates the desensitization of ASIC1a and binds to several ASIC conformations, supporting the view that spider venoms offer a diverse range of mechanisms beyond simple pore inhibition. Beyond analgesia, the ASIC1a program now encompasses ischemic neuroprotection and/or cardioprotection. Hi1a is a potent neuroprotective and cardioprotective agent *via* ASIC1a, currently under investigation for acute ischemic diseases such as stroke, myocardial infarction and organ transplantation injury [16]. Synthetic advancements have been particularly significant: total synthesis and ligation-based methods now enable the production of bioactive Hi1a in 'useful scale' quantities [27]. Recent evaluations position Hi1a among the top contenders to become the first spider-based therapeutic, with future efforts focusing on acute tissue protection in the periphery.

Anti-infectives

The development of anti-infectives is currently among the most rapidly expanding applications for spider peptides. This surge is driven by the increasing challenge of antibiotic resistance, which has created a significant unmet need for novel, membrane-active scaffolds capable of rapidly eradicating bacteria without promoting the selection of resistant variants [57]. Unlike the disulfide-rich ion-channel toxins prevalent in analgesic research, this field is largely characterized by linear, cationic, amphipathic peptides that disrupt microbial membranes. These peptides can often be optimized through sequence truncation, charge redistribution, amidation or hybrid design [32]. A prominent theme in this area is the broad spectrum of anti-infective functions. Recently discovered spider peptides demonstrate antibacterial, antifungal, anti-biofilm and anti-inflammatory properties, with many scaffolds exhibiting multiple activities. This multiplexity is exemplified by lycotoxin-Pa2a, which displays antibacterial effects comparable to or superior to melittin. It achieves this by disrupting bacterial membranes, promoting intracellular reactive oxygen species generation and inhibiting the expression of inflammatory mediators, all while exhibiting low toxicity to primary mammalian cells and erythrocytes. Similarly, rationally designed peptides that combine direct disruption of bacterial cell membranes with the blockage of inflammatory signalling *via* TLR4 offer a more attractive therapeutic strategy for infected inflammatory lesions than mere sterilization [132]. The most prominent theme is the "Breadth of anti-infective function." Recently discovered spider peptides exhibit antibacterial, antifungal, anti-biofilm and anti-inflammatory properties, with many possessing multiple activities within the same scaffold. This multiplexity is exemplified by lycotoxin-Pa2a, which shows antibacterial effects comparable to or better than melittin, disrupts bacterial membranes, promotes intracellular reactive oxygen species (ROS) generation and inhibits inflammatory mediator expression, all with low toxicity to primary mammalian cells and erythrocytes. Similarly, rationally designed peptides that combine direct bacterial cell membrane disruption with the blockage of inflammatory signalling *via* TLR4 offer a compelling approach for infected inflammatory lesions, surpassing mere sterilization. Another significant trend is the shift from generic Gram-positive testing toward priority multidrug-resistant pathogens. AATX-Ab2a and AATX-Ab3a inhibit both Gram-positive and Gram-negative bacteria, exhibit activity against multidrug-resistant

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Pseudomonas aeruginosa, and suppress fungi while maintaining low toxicity to normal human cells [51]. LC-AMP-F1 and LC-AMP-I1 extend this pattern, demonstrating activity against panels of clinically relevant MDR bacteria, inhibiting biofilm formation, disrupting established biofilms, exhibiting low haemolysis and showing additive or synergistic effects with conventional antibiotics [66]. PA-Win2 further incorporates a quorum-sensing dimension, suppressing P. aeruginosa growth while downregulating Las, Pqs and Rhl circuitry and degrading pre-formed biofilms [132].

The field is also beginning to generate *in vivo* proof-of-concept, a critical advancement beyond much of the existing AMP literature. LC-AMP-I1 reduced bacterial proliferation in a neutropenic mouse thigh infection model. LyeTx I mnΔK outperformed vancomycin in rapid bactericidal action and anti-biofilm effects against MRSA, and when formulated as a gel, was superior to vancomycin in a mouse wound model [74]. LyeTx I also reduced bacterial load and lung inflammation in a murine carbapenem-resistant Klebsiella pneumoniae pneumonia model, demonstrating that spider-derived AMPs can be effective in deep-tissue infection settings as well as superficial wounds [133].

Mechanistically, anti-infective spider peptides are no longer viewed as nonspecific detergents. Biophysical studies of latarcins reveal that some family members are aggressively membrane-permeabilizing and haemolytic, whereas others kill bacteria more selectively through subtler lipid interactions or possible intracellular targeting [40]. Anti-staphylococcal selectivity also relies on distinct sequence characteristics, as evidenced by neural-network-guided fragments derived from linear spider toxins, such as N-terminal hydrophobic anchors and C-terminal amidation, both of which promote interaction with the bacterial membrane without causing haemolysis [52]. This mechanistic resolution holds significant promise for more predictive peptide engineering.

The most important constraint remains the high level of translation costs. Stability, cytotoxicity and delivery are consistently identified as critical areas of concern in reviews [57]. LyeTx I exemplifies this trade-off, demonstrating excellent antibacterial activity against CRKP but exhibiting high toxicity and suboptimal plasma stability, indicating the need for formulation or chemical modification to improve pharmacokinetics or toxicity and achieve a therapeutic window. However, the anti-infective field is progressing through both the identification of new peptides and their acetylation, amidation, helicity tuning, hybridization and combination therapy in an effort to discover orally bioavailable molecules [133].

Oncology

Limited, though growing, preclinical cancer literature of spider-venom peptides exists. The majority of data are from *in vitro* cytotoxicity testing, mechanistic membrane interaction studies and from optimised material delivery early in the development process, not from strong efficacy packages in animal models or clinically advanced candidates [134]. That's why this difference has relevance to review framing: While oncology does not have a spider-peptide program anywhere near the level of translational maturity that has been found in pain and anti-infectives are even more distant. One of the main scaffolds in this area is an amphipathic, cell-penetrating, 25-residue α-helical peptide from *Lycosa vittata*, called LVTX-8. The advance is that rational modification was used in creating the 7 synthetic derivatives of LVTX-8 that showed either the same or improved anticancer activity in cell assays, and that the 2 *in vivo* modified analogues possessed increased proteolytic stability, less pro-haemolytic activity and more prolonged activity in cells compared to the parent peptide. LVTX-8 is not a monomodular drug, acting on a single target, but is a combination of cell-disarying, mitochondrial targeting and mitochondrial induction of membrane potential, indicating a multimodal cell-death mechanism [42,59].

Other linear peptides derived from spiders also predominantly support the oncology paradigm, which is largely surface-based. Combined antibacterial, anti-biofilm and antitumor activities were presented by LtM, *i.e.* the most promising antitumour analogue, and by synthetic analogues based on latarcin-3a, in which Lt-MAP2 was the most promising antitumour analog, without haemolysis at the maximum tested concentration [59]. Liposome-encapsulated LyeTx III was more cytotoxic against HN13 tumour cells than free peptide, and was suggested to increase the half-life and increase the ability to target the tumour [104]. The data described here strongly indicate that delivery engineering is far more relevant than sequence potency in the process of translating into useful oncologic leads membrane-active spider peptides. From a conceptual perspective, spider peptides are also members of the larger family of anticancer peptides where many examples are cationic amphiphiles which bind to altered tumour-cell membranes, and some bind to ion channels or immunoregulatory pathways that are important in cancer [134]. Current evidence is, however, diffused in cell lines, formulations and mechanistic assays and is poorly compared side-by-side with the standard chemotherapeutic drugs; moreover, *in vivo* validation of the drugs is rare. This largely unvalidated space is best described as the oncology domain: it is well suited for exploring mechanisms and engineering, but not yet robust enough to support near-term clinical translation [42].

Cardiovascular and coagulation targets

Cardiovascular and coagulation applications are emerging as a significant expansion beyond the historically dominant neuropharmacology of spider venom. The capacity of spider peptides to modulate mammalian ion channels, beyond the NaV pain targets, including potassium and calcium channels linked to excitability, arrhythmia and vascular biology, represents a crucial enabling concept [20]. This broadens the therapeutic spectrum beyond analgesia to encompass rhythm control, tissue protection and thrombosis. One notable area of focus is cardiac ion channels. Screening animal venoms against hKv1.5, a promising target for atrial fibrillation therapy, identified Osu1, a spider peptide that modulates human hKv1.5 while retaining its insecticidal activity. As Osu1 appears to represent a novel structural template and likely a novel family of toxins, its current therapeutic value is primarily as a lead scaffold and channel probe. Concurrently, venom composition studies of *Lycosa coelestis* revealed superior efficacy in blocking CaV channels in the dorsal root ganglion, suggesting the existence of numerous unexplored CaV channel modulators in plant and spider venoms [45]. The most direct therapeutic advancement in this domain is anticoagulation. GC38, a cystine-knot peptide identified through spider venom gland transcriptomics, potentiates activated protein C, prolongs plasma clotting time and allosterically binds APC with micromolar affinity [135]. Critically, GC38 demonstrated efficacy across three *in vivo* thrombosis models, arterial occlusion, deep vein thrombosis and photothrombotic stroke, while exhibiting low haemorrhagic risk in tail-bleeding assays. This combination of dual arterial/venous antithrombotic action and limited bleeding liability positions GC38 as one of the most compelling newly reported non-neuro spider-peptide leads. Furthermore, ASIC1a inhibition establishes a link between neuroprotection and cardioprotection. In response to the proposed role of proton-sensing pathways in organ-transplant ischemia, Hi1a is under development for use in myocardial infarction and, alongside stroke, addresses acute cardiovascular injury through various mechanisms unrelated to haemostasis or electrophysiology (Table 1) [16]. While the cardiovascular/coagulation field is smaller and less validated than the analgesic field, it is not merely anecdotal. It now encompasses at least three distinct mechanistic subprograms, hKv1.5 modulation, ASIC1a-mediated cardioprotection and APC-potentiating anticoagulation, which are equivalent to, rather than simply extensions of, its role in pain research, thereby establishing it as a veritable emerging application area rather than a useful peripheral add-on [20].

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Synthesis and outlook

The literature provides evidence for a clear hierarchy of maturity across therapeutic domains for spider venom applications (Table 4). Analgesia and ion-channel disorders represent the two most advanced areas, attributed to the exceptionally robust biology of this target class. This includes exquisite pharmacology and peptide engineering, which have led to the generation of highly optimized NaV1.7 and ASIC1a ligands [131]. Currently, most new activity is observed in anti-infectives, a growth area driven by the pressing need for novel drugs and the abundance of linear membrane-active peptides in spider venoms that can be rapidly discovered, redesigned and tested. While cardiovascular and coagulation applications are nascent, their plausibility has increased due to compounds like Hi1a and GC 38 [21]. Across all four domains, a consistent lesson emerges: chemistry-enabled reprogramming is now as crucial as natural "discovery." Research advances are overcoming classic limitations such as scarcity, instability, poor selectivity and delivery. This progress is evident through advancements in transcriptomics, total synthesis, ligation chemistry, analogue design, encapsulation and AI-assisted optimization [2]. Consequently, spider toxins should no longer be viewed solely as a source of exotic neurotoxins; rather, they are emerging as a highly defensible conclusion of review articles. Increasingly, they represent a programmable peptide armoury. The most significant payoff is shown in pain management, with substantial innovation also seen in anti-infectives. Additionally, promising, albeit less mature, opportunities are appearing in cancer, cardioprotection and anticoagulation.

Table 4. Leading therapeutic applications of spider venom peptides

Indication	Peptide or platform	Target or mechanism	Quantitative or functional signal	Ref.
Pain	Pn3a	Selective NaV1.7 inhibition	IC50 0.9 nM with 40-1000-fold selectivity over other NaV subtypes	[136]
Pain	mGpTx1-SA	Engineered NaV1.7 inhibitor with reduced rKv4.2/4.3 activity	Retained hNav1.7 inhibition, reduced off-target Kv activity and eliminated cardiotoxicity while preserving analgesia in inflammatory and neuropathic models	[122]
Ischemic stroke	Hi1a	ASIC1a inhibition by stabilizing the closed channel state	A single 2 ng/kg dose given up to 8 h after stroke induction reduced infarct size and improved neurological outcomes in rats	[137]
Cancer	Lycosin-I	Selective membrane binding plus apoptosis, G1 arrest and ferroptosis in leukaemia models	*In vivo* intraperitoneal treatment inhibited xenograft growth without detected side effects	[78]
Infection	GK37 and Lycosin-II	Membrane-disruptive antimicrobial peptides	GK37 inhibited *S. aureus* with MIC 1.552 μM and showed little haemolysis at 4× MIC; Lycosin-II inhibited drug-resistant clinical isolates, including MDR *A. baumannii*	[138]

Translational barriers of spider venom peptides

The primary translational barriers for spider venom peptides typically stem not from a lack of potency, but from challenges in developability. Rapid proteolysis and short half-lives remain persistent issues, particularly with linear or amphipathic peptides, directly impacting the route of administration, systemic exposure and duration of action [87]. Despite achieving high levels of target engagement, the pharmacokinetics, site of action, tissue distribution, plasma protein binding, efflux, metabolism and immunogenicity of most spider venom leads are poorly understood, yet these factors are clinically crucial for long-term therapeutic use [139]. Another significant barrier is off-target activity. Some venom peptides are multitarget compounds, interacting with one or more channel or receptor subtypes beyond the intended target. This promiscuity poses a risk of unintended side effects in excitable tissues [83]. This concern is not merely theoretical because: (a) insecticidal or analgesic spider toxins can exhibit measurable activity on mammalian ion channels and (b) membrane-active peptides, while displaying desired antimicrobial or anticancer properties, can also induce haemolysis or nonspecific cytotoxicity [20]. Furthermore, limited tissue access

presents a challenge, especially for targets located in intracellular spaces, the gut, or the central nervous system. Poor oral bioavailability, restricted epithelial permeability and the difficulty of blood-brain barrier penetration impede access, necessitating advanced delivery engineering [57].

A second barrier involves material access and manufacturing. Native venom is inherently scarce, and the typically low venom yields from most species make it challenging to isolate sufficient quantities for comprehensive bioactivity testing, preclinical development and the characterization of minute structures [1]. This scarcity complicates recombinant production and chemical synthesis during preclinical development [140]. A critical limitation is the ability to achieve scalable production. Disulfide-rich spider peptides often suffer from poor expression, folding, refolding, secretion and purification, leading to low yields, irrespective of the peptide's native disulfide pattern. While bacterial systems are inexpensive and robust, they struggle with correct oxidation and post-translational processing for disulfide-rich peptides. Yeast systems offer a superior secretion environment compared to bacterial systems, but at an increased cost, thus requiring an optimization step. Consequently, the field is moving toward a "cocktail" approach, combining recombinant expression, synthetic access and sequence engineering (*e.g.* cyclization, terminal protection, D-amino-acid substitution, carrier conjugates) to enhance stability, prolong half-life and facilitate manufacture without compromising activity [87].

The barrier profile extends from clinical pharmacology to environmental stability, formulation, delivery and production costs for spider venom biopesticides. The efficacy of commercial and near-commercial insecticidal peptides underscores the importance of intrinsic toxicity alongside the ability to withstand solar heat and exposure, be consistently reproduced in the laboratory, and be assimilated orally or topically by pests [141]. Oral delivery, in particular, is challenging for peptide toxins, as they may not act as rapidly as some small molecule insecticides and have limited membrane permeability, making formulation a key determinant of product performance. Strategies to enhance persistence in the insect gut, such as surfactant-treated lectins, nanocapsulation and fusion to snowdrop lectin or cell-penetrating peptides, could significantly improve aphidicidal or larvicidal activity and gut passage. However, achieving sustainable recombinant yields, process repeatability and fermentation optimization remains elusive for commercial products, even for promising constructs. Optimizing gene copy number, enzyme structures, signal sequences, strain background and cell culture conditions is a necessary process to improve yields compatible with scale-up.

Future directions for spider venom therapeutics

The current hunts that are based on the potency of a venom extract tend to be restricted to *in vitro* activity, whereas future snake venom chemical studies need to focus on developing the venom to better become a candidate for human use, creating the venomics criteria of discoverability first and then potency. In other words, key methods used early in the translational process, such as proteolytic stability, targetability, toxicity, PK, tissue delivery and manufacturability, come first, with *in vivo* activity second, not the other way around [142]. The strongest discovery model is now an integrated pipeline that combines proteo-transcriptomics for broad and low-input toxin discovery, protein-level validation to avoid transcript-only overcalling, structural biology for mechanism and redesign, AI and machine learning for ranking candidates by predicted activity, stability, toxicity and cell penetration, medicinal chemistry for sequence minimization and conformational stabilization and delivery engineering such as CPP fusion, nanocarriers and advanced formulations to convert bioactive scaffolds into drug-like molecules [2]. This pipeline is already technically feasible: transcriptomics and cDNA-guided venomics accelerate discovery from tiny venom samples and poorly characterized species, in silico and AI-guided design can identify or optimize multifunctional antimicrobial leads from spider toxin templates [143], and rational engineering can materially improve peptide performance, as shown by stabilized LVTX-8 analogues with higher proteolytic resistance and lower haemolysis, by Ssp1a analogues with altered NaV subtype selectivity, and by

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formulation-enabled peptide delivery platforms [63]. Just as importantly, the next wave of discovery should expand beyond the field's traditional focus on disulfide-rich ion-channel peptides toward neglected venom modalities with distinct translational promise. Spider venom enzymes remain underexplored despite growing evidence that their diversity has been systematically underestimated and that protein-validated enzyme inventtories may open new industrial and biomedical applications, while recombinant studies on hyaluronidases, metalloproteases and related enzymes show that expression technology is now beginning to make these scarce components experimentally accessible [144]. Another major opportunity is derived from venom's CPPs, which could be used as delivery vehicles as well as bioactive agents and natural and engineered CPP frameworks are being recognized as vehicles for intracellular transport and tissue-directed cargo delivery [145]. Spider acylpolyamines deserve restimulation as well, being chemically modular, first targeting ionotropic glutamate and nicotinic receptors, with the ability to stimulate synthetic analogues and molecular probes, and additionally because they can provide a means for delivering targeted agents with polyamine transporters [146]. GPCR-active venom peptides are not very well represented within the venom translational programs, and although GPCRs represent an established class of pharmacological targets, they are also found to be modifiable *via* different scaffolds, by both agonist and antagonist peptides and also by allosteric peptides [12]. Altogether, the literature justifies a future discovery-to-development approach based on omics-driven cheminformatics throughout the venom mining, validation, rank ordering, medicinal chemistry re-design, delivery and solution of the venom exposure and a deliberate expansion of the searchable chemical space from enzymes to CPP-like toxins and acylpolyamines to GPCR modulators, where many of the most compelling next-generation spider venom therapeutics may be found.

Conclusion

Spider toxins are an abundant source of therapeutic leads but lack clinical promise in their native form. However, their native scaffolds can be reprogrammed. Disulfide-rich inhibitor cystine knot peptides are the most mature platform due to their thermal, chemical and proteolytic stability. Linear peptides, enzymes and acylpolyamines represent an expanded chemical space for future lead classes. Advances in venomics, combining transcriptomics, proteomics and machine learning, enable faster discovery, though functional annotation remains a major limitation. The most promising approach is complex design, not just peptide discovery, combining sequence design, motif grafting, conjugation, formulation, total synthesis and nanocarrier design to enhance selectivity, stability, delivery and cytotoxic tuning for pain, ischemia, infection and cancer. The field is transitioning from venom diversity cataloguing to 'developability-first venomics'. Future success relies on leveraging developments in structural biology, medicinal chemistry and delivery science while overcoming venom scarcity, off-target activity and short half-life.

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