

# Specialized insulin is used for chemical warfare by fish-hunting cone snails

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**More than 100 species of venomous cone snails (genus *Conus*) are highly effective predators of fish. The vast majority of venom components identified and functionally characterized to date are neurotoxins specifically targeted to receptors, ion channels, and transporters in the nervous system of prey, predators, or competitors. Here we describe a venom component targeting energy metabolism, a radically different mechanism. Two fish-hunting cone snails, *Conus geographus* and *Conus tulipa*, have evolved specialized insulins that are expressed as major components of their venoms. These insulins are distinctive in having much greater similarity to fish insulins than to the molluscan hormone and are unique in that posttranslational modifications characteristic of conotoxins (hydroxyproline,  $\gamma$ -carboxyglutamate) are present. When injected into fish, the venom insulin elicits hypoglycemic shock, a condition characterized by dangerously low blood glucose. Our evidence suggests that insulin is specifically used as a weapon for prey capture by a subset of fish-hunting cone snails that use a net strategy to capture prey. Insulin appears to be a component of the nirvana cabal, a toxin combination in these venoms that is released into the water to disorient schools of small fish, making them easier to engulf with the snail's distended false mouth, which functions as a net. If an entire school of fish simultaneously experiences hypoglycemic shock, this should directly facilitate capture by the predatory snail.**

insulin shock | cone snails | conotoxins | nirvana cabal | venom

The venoms of predatory marine cone snails are remarkably potent and diverse (1). Most bioactive venom components are small disulfide-rich peptides, termed conotoxins (1), that target specific receptors and ion channel subtypes located in the prey's nervous system (2, 3). Here, we provide evidence for a specialized insulin in the venom of *Conus geographus* that is part of the chemical arsenal used by the snail for capturing prey. This finding significantly extends known molecular mechanisms of envenomation beyond conventional neurotoxins.

Unlike other fish-hunting cone snails that inject venom as they tether the prey, *C. geographus* engulfs its fish prey with its highly distended false mouth before venom injection (1). It has been suggested that *C. geographus* releases specialized toxins, called the "nirvana cabal," into the water to suppress the sensory circuitry of schools of small fish prey (4). Our data strongly suggest that the specialized *C. geographus* insulin we characterized, Con-Ins G1, forms part of this venom cabal.

Vertebrate insulin, synthesized in pancreatic  $\beta$  cells, is the key hormone regulator of carbohydrate and fat metabolism (5); in the brain, it functions as a neuromodulator of energy homeostasis and cognition (6). Insulin is initially synthesized as a precursor comprising three regions (A, B, and C) (7), from which proteolytic

cleavage of the C peptide in the Golgi releases the mature insulin heterodimer with an A and B chain connected by two disulfide bonds. The A chain contains an additional intramolecular disulfide. The primary sequence and arrangement of cysteines that form disulfides are highly conserved in all vertebrates (5). In contrast, invertebrate insulin family members are more variable and serve in neuronal signaling, memory, reproduction, growth, and metabolism (8, 9). In molluscs, insulins are primarily expressed in neuroendocrine cells, including neurons and cerebral ganglia (9, 10). Molluscan and most other invertebrate insulins differ from the vertebrate hormone in containing two additional cysteines (one in each chain) that are assumed to form an additional disulfide between the A and B chain (9). Moreover, the mature molluscan insulin chains are generally larger than vertebrate insulins. Thus, sea hare (*Aplysia californica*) insulin (AI) has a molecular mass of 9,146 Da (10) (compared with 5,808 Da for human insulin).

The venom gland of cone snails is highly specialized for conotoxin biosynthesis and secretion (11). Our discovery that an insulin is expressed in the venom gland of *C. geographus* at levels

## Significance

**The discovery and characterization of insulin, a key hormone of energy metabolism, provided a life-saving drug for diabetics. We show that insulin can be subverted for nefarious biological purposes: Venomous cone snails use specialized insulins to elicit hypoglycemic shock, facilitating capture of their fish prey. This finding extends our understanding of the chemical and functional diversity of venom components, such that the snail's arsenal includes a diverse set of neurotoxins that alters neuronal circuitry, as well as components that override glucose homeostasis. The highly expressed venom insulins are distinct from molluscan insulins and exhibit remarkable similarity to fish insulins. They are the smallest of all insulins characterized from any source, potentially providing new insights into structure-function elements of insulin action.**

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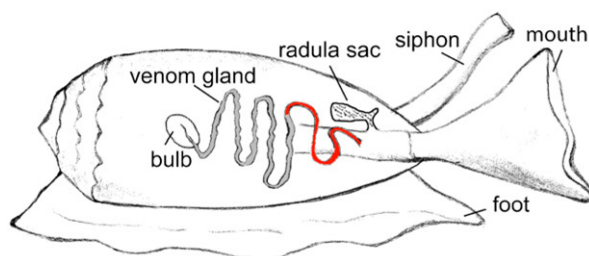
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**Table 1. Expression levels and distribution of *C. geographus* insulins across the length of the venom gland**

	Total reads	Distribution of reads in four sections of the <i>C. geographus</i> venom gland (%)			
		1 (proximal)	2 (proximal central)	3 (distal central)	4 (distal)
<b>Insulins</b>					
Con-Ins G1	1,242	0	0.1	0	<b>99.9</b>
Con-Ins G2	1,120	49.5	30.8	18.8	0.9
<b>Nirvana cabal toxins</b>					
Conantokin G	38,854	0.4	0.1	36.9	<b>62.6</b>
Conopressin G	310	4.5	10.2	9.1	<b>76.2</b>
Contulakin G	899	0.1	0.1	21.1	<b>78.7</b>
<b>Motor cabal toxins</b>					
μ-GIIIB	114,509	42.5	43.0	14.4	0.2
ω-GVIA	3,655	74.0	17.3	7.2	1.5
α-GII	19,059	47.5	36.1	15.0	1.4



The transcriptomes of four regions of the venom gland were sequenced by 454 RNA-Seq and differential expression of insulin transcripts and selected conotoxins was determined by mapping all reads to the transcripts of interest. The (distal) region of the venom gland is highlighted in red in the anatomical schematic. Read numbers for transcripts with highest expression in this region are also shown in red.

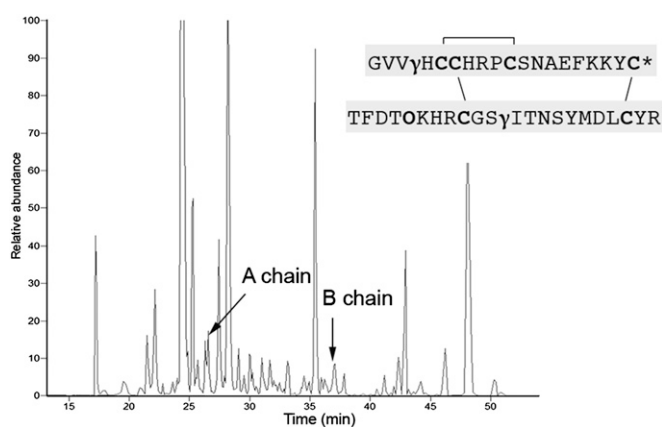
from 83% to 87%. The predicted mature peptide of the most similar transcript, Con-Ins T1, differs by only one conservative substitution in the A chain (Tyr-19 to Phe) and five substitutions in the B chain, of which three are conservative (Fig. S2). No sequences resembling endogenous molluscan insulins were found in the venom gland of *C. tulipa*, despite deep sequencing of PCR products (a total of 274,520, 187,930, and 132,975 reads were obtained for Con-Ins T1, T2, and T3, respectively).

Five additional fish-hunting cone snails were examined by RT-PCR using the same oligonucleotides and PCR conditions used for *C. geographus* and *C. tulipa* (Table S1). Unlike *C. geographus* and *C. tulipa*, which use a “net-hunting” strategy for prey capture, these species belong to the “hook and line hunters” (1). RT-PCR did not identify insulin transcripts in any of the five tested species. For two of these snails, *C. bullatus* and *C. consors*, the absence of insulin transcripts was also confirmed by mining next-generation transcriptome data (15, 16) [*C. consors* transcriptome data have not been made publically available, but Violette et al. (16) do not report any insulin expression in the venom gland of this species].

The venom glands of two mollusc and four worm-hunting cone snails were also investigated; all expressed molluscan-like insulins, but not the fish-like peptides (Table S1 and Fig. S1). As observed for Con-Ins G2, these transcripts were characterized by an additional cysteine residue in each chain and longer predicted A and B chains. Sequence diversity ranged from one transcript for *Conus marmoreus*, *Conus textile*, and *Conus memiae* to four in *Conus floridulus*. These findings suggest that insulin expression in the venom gland of a particular species can be correlated with its feeding strategy. Molluscan-like insulins are broadly distributed among snail and worm hunters, whereas the expression of fish-like insulins appears to be limited to a subset of fish hunters.

### Con-Ins G1: High Abundance and Novel Posttranslational Modifications.

To determine whether *C. geographus* insulin transcripts were translated and posttranslationally processed into mature insulins, the venom of this species was interrogated by MS. Mature peptides derived from the Con-Ins G1 precursor but not G2 were identified by MS sequencing performed on reduced and alkylated (RA) venom using a high-resolution Orbitrap Elite instrument.



**Fig. 3.** MS sequencing of Con-Ins G1 in the venom of *C. geographus* identifies the A and B chain and several modifications unusual for insulins. (A) Venom was reduced and alkylated and subjected to reverse-phased chromatography and tandem MS sequencing on a high-resolution Orbitrap Elite mass spectrometer. Tandem MS data are provided in Fig. S3. Posttranslational modifications are provided in their single letter code. γ, γ-carboxylated glutamate; O, hydroxyproline; \*, C-terminal amidation. (Right) The resulting Con-Ins G1 heterodimer. Cysteines and modified residues are shown in bold.

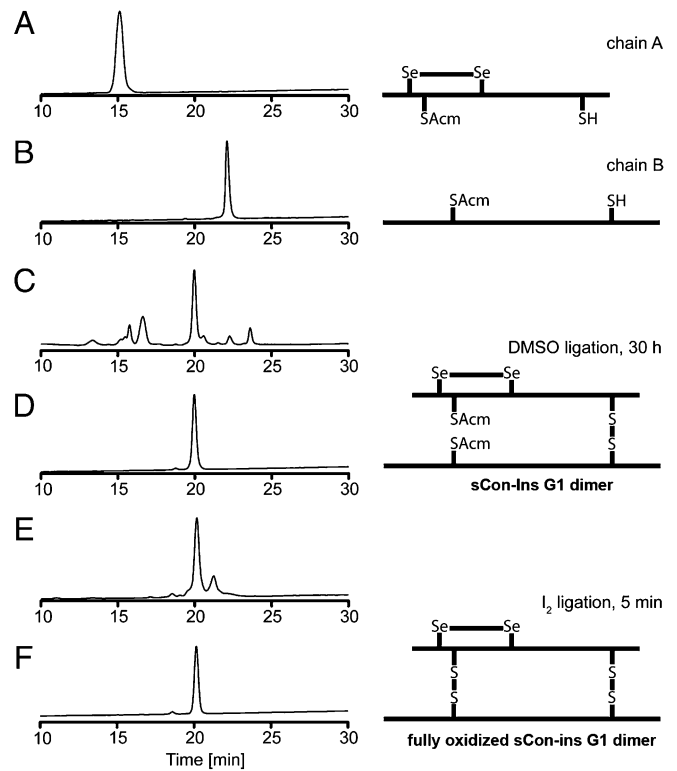
Tandem MS identified the individual A and B chains of Con-Ins G1, demonstrating that cleavage of the precursor peptide at basic or dibasic sites liberates an A and B chain, as observed for other insulins (Fig. 3; MS/MS spectra in Fig. S3). Con-Ins G1 carries several posttranslational modifications that are unprecedented in insulins but commonly observed in other cone snail venom peptides (17). Glu4 of the A chain, one of the most conserved amino acids in vertebrate insulin (18), is modified to  $\gamma$ -carboxylated glutamic acid (Gla or  $\gamma$ ). Another Gla modification (Gla12) was also identified in the B chain. Moreover, Pro5 in the A chain is modified to hydroxyproline (Hyp5), and the C terminus of this chain is amidated (Fig. 3).

MS sequencing further revealed the presence of several variants of Con-Ins G1 in fractionated venom (Fig. S4). Modifications in these variants include Gla15 and Hyp10 in the A chain, Hyp5 in the B chain, and alternative proteolytic cleavage at the C terminus of the B chain. The presence of multiple variants deriving from the same peptide precursor has recently been described for several conotoxins (16, 19) but is exceptional for insulins. The other vertebrate-like insulin, Con-Ins G3, was also identified by MS sequencing (Fig. S5). Similar to Con-Ins G1, this peptide is modified, and several variants could be identified in the venom (Fig. S4).

Fractionated *C. geographus* venom was analyzed by high-resolution LC-MS and searched for masses corresponding to the intact insulin dimers. A heterodimer that consisted of the originally sequenced A and B chains (Fig. 3) was not identified, but MS sequencing successfully identified one of the variants that contained an additional  $\gamma$ -carboxylated glutamic acid in the A chain (Gla15) and did not have the N-terminal arginine in the B chain (Fig. S6A). The molecular mass of this variant was 5,030.06 Da, consistent with the presence of three disulfide bonds. A second variant containing an additional hydroxyproline (Hyp10) in the A chain (5,046.05 Da; Fig. S6B) and a third variant that had no hydroxylated prolines in either chain were also identified (5,014.05 Da; Fig. S6C). Fractions were reduced and alkylated, and sequences were confirmed by tandem MS sequencing (Fig. S5). These data clearly show that Con-Ins G1 forms a heterodimer consisting of A and B chains that are linked by disulfide bonds. The intact heterodimer of the other vertebrate-like peptide, Con-Ins G3, was also identified by MS sequencing. Intact Con-Ins G3 has a molecular mass of 4,817.10 Da, consistent with the presence of three disulfide bonds (Fig. S6D). With a total length of 43 amino acids, to our knowledge, native Con-Ins G1 and G3 are the shortest native insulins reported to date (human, zebrafish, and *Aplysia* insulins are 51, 50, and 80 residues long, respectively).

Venom fractionation and sequencing further confirmed that Con-Ins G1 and its variants are abundant peptides in the venom of *C. geographus*. The venom fraction containing Con-Ins G1 was 1 of 25 collected fractions and predominantly consisted of venom insulins.

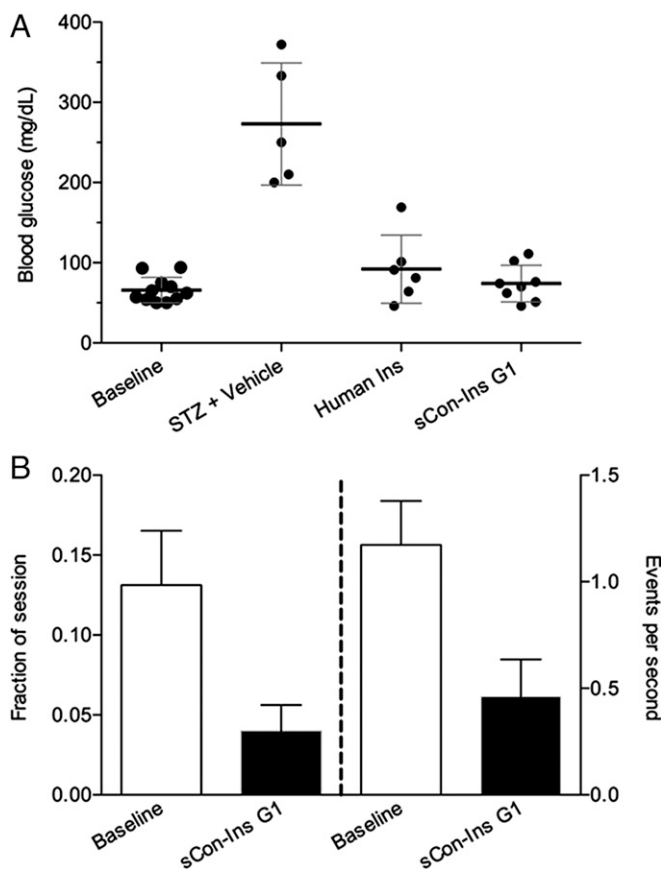
**Con-Ins G1 Was Successfully Synthesized Using a Selenocysteine-Based Strategy.** To obtain sufficient material to investigate its bioactivity in fish, Con-Ins G1 was chemically synthesized. Since the first chemical synthesis in 1963 (20), insulin has remained challenging to synthesize with the correct intra- and intermolecular disulfide bonds. A strategy incorporating a pair of selenocysteines in the A chain was used to synthesize Con-Ins G1. Replacement of one pair of cysteines with selenocysteines (Sec) has been used for the synthesis of various bioactive peptides, including conotoxins (21). Diselenide bond-containing peptide analogs have similar biological activities to their native peptides and, in some cases, even improved potency or selectivity (21). Due to the lower redox potential, diselenide bond formation is favored over the disulfide bond formation under acidic conditions (21). This Cys-to-Sec replacement strategy was combined with



**Fig. 4.** Synthesis of sCon-Ins G1. (A) Purified chain A with the intramolecular Sec-Sec bridge formed and Cys-7 protected with the AcM group. (B) Purified chain B with Cys-9 protected with the AcM group. (C) sCon-Ins G1 heterodimer formation by treatment with 20% DMSO for 30 h in 0.1 M Tris-HCl containing 1 mM EDTA, pH 7.5. (D) Purified sCon-Ins G1 heterodimer. (E) Oxidation of remaining disulfide bond of the heterodimer of sCon-Ins G1 by treatment with  $I_2$  in the mixture of acetonitrile, water, and TFA at 200  $\mu$ M for 5 min. (F) Purified final sCon-Ins G1.

orthogonal protection of the remaining two pairs of cysteines to sequentially form intra- and intermolecular disulfide bridges, as shown in Fig. 4. Cys6 and Cys11 of chain A were replaced with Sec residues, which formed an intradiselenide bridge after peptide cleavage and reduction (21, 22) (Fig. 4A). Cys20A and Cys21B of the respective chains [synthesized with a trityl (Trt) protecting group] were used to form the first intermolecular disulfide bridge on DMSO treatment (Fig. 4C). Cys7A and Cys9B, bearing acetaminomethyl (AcM) groups, were subjected to iodine oxidation to remove AcM protection and simultaneously form the second disulfide bridge (Fig. 4E). The two-step ligation protocol led to the final purified product, synthetic selenocysteine-Con-Ins G1 (sCon-Ins G1), in a total yield of 10.5%, based on the starting amount of purified chain A. RP-HPLC confirmed the purity (95%), and electrospray ionization MS sequencing confirmed the correct identity of the product (Fig. S7). To our knowledge, this is the first time a selenocysteine-based strategy has been applied to the synthesis of insulin.

**sCon-Ins G1 Lowers Blood Glucose and Alters Swimming Activity in Fish.** The streptozotocin (STZ)-induced model of hyperglycemia was used to assess whether sCon-Ins G1 could effectively lower blood glucose levels in a model prey: adult zebrafish. Animals were first rendered hyperglycemic through i.p. injection of the  $\beta$ -cell poison STZ (1.5 g/kg) (23), and the effect of subsequent injection of sCon-Ins G1 was examined. Following STZ treatment, blood glucose levels were significantly elevated from  $65.9 \pm 4.75$  ( $n = 11$ ) to  $273.0 \pm 34.08$  mg/dL ( $n = 5$ ,  $P < 0.01$ ; Fig. 5A). Administration of sCon-Ins G1 at 65 ng peptide/g body weight



**Fig. 5.** sCon-Ins G1 lowers blood glucose and disrupts swimming behavior in zebrafish. (A) Insulin activity was determined using the streptozotocin (STZ)-induced model of hyperglycemia. Injection of 1.5 g/kg STZ into adult zebrafish caused hyperglycemia as evident by a significant increase of blood glucose ( $n = 5$  fish,  $P = 0.003$ ) compared with controls ( $n = 11$ ). Hyperglycemia was successfully reversed on administration of 65 ng/g sCon-Ins G1 ( $n = 8$ ,  $P = 0.003$ ) and human insulin (65 ng/g,  $n = 6$ ,  $P < 0.003$ ). Data were analyzed in Prism Graphpad software (version 6.0) using unpaired  $t$  tests with Welch's correction. Plotted dots represent individual fish. Bold lines and error bars represent means  $\pm$  SD. (B) Application of sCon-Ins G1 into the water column (25 nmol/mL) significantly reduced overall locomotor activity, observed as a significant decrease in the percentage of time spent swimming ( $P = 0.038$ , left  $y$  axis) and movement frequency ( $P = 0.003$ , right  $y$  axis) compared with baseline values for the same animals. Values represent means  $\pm$  SEM ( $n = 3$ ).

significantly lowered blood glucose to  $74.0 \pm 8.051$  mg/dL ( $n = 8$ ,  $P < 0.01$ ). The ability to effectively lower blood glucose was similar to human insulin ( $92.0 \pm 17.35$  mg/d,  $n = 6$ ,  $P < 0.01$ ).

To determine whether sCon-Ins G1 elicits an effect when applied to the water column, the spontaneous swimming behavior of fish larvae (7 d after fertilization,  $n = 3$ ) was monitored before (baseline) and following application of 25 nmol/mL peptide. Administration of sCon-Ins G1 reduced overall locomotor activity, observed as a significant decrease in the percentage of time spent swimming ( $P = 0.038$ ) and movement frequency ( $P = 0.003$ ) compared with baseline values for the same animals (Fig. 5B and Movie S1). It has previously been reported that externally applied human insulin can induce insulin shock in fish via direct absorption through the gills into the bloodstream (24). Our findings suggest that sCon-Ins G1 also efficiently crosses the endothelium of the gill plexus.

## Discussion

Insulin is an essential hormone, but overdoses cause hypoglycemia, and in extreme cases, fatality. The deliberate use of insulin for nefarious purposes is both the material of fiction and

regularly rumored to occur in real life. In the famous von Bülow case, an alleged overdose of insulin became the basis for a sensational criminal trial. Remarkably, evolution has developed a parallel story line, with the normal physiological function of insulin being subverted by a lineage of cone snails as part of their strategy to overcome their fish prey. Our results establish that species in the Gastridium clade, one of five to six clades of fish-hunting cone snails, use insulin as an offensive weapon, part of the chemical armamentarium evolved for maximizing the probability of prey capture.

Cone snails, like all molluscs, have endogenous insulin, and we identified a sequence resembling endogenous molluscan insulins in the venom glands of *C. geographus* and several vermivorous and molluscivorous species. These sequences differ from fish insulins in highly distinctive ways, notably the presence of an additional pair of cysteines. We demonstrate that cone snails in the Gastridium clade have, in addition to molluscan type insulin, a unique class of insulins that is expressed at very high levels in their venom. These specialized insulins share much greater similarity to the vertebrate insulin found in fish, the prey of these snails. We directly demonstrate that Con-Ins G1 is not only bioactive in fish, but, when this insulin is applied to the water, the fish become hypoactive.

Larger cone snails in the Gastridium clade, including *C. geographus* and *C. tulipa* use a net strategy for prey capture. It has been suggested that in the wild, these snails primarily stalk schools of small fish hiding in reef crevices at night. By extruding a subset of venom components, known as the nirvana cabal, as they approach the school of fish, they are able to elicit a state of sensory deprivation and general hypoactivity (4). Suggested members of this nirvana cabal include conantokin-G, a selective antagonist of the NMDA receptor (25); contulakin-G, a neurotensin receptor agonist (26); and Lys-conopressin-G, an agonist of the vasopressin receptor (27). Like Con-Ins G1, these peptides are most highly expressed in the distal section of the long and convoluted venom gland, closest to the injection apparatus (Table 1). In contrast, venom components that cause irreversible neuromuscular block (motor cabal toxins) (4), including the nicotinic acetylcholine antagonist  $\alpha$ -GII and the sodium channel blocker  $\mu$ -GIIIB, display very low expression levels in this region.

The present evidence shows that the nirvana cabal targets not only the circuitry of the fish nervous system but also energy metabolism because Con-Ins G1, when released, results in hypoglycemic shock in the fish. The symptoms of low blood glucose, which include weakness and neuronal dysfunction, are characteristic of physiological impairments induced by the nirvana cabal (4). Three additional fish-like insulin sequences were also identified in the venom gland of *C. tulipa*, a close relative of *C. geographus*. However, specialized insulins that are venom components in these fish-hunting species are absent in venoms of other fish hunters that do not use a net strategy, but instead harpoon their prey. Moreover, cone snails that prey on snails and worms do not express the fish-like sequence but insulins that are similar to endogenous molluscan and worm insulins, suggesting that these insulins also play a role in prey capture.

High expression levels of the molluscan-like insulin Con-Ins G2 in the proximal region of the venom gland of *C. geographus* are consistent with recent observations on the potential role of this region for the expression of toxins destined for defense rather than predation (13).

To our knowledge, insulin has not been reported in any other animal venom. However, venom components that target the prey's metabolism and, more specifically, glucose homeostasis, appear to be more widespread. Although mechanistically different to cone snail venom insulins, exendin-4 isolated from the saliva venom of the gila monster *Heloderma suspectum* likely elicits a similar physiological response. Exendin-4 is a potent agonist of the glucagon-like peptide receptor and promotes the

secretion of endogenous insulin in the prey (28). Exendin-4 has been developed as a commercial drug (exenatide) and effectively treats diabetes mellitus, highlighting the therapeutic potential of venom hormone mimics (28).

Evolving insulin to be used as a pharmacological weapon presents a novel and unexpected type of event in chemical biology. Many of the underlying mechanisms of the chemical interactions between animals are likely to be as surprising as the scenario detailed in this study. Our study reveals a class of insulins that have evolved to act rapidly and potently to cause severe hypoglycemia. It is therefore not so surprising that this is the shortest insulin yet discovered; at the molecular level, all regulatory aspects embedded in the insulin structure are nonessential details for this class of insulins that may be impervious to the subtle aspects of regulatory physiology. Their short length and posttranslational modifications may well reflect their streamlined physiological role in disrupting glucose homeostasis. Consequently, although normal vertebrate insulin signaling needs to be sensitive to downstream effects, these insulins may not be so constrained. A more comprehensive study of the structure and function of this special class of insulins, and a comparison with normal insulins,

may provide insight into the structural elements of insulins, which are necessary for the more subtle regulation of these important hormones in normal physiology.

## Materials and Methods

Detailed material and methods are provided in *SI Material and Methods*. Briefly, insulin transcripts were sequenced from cone snail venom glands by RT-PCR and/or next generation transcriptome sequencing. The two fish-like insulins, Con-Ins G1 and G3, were identified in the venom of *C. geographus* by reversed-phase chromatography combined with high-resolution mass spectrometry. Con-Ins G1 was chemically synthesized using a selenocysteine-based strategy and tested on zebrafish using the STZ-induced model of hyperglycemia and by direct application of the peptide into the water column.

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