Selecting Potential Neuronal Drug Leads from Conotoxins of Various Venomous Marine Cone Snails in Bali, Indonesia

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ABSTRACT: Many conotoxins, natural peptides of marine cone snails, have been identified to target neurons. Here, we provide data on pharmacological families of the conotoxins of 11 species of cone snails collected in Bali. The identified definitive pharmacological families possibly targeting neuronal tissues were α (alpha), τ (tau), ρ (rho), and ω (omega). These classes shall target nicotinic acetylcholine receptors, voltage-gated Na channels, voltage-gated K channels, and α1-adrenoceptors, respectively. The VI/VII-O3 conotoxins might be prospected as an inhibitor of N-methyl-D-aspartate. Con-ikot-ikot could be applied as an α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor blocker medicine. The definitive pharmacology classes of conotoxins as well as those yet to be elucidated need to be further established and verified.

INTRODUCTION

Conotoxins are natural peptide components of the venoms of marine cone snails of the Conus genus, which are remarkably diverse in terms of structure and function. Many conotoxins have been identified to have neuronal targets. The snails capture prey using a diverse array of toxins, mainly neurotoxins, although a few can be cardioactive. Unique potency and selectivity profiles for a range of neuronal targets have made several conotoxins valuable as drug leads of analgesics, neuropsychiatric, and other neuropharmacologicals. Neurologic application for pain reduction is the most common ongoing approved, preclinical, or clinical trial of conotoxins or their derivatives. The α-MVIIA conotoxin, marketed as ziconotide, was approved by the U.S. Food and Drug Administration in 2004 to treat chronic pain. The γ-MrIA, α-CVID, contulakin-G, α1-Vc1.1, and μ-O-MrVIB conotoxins were in preclinical and clinical trials to cure neuropathic pain or neuroprotection. Only one conotoxin, namely, κ-PVIIA, was in the preclinical phase to treat non-neurological complaints. This conotoxin was on trial to cure myocardial infarction.

The classical organization of a conopeptide precursor is ER signal sequence, N-proregion, mature peptide region, and C-terminal prorregions. The precursor protein is then cleaved by proteases, generating active conotoxins that form key constituents of the venom. The conotoxins are classified according to gene superfamily, cysteine framework, and pharmacological class. The gene superfamily is based on the signal sequences, the cysteine framework is determined from the number of cysteine residues with estimated disulfide bonds of the mature peptide, and the pharmacological class is based on established pharmacological proof of certain conotoxins. The pharmacological families are annotated according to gene superfamily, cysteine framework, and pharmacological class. Neurologic application for pain reduction is the most common ongoing approved, preclinical, or clinical trial of conotoxins or their derivatives. The α-MVIIA conotoxin, marketed as ziconotide, was approved by the U.S. Food and Drug Administration in 2004 to treat chronic pain. The γ-MrIA, α-CVID, contulakin-G, α1-Vc1.1, and μ-O-MrVIB conotoxins were in preclinical and clinical trials to cure neuropathic pain or neuroprotection. Only one conotoxin, namely, κ-PVIIA, was in the preclinical phase to treat non-neurological complaints. This conotoxin was on trial to cure myocardial infarction.

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and systematic biology points of view of the findings have been published.\textsuperscript{30–32} However, the pharmacological actions of discovered conotoxins have not been reported. Here, we provide data on the pharmacological families of the species published previously to provide insights into which to select and further study for bioprospecting potential drug leads from Indonesian marine snails.

\section*{MATERIALS AND METHODS}

Published prosequences of conotoxins from 11 species of snails found in Bali\textsuperscript{1,16,17} were analyzed. The species were \textit{Conus arenatus}, \textit{Conus coronatus}, \textit{Conus elisaeus}, \textit{Conus imperialis}, \textit{Conus lividus}, \textit{Conus marmoratus}, \textit{Conus quercinus}, \textit{Conus rattus}, \textit{Conus sponsalis}, \textit{Conus varie}, and \textit{Conus virgo}.\textsuperscript{30} The ER signal sequence, N-proregion, mature peptide region, and C-terminal proregions, as well as cysteine framework and gene superfamilies, were identified using ConoServer (http://www.conoserver.org/).\textsuperscript{1,16,17} The pharmacological families were predicted using statistics on pharmacological families available on the server based on previously published cysteine frameworks and gene superfamilies.\textsuperscript{1} The data were further clustered as definitive pharmacological family (DPF), definitive combined pharmacological family (DCPF), nonalphabetical pharmacological family (NAPF), divergent gene family (DGF), newly proposed gene family,\textsuperscript{30} novel gene family and cysteine framework combination (NGFCFC), unassigned gene family (UGF) SF,\textsuperscript{1} and unknown conotoxin. The DCPF cluster was further assembled based on the cysteine framework and gene family. Protein modeling, prediction, and analysis of the representative mature toxin sequences were conducted using the Phyre2 server (http://www.sbg.bio.ic.ac.uk).\textsuperscript{33}

\section*{RESULTS}

The pharmacological classes of conotoxins discovered in various venomous marine cone snails in Bali, Indonesia, based on pharmacological family and cysteine framework, are listed in Table 1. Meanwhile, Table 2 shows the detailed list of conotoxin peptides clustered in DCPF and NAPF identified in various species. The result shows that the DPFs, listed from the most frequent, were $\alpha$, $\kappa$, $\iota$, and $\rho$ with 66, 54, 37, and 4 conotoxin sequences, respectively. The total number of conotoxins annotatable to definitive families was 161. There were 400, 121, 119, 71, 63, 35, 12, and 3 conotoxin sequences ascribed to DCPF as follows, listed in the order of frequency: $\delta/\gamma/\kappa/\mu/\omega$, $\alpha/\iota/\kappa/\mu$, $\epsilon/\mu/\tau$, $\gamma/\omega$, $\alpha/\rho$, $\alpha/\kappa$, $\alpha/\sigma$, and $\alpha/\kappa/\mu$, respectively. A total of 824 conotoxins were assigned to these clusters. The number of conotoxins in the clusters of NAPFs of con-ikot-ikot, conkunitzin, conodipin, conoporin, and conophyisin was 101, 58, 30, 28, and 20, respectively. The other 90 conotoxins were clustered into DGF. NGFCFC consists of 315 sequences. UGFs of SF-04, mi1, and mi2 conotoxins were 50, while 319 were ungrouped conotoxins with certain gene families with poor cysteine residue. Each cluster was further subclustered based on the cysteine framework and gene family.

The conotoxins of NGFCFC are presented in Table 3. The most common combination was IX-P, followed by XV-V, XI-I3, XV-N, VI/VII-O3, and XXII-E. The combinations of conotoxins in the clusters of nonalphabetical families with a certain or novel cysteine framework (NCF) were V, XXI, and NCF—con-ikot-ikot; IX, XII, XIV, and NCF—conkunitzin; VIII and NCF—conodipin; NCF—conoporin; as well as

\begin{table}[h]
\centering
\caption{Pharmacological Classes of Conotoxins Discovered in Various Venomous Marine Cone Snails in Bali, Indonesia, Based on Pharmacological Family and Cysteine Framework.\textsuperscript{30}}
\begin{tabular}{lcccccccccccccccc}
\hline
\textbf{Framework} & \textbf{A} & \textbf{I} & \textbf{K} & \textbf{R} & \textbf{DPF} & \textbf{DCPF} & \textbf{NAPF} & \textbf{DivMKFPLLFISL} & \textbf{DivMKVAVVLLVS} & \textbf{NPGF} & \textbf{SF-04} & \textbf{SF-mi1} & \textbf{SF-mi2} & \textbf{UKC} \\
\hline
\textbf{C. arenatus} & 5 & 10 & 2 & 0 & 199 & 31 & 5 & 0 & 0 & 3 & 91 & 2 & 0 & 0 & 1 & 319 \\
\textbf{C. coronatus} & 10 & 0 & 0 & 0 & 138 & 46 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
\textbf{C. ebraeus} & 12 & 2 & 1 & 0 & 20 & 0 & 13 & 0 & 3 & 0 & 0 & 1 & 0 & 0 & 0 & 0 \\
\textbf{C. imperialis} & 10 & 0 & 0 & 0 & 138 & 46 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
\textbf{C. lividus} & 14 & 0 & 0 & 0 & 20 & 0 & 13 & 0 & 3 & 0 & 1 & 0 & 0 & 0 & 0 & 0 \\
\textbf{C. marmoreus} & 11 & 0 & 0 & 0 & 20 & 0 & 13 & 0 & 3 & 0 & 1 & 0 & 0 & 0 & 0 & 0 \\
\textbf{C. quercinus} & 3 & 5 & 0 & 0 & 138 & 46 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
\textbf{C. varius} & 66 & 37 & 54 & 4 & 824 & 237 & 17 & 2 & 21 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
\textbf{C. virgo} & 323 & 0 & 7 & 1 & 2 & 5 & 10 & 0 & 1 & 0 & 8 & 0 & 4 & 8 & 0 & 0 \\
\textbf{C. arenatus} & 0 & 0 & 15 & 0 & 53 & 9 & 0 & 0 & 3 & 1 & 1 & 0 & 0 & 14 & 1 & 1 \\
\textbf{C. coronatus} & 0 & 0 & 15 & 0 & 53 & 9 & 0 & 0 & 3 & 1 & 1 & 0 & 0 & 14 & 1 & 1 \\
\textbf{C. ebraeus} & 0 & 0 & 15 & 0 & 53 & 9 & 0 & 0 & 3 & 1 & 1 & 0 & 0 & 14 & 1 & 1 \\
\textbf{C. imperialis} & 0 & 0 & 15 & 0 & 53 & 9 & 0 & 0 & 3 & 1 & 1 & 0 & 0 & 14 & 1 & 1 \\
\textbf{C. lividus} & 0 & 0 & 15 & 0 & 53 & 9 & 0 & 0 & 3 & 1 & 1 & 0 & 0 & 14 & 1 & 1 \\
\textbf{C. marmoreus} & 0 & 0 & 15 & 0 & 53 & 9 & 0 & 0 & 3 & 1 & 1 & 0 & 0 & 14 & 1 & 1 \\
\textbf{C. quercinus} & 0 & 0 & 15 & 0 & 53 & 9 & 0 & 0 & 3 & 1 & 1 & 0 & 0 & 14 & 1 & 1 \\
\textbf{C. varius} & 0 & 0 & 15 & 0 & 53 & 9 & 0 & 0 & 3 & 1 & 1 & 0 & 0 & 14 & 1 & 1 \\
\textbf{C. virgo} & 0 & 0 & 15 & 0 & 53 & 9 & 0 & 0 & 3 & 1 & 1 & 0 & 0 & 14 & 1 & 1 \\
\hline
\textbf{Total} & 66 & 37 & 54 & 4 & 824 & 237 & 17 & 2 & 21 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
\hline
\end{tabular}
\end{table}
The divergent DivMKFPLLFISL occurred with the cysteine framework VI/VIII, while DivMKFPLAVVLLVS occurred with XIV. The new proposed gene families and cysteine framework combinations were IX MEFR, VI/VII MKFLL, IX and VI/VII MKISL, VIII and XIV MMLFM, as well as VI/VII MRFYM. The UGFs are presented in combinations of XIII SF-04, XIII SF-mi1, and NCF SF-mi2.

We clustered the mature toxins of the combined families α/ι/κ/μ, α/κ, α/ρ, α/σ, δ/γ/κ/μ/ω, ε/μ/τ, and γ/ω and conducted protein prediction with some representatives of each group. The PDB data for >50% identity and homology show that only γ/ω representative resulted in 78.8% homology and 60% identity in the established pharmacological class.

Those with cysteine framework VI/VII and gene superfamily O2 are close to ω-conotoxin MVII. The other representatives could not be estimated in any established pharmacological class (not shown).

The identified DPFs possibly targeting neuronal tissues were α (alpha), ρ (rho), and β (beta), while those of other groups were the VI/VII-O3 conotoxins as an inhibitor of N-methyl-D-aspartate (NMDA) and the con-ikot-ikot as an α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor blocker. The representatives of the conotoxins possibly targeting neuronal tissues found in Bali, Indonesia, are listed in Table 4, which were selected based on cysteine framework and/or gene superfamily. PDB search of some conotoxins found no template, which has high percentage of identity.

Table 2. Number of Conotoxin Peptides Clustered in DCPF and NAPF Identified in Various Species Marine Cone Snails Found in Bali, Indonesia

| species          | DCPF | NAPF |
|------------------|------|------|
|                  | α/ι/κ/μ | α/κ/μ | α/κ | α/ρ | α/σ | δ/γ/κ/μ/ω | ε/μ/τ | γ/ω | α/ι/κ/μ | α/κ/μ | α/κ | α/ρ | α/σ | δ/γ/κ/μ/ω | ε/μ/τ | γ/ω |
| C. arenatus      | 2     | 11   | 3   | 14  | 4   | 80    | 7     | 7   | 26    | 13   | 4   | 0   | 2   |
| C. coronatus     | 45    | 13   | 0   | 9   | 0   | 67    | 12    | 12  | 21    | 10   | 3   | 8   | 2   |
| C. erubescens    | 4     | 0    | 0   | 0   | 0   | 8     | 0     | 1   | 1     | 2    | 5   | 1   | 2   |
| C. imperialis    | 6     | 0    | 0   | 1   | 1   | 9     | 7     | 5   | 0     | 0    | 2   | 1   | 2   |
| C. lividus       | 20    | 2    | 0   | 16  | 0   | 31    | 16    | 7   | 14    | 11   | 0   | 2   | 0   |
| C. marmoratus    | 6     | 0    | 0   | 0   | 0   | 1     | 9     | 2   | 0     | 1    | 0   | 0   | 0   |
| C. quercinus     | 8     | 1    | 0   | 8   | 0   | 16    | 1     | 8   | 4     | 0    | 2   | 0   | 1   |
| C. rattus        | 2     | 2    | 0   | 0   | 1   | 11    | 3     | 1   | 17    | 6    | 2   | 8   | 1   |
| C. sponalis      | 14    | 5    | 0   | 3   | 0   | 125   | 32    | 20  | 9     | 12   | 7   | 0   | 3   |
| C. varius        | 12    | 1    | 0   | 7   | 5   | 24    | 19    | 3   | 8     | 2    | 4   | 7   | 4   |
| C. virgo         | 2     | 0    | 0   | 5   | 0   | 28    | 13    | 5   | 1     | 1    | 3   | 0   | 4   |
| total            | 121   | 35   | 3   | 63  | 12  | 400   | 119   | 71  | 101   | 58   | 30  | 28  | 20  |

αCII = con-ikot-ikot; CKNZ = conkunitzin; CNDP = conodipin; CNPR = conoporin; CNPS = conophysin.

Table 3. Number of Conotoxin Peptides of Novel Pharmacological Family with Definitive Cysteine Framework and Gene Superfamilies Identified in Each Species

| CF and GF combinations | species          | CF | GF | C. arenatus | C. coronatus | C. erubescens | C. imperialis | C. lividus | C. marmoratus | C. quercinus | C. rattus | C. sponalis | C. varius | C. virgo | total |
|------------------------|------------------|----|----|-------------|--------------|---------------|---------------|-------------|---------------|--------------|-----------|-------------|-----------|----------|-------|
| IX                     | P                | 17 | 9  | 1           | 11           | 4             | 0             | 0           | 2             | 16          | 12        | 0           | 72       |
| XV                     | V                | 4  | 3  | 0           | 0            | 19            | 0             | 6           | 0             | 2           | 0         | 3           | 37       |
| XI                     | L3               | 3  | 2  | 2           | 0            | 0             | 0             | 0           | 0             | 1           | 16        | 0           | 24       |
| XV                     | N                | 4  | 3  | 2           | 1            | 4             | 0             | 1           | 2             | 1           | 0         | 6           | 24       |
| XXII                   | E                | 4  | 3  | 0           | 1            | 2             | 3             | 3           | 1             | 2           | 2         | 0           | 21       |
| XIX                    | N                | 2  | 0  | 0           | 0            | 0             | 0             | 0           | 3             | 4           | 8         | 0           | 17       |
| XII                    | 14               | 2  | 8  | 0           | 0            | 1             | 0             | 0           | 0             | 3           | 0         | 0           | 14       |
| XV                     | O2               | 2  | 0  | 0           | 0            | 0             | 0             | 0           | 1             | 10          | 1         | 0           | 14       |
| XVII                   | Y                | 2  | 0  | 1           | 0            | 2             | 0             | 1           | 0             | 5           | 0         | 2           | 13       |
| XII                    | U                | 0  | 0  | 0           | 0            | 2             | 0             | 5           | 0             | 4           | 0         | 1           | 12       |
| VI/VII                 | V                | 0  | 0  | 1           | 0            | 9             | 0             | 0           | 0             | 1           | 0         | 0           | 11       |
| VI/VII                 | U                | 0  | 0  | 1           | 0            | 0             | 0             | 0           | 1             | 5           | 0         | 0           | 7        |
| XVI                    | Q                | 0  | 0  | 0           | 0            | 4             | 0             | 3           | 0             | 0           | 0         | 0           | 7        |
| XVI                    | T                | 7  | 0  | 0           | 0            | 0             | 0             | 0           | 0             | 0           | 0         | 0           | 7        |
| IX                     | M                | 0  | 0  | 3           | 1            | 0             | 0             | 0           | 0             | 2           | 0         | 6           | 6        |
| XVI                    | M                | 0  | 0  | 0           | 0            | 0             | 0             | 0           | 0             | 6           | 0         | 6           | 0        |
| XXIII                  | K                | 0  | 0  | 0           | 5             | 0             | 0             | 0           | 0             | 1           | 0         | 6           | 6        |
| XIV                    | T                | 0  | 0  | 0           | 0            | 0             | 0             | 0           | 0             | 4           | 0         | 0           | 4        |
| XVIII                  | 12               | 0  | 0  | 0           | 0            | 0             | 0             | 1           | 0             | 0           | 1         | 2           | 4        |
| XVIII                  | O1               | 2  | 0  | 0           | 0            | 0             | 0             | 0           | 0             | 0           | 0         | 0           | 2        |

αListed from the most frequent; CF = cysteine framework; GF = gene superfamily; single peptide combinations were not shown. These were XX D, IX E, XII I2, VI/VII I4, IX N, I O1, XII O1, XVIII O1, XIV O3, III Q.
confidence, while others found template database with confidence levels of 47.5–100%, with percentages of identity of 38.5–88%. The results of protein modeling, prediction, and analysis of the representative of mature toxin sequences are presented in Figure 1. The figure explains that some peptides consist of random coil and \( \alpha \) helix, while LiO32 is merely random coiled. Sequences of all species are available at dryad (doi:10.5061/dryad.1v5d3).30 The cDNAs of the representatives of the conotoxins possibly targeting neuronal tissues described in this article are available in GenBank with Acc. no. MN580095-MN580108.

Table 4. Representatives of the Conotoxins Possibly Targeting Neuronal Tissues Found in Bali, Indonesia, and the Result of PDB Search

| conotoxin name | species | cysteine framework | gene superfamily | pharmacological class | PDB search (% confidence/PID) |
|---------------|---------|--------------------|------------------|------------------------|-----------------------------|
| CoM22 | C. coronatus | I | M | \( \alpha \) | none |
| RtM11 | C. rattus | II | M | \( \alpha \) | metallothionein mt_nc (65.3/67) |
| ArL1 | C. arenatus | XIV | L | \( \alpha \) | none |
| ArD1 | C. arenatus | XX | D | \( \alpha \) | \( \alpha \)-conotoxin gexxa (99.9/49) |
| ArI110 | C. arenatus | XI | I | \( \alpha \) | conotoxin g117 (85.9/41) |
| LiA58 | C. lividus | VI/VII | A | \( \kappa \) | \( \alpha \)-conotoxin vca1 (85.4/60) |
| ArI21 | C. arenatus | XI | I2 | \( \kappa \) | none |
| ArO130 | C. arenatus | XI | O1 | \( \kappa \) | | |
| LiM43 | C. lividus | XXVII | M | \( \kappa \) | none |
| LiT8 | C. lividus | I | T | \( \rho \) | none |
| LiO32 | C. lividus | VI/VII | O3 | | NMDA blocker none |
| ArCII1 | C. arenatus | V | UK | CII/AMPA blocker | defensin, \( \alpha \) (47.5/55) |
| ArCII16 | C. arenatus | XXI | UK | CII/AMPA blocker | con-ikot-ikot (100/38) |
| ArCII12 | C. arenatus | UK | UK | CII/AMPA blocker | none |

"The first two characters are abbreviated species name, followed by gene superfamily and the number of the sequence in the database as previously published;30 PID = percentage of identity; NMDA blocker = putative NMDA blocker; AMPA blocker = putative AMPA blocker; UK = unknown; CII = con-ikot-ikot. Only search results of confidence level of >40% are shown; PDB search was conducted in website http://www.sbg.bio.ic.ac.uk/?phyre2/html/page.cgi?id=index."

Figure 1. Final model of the mature peptide region of the representatives of the conotoxins possibly targeting neuronal tissues found in Bali, Indonesia. The peptide names are the same as described in Table 4. The N-proregion was included in the modeling for the mature peptides of less than 30 residues. Modeling was conducted at http://www.sbg.bio.ic.ac.uk/?phyre2/html/page.cgi?id=index.33

Confidence, while others found template database with confidence levels of 47.5–100%, with percentages of identity of 38.5–88%. The results of protein modeling, prediction, and analysis of the representative of mature toxin sequences are presented in Figure 1. The figure explains that some peptides consist of random coil and \( \alpha \) helix, while LiO32 is merely random coiled. Sequences of all species are available at dryad (doi:10.5061/dryad.1v5d3).30 The cDNAs of the representatives of the conotoxins possibly targeting neuronal tissues described in this article are available in GenBank with Acc. no. MN580095-MN580108.

**DISCUSSION**

As expected, the total number of conotoxins identified in our study was large or 1996. Such an abundance is very common in marine cone snails. Each sea snail species typically possesses an average of 100–200 conotoxins,54 which are employed to paralyze prey.55 Of the total, only 161 (8.1%) can be assigned to an established pharmacological family. Another 824 (41.3%) are assigned to possible combinations of established families. The number of conotoxins in the clusters of nonalphabetical families of con-ikot-ikot, conkunitzin, conodipin, conoporin, and conophysin was 237 (11.9%). The other 90 conotoxins were clustered into divergent groups and 71 to new proposed families.

The last group was further classified as unknown pharmacological class of conotoxins with definitive gene family and cysteine framework, nonalphabetical family, divergent, variant MEF, variant MKFL, variant MMLFM, variant MRFYM, SF-04, and SF-mi1, as well as SF-mi2 gene families with definitive or NCFs. NGFCFC consists of 315 sequences. UGFs of SF-04, mi1, and mi2 conotoxins were 50, while 319 were ungrouped conotoxins with certain gene families but NCF.
conotoxins assigned to them have been identified as stimulating or blocking receptors, ion channels, or transporters. The α family works at nicotinic acetylcholine receptors (nAChRs), the β family at voltage-gated Na channels, the κ family at voltage-gated K channels, and the ρ family at 1-adrenoceptors. The ω family is a voltage-gated Ca channel blocker, which might be useful for cardiovascular disorder.

To further predict the pharmacological action of uncertain or unknown conotoxins, we clustered the data based on gene superfamily and cysteine framework. The five most common combination of conotoxins with DCFP was IX—P, followed XV—V, XI—I3, XV—N, and XXII—E. The combination of IX—P has been described in TxFXA as a prototype of P-superfamily conotoxin, which causes “spasmodic” symptoms on intracerebral injection in mice. The known O3-gene superfamily conotoxins have the VI/VII cysteine framework. The only characterized O3 superfamily is “bromosleeper”, which causes lethargy, drowsiness, and sleep in mice. This conotoxin is thought to be similar to conantokin, an inhibitor of NMDA receptors. The pharmacological actions of other combinations are yet to be described.

Assembling the conotoxins annotated as belonging to nonalphabetical gene superfamilies, we listed the combination of gene superfamilies with an established framework or NCF as conkunitzin combined with the frameworks IX, XII, XIV, and NCF; con-ikot-ikot with the frameworks V, XXI, and NCF; and SF-04 and SF-mi1 with the framework XIII. Meanwhile, SF-mi2, conopisin, conophysin, and conoporin have novel frameworks. Conkunitzin of conus snails displayed high sequence similarity to the kunitz domain of dendrotoxin superfamily with the frameworks VI/VII and XIII. Our SF-mi1 sequence data show framework XIII; however, the SF-mi3 data show an undescribed cysteine framework. The pharmacological effects of these conotoxins are yet to be elucidated.

The number of potential neuronal drugs from conotoxins discovered in various venomous marine cone snails in Bali, Indonesia, is huge. The marine cone snails seem to be well equipped with mainly neurotoxic venoms, but also a very few cardio toxic venoms, to immobilize the prey. A small portion of the conotoxins from Bali could be annotated to specific pharmacological classifications, which could be the first stepping stone to develop neurological drugs. A much larger portion has yet to be assigned, but the pharmacological action can be predicted based on the published literature. PDB searches of mature toxins with an undescribed cysteine framework or novel framework pattern combinations should give an insight into their possible pharmacological actions.

Conotoxins that work on nAChRs might be developed as antidepressants. nAChR modulation is an area with significant promise for future antidepressant drug development.

Furthermore, this group of cholinergic receptors has been recently known to be involved in the nicotine reward effect. Because ACh is known as a dopamine release regulator, α-conotoxins may potentially exhibit a salutary effect in psychoses and Tourette’s syndrome treatment. We assigned 27 peptides from six species to the α pharmacological class, which acts upon this receptor. Another 14 peptides are annotated to β (iota), with molecular targets of voltage-gated Na channels. It is intriguing to discuss the therapeutic potentials of an α-conotoxin found in our current study. This conotoxin has been revealed to possess agonistic activity against three sodium channels, namely, NaV1.2, NaV1.6, and NaV1.7. Because these sodium channels are implicated in many diseases such as migraine, epilepsy, autism, ataxia, pain disorders, paroxysmal itch, and anosmia, we can utilize this group as a chemical tool to support the research on developing novel drugs against these pathological conditions. The β family could be developed into drugs targeting chronic pain, epilepsy, and cardiac arrhythmias. The κ targets the voltage-gated K channels, which might be beneficial to be developed as new drugs for cancer; autoimmune diseases; and metabolic, neurological, and cardiovascular disorders. The ρ class was identified in three peptides from two species. This class specifically targets the α1-adrenoceptors, which play a key role in the modulation of sympathetic nervous system activity, as well as being a site of action for many therapeutic agents.

The NMDA blocker framework VI/VII—O3 was identified in this study. This could have the potential to treat some neurodegenerative disorders, such as Alzheimer’s disease, Parkinson’s disease, Huntington’s disease, and amyotrophic lateral sclerosis. Con-ikot-ikot was identified in 10 species (but not C. imperialis), and the number of peptides was 101. This class is reported to exhibit an effect on AMPA receptors, inhibiting channel desensitization. This can be a potential antiepileptic drug.
PDB search of conotoxins shows that the representatives of the conotoxins possibly targeting neuronal tissues found in this study are novel. Some have no confidence template in the database, while others have percentages of identity of 38.5–88%. The variety of secondary structures of random coil and α helix might explain the mechanism of action and protein targets.

The pharmacological effect of the definitive as well as uncertain pharmacological classes of conotoxins should be determined and proven. Being peptides, the production and purification of conotoxons should be straightforward. For example, conotoxins can be produced using recombinant DNA technology or synthetic peptides. Simple clustering based on suspected pharmacological families or gene families and cysteine framework conducted in our study should be a simple approach to select conotoxin(s) of interest.

## CONCLUSIONS

The identified DPFs possibly targeting neuronal tissues were α (alpha), τ (tau), κ (kappa), and ρ (rho) as well as NMDA and AMPA receptor blocker. The definitive pharmacological classes of conotoxins as well as those yet to be elucidated need to be further established and verified.

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The authors declare no competing financial interest.

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## ABBREVIATIONS

Ach, acetylcholine; AMPA, α-aminooxy-3-hydroxy-5-methyl-4-isoxazole propionic acid; DCPF, definitive combined pharmacological family; DPF, definitive pharmacological family; nAChRs, nicotinic acetylcholine receptors; NAPF, non-alphabetical pharmacological family; NCF, novel cysteine framework; NGFCFC, novel gene family and cysteine framework combination; NMDA, N-methyl-D-aspartate; PDB, Protein Data Base; UGF, unassigned gene families

## REFERENCES

1. Robinson, S.; Norton, R. Conotoxin gene superfamilies. Mar. Drugs 2014, 12, 6058–6101.
2. Gao, B.; Peng, C.; Yang, J.; Yi, Y.; Zhang, J.; Shi, Q. Cone Snails: A Big Store of Conotoxins for Novel Drug Discovery. Toxins 2017, 9, 397.
3. Möller, C.; Melaun, C.; Castillo, C.; Diaz, M. E.; Renzelman, C. M.; Estrada, O.; Kuch, U.; Lokey, S.; Mari, F. Functional hypervariability and gene diversity of cardioactive neuropeptides. J. Biol. Chem. 2010, 285, 40673–40680.
4. Bingham, J.-P.; Mitsuenga, E.; Bergeron, Z. L. Drugs from slugs—past, present and future perspectives of α-conotoxin research. Chem. Biol. Interact. 2010, 183, 1–18.
5. Gonzales, D. T. T.; Saloma, C. P. A bioinformatics survey for conotoxin-like sequences in three turrid snail venom duct transcriptomes. Toxicon 2014, 92, 66–74.
6. Miljanich, G. Ziconotide: neuronal calcium channel blocker for treating severe chronic pain. Curr. Med. Chem. 2004, 11, 3029–3040.
7. Nielsen, C. K.; Lewis, R. J.; Alewood, D.; Drinkwater, R.; Palant, E.; Patterson, M.; Yaksh, T. L.; McCumber, D.; Smith, M. T. Anti-allodynic efficacy of the ρ-conopeptide, Xen2174, in rats with neuropathic pain. Pain 2005, 118, 112–124.
8. Adams, D. J.; Smith, A. B.; Schroeder, C. I.; Yasuda, T.; Lewis, R. J. α-Conotoxin CVID Inhibits a Pharmacologically Distinct Voltage-sensitive Calcium Channel Associated with Transmitter Release from Pre- and Post-ganglionic Nerve Terminals. J. Biol. Chem. 2003, 278, 4057–4062.
9. Craig, A. G.; Norberg, T.; Griffin, D.; Hoefer, C.; Ahlström, M.; Schmidt, K.; Low, W.; Dykert, J.; Richelson, E.; Navarro, V.; Mazella, J.; Watkins, M.; Hillyard, D.; Imperial, J.; Cruz, L. J.; Olivera, B. M. Conulatin-G, an α-Glycosylated Invertebrate Neurotensin. J. Biol. Chem. 1999, 274, 13752–13759.
10. Malmberg, A. B.; Gilbert, H.; McCabe, T. R.; Basbaum, A. I. Powerful antinoceptive effects of the cone snail venom-derived subtype-selective NMDA receptor antagonists conantokins G and T. Pain 2003, 101, 109–116.
11. Satkunanathan, N.; Livett, B.; Gayler, K.; Sandall, D.; Down, J.; Khalil, Z. Alpha-conotoxin Vc1.1 alleviates neuropathic pain and accelerates functional recovery of injured neurons. Brain Res. 2005, 1059, 149–158.
12. Ekberg, J.; Jayamanne, A.; Vaughan, C. W.; Aslan, S.; Thomas, L.; Mould, J.; Drinkwater, R.; Baker, M. D.; Abrahamson, B.; Wood, J. N.; Adams, D. J.; Christie, M. J.; Lewis, R. J. O-conotoxin MrVIB selectively blocks Nav1.8 sensory neuron specific sodium channels and chronic pain behavior without motor deficits. Proc. Natl. Acad. Sci. U.S.A. 2006, 103, 17030–17035.
13. Lubbers, N. L.; Campbell, T. J.; Polakowski, J. S.; Bulaj, G.; Layer, R. T.; Moore, J.; Gross, G. J.; Cox, B. F. Postsynaptic administration of CGX-1051, a peptide from cone snail venom, reduces infract size in both rat and dog models of myocardial ischemia and reperfusion. J. Cardiovasc. Pharmacol. 2005, 46, 141–146.
14. Kaas, Q.; Westermann, J.-C.; Craik, D. J. Conopeptide characterization and classifications: an analysis using ConoServer. Toxicon 2010, 55, 1491–1509.
15. Knapp, O.; McArthur, J. R.; Adams, D. J. Conotoxins targeting neuronal voltage-gated sodium channel subtypes: potential analgesics? Toxins 2012, 4, 1236–1260.
16. Kaas, Q.; Westermann, J.-C.; Halai, R.; Wang, C. K. L.; Craik, D. J. ConoServer, a database for conopeptide sequences and structures. Bioinformatics 2008, 24, 445–446.
17. Kaas, Q.; Yu, R.; Jin, A.-H.; Dutertre, S.; Craik, D. J. ConoServer: updated content, knowledge, and discovery tools in the conopeptide database. Nucleic Acids Res. 2012, 40, D325–D330.
18. Gray, W. R.; Luque, A.; Olivera, B. M.; Barrett, J.; Craik, L. J. Peptide toxins from Conus textile neovicarius. J. Biol. Chem. 1981, 256, 4734–4740.
19. Fainzilber, M.; Gordon, D.; Hasson, A.; Spira, M. E.; Zlotkin, E. Mollusc-specific toxins from the venom of Conus textile neovicarius. Eur. J. Biochem. 1991, 202, 589–595.
(20) Fainzilber, M.; Nakamura, T.; Lodder, J. C.; Zlotkin, E.; Kits, K. S.; Burlingame, A. L. gamma-Conotoxin-PnVIIA, a gamma-carboxyglutamate-containing peptide agonist of neuronal calcium current. Biochemistry 1998, 37, 1470–1477.

(21) Rigby, A. C.; Lucas-Meunier, E.; Kalume, D. E.; Czerwic, E.; Hambe, B.; Dahlqvist, I.; Fossier, P.; Baux, G.; Roepepoft, P.; Balea, J. D.; Furie, B. C.; Furie, B.; Stenflo, J. A conotoxin from Conus textilis with unusual posttranslational modifications reduces presynaptic Ca²⁺ influx. Proc. Natl. Acad. Sci. U.S.A. 1999, 96, 5758–5763.

(22) Buczek, O.; Wei, D.; Babon, J. J.; Yang, X.; Fiedler, B.; Chen, P.; Yoshikami, D.; Olivera, B. M.; Bulaj, G.; Norton, R. S. Structure and sodium channel activity of an excitatory 11-superfamily conotoxin. Biochemistry 2007, 46, 9929–9940.

(23) Terlau, H.; Stocker, M.; Shon, K. J.; McIntosh, J. M.; Olivera, B. M. Micro-o-conotoxin MtVIA inhibits mammalian sodium channels, but not through site I. J. Neurophysiol. 1996, 76, 1423–1429.

(24) Cruz, L. J.; Olivera, B. M. Calcium channel antagonists. Omega-conotoxin defines a new high affinity site. J. Biol. Chem. 1986, 261, 6230–6233.

(25) Cruz, L. J.; Gray, W. R.; Olivera, B. M.; Zeikus, R. D.; Kerr, L.; Yoshikami, D.; Moczylowski, E. Conus geographus toxins that discriminate between neuronal and muscle sodium channels. J. Biol. Chem. 1985, 260, 9280–9288.

(26) Sharpe, I. A.; Gehmann, J.; Loughnan, M. L.; Thomas, L.; Adams, D. A.; Atkins, A.; Palant, E.; Craik, D. J.; Adams, D. J.; Alewood, P. F.; Lewis, R. J. Two new classes of conopeptide inhibit the α1-Adrenoceptor and noradrenaline transporter. Nat. Neurosci. 2001, 4, 902–907.

(27) England, L. J.; Imperial, J.; Jacobsen, R.; Olivera, B.; Maricq, A. V. A novel Conus snail polypeptide causes excitoxicity by blocking desensitization of AMPA receptors. Curr. Biol. 2009, 19, 900–908.

(28) Ahren, J. D.; Vetter, I.; Mohaldeen, H.; Vetter, I.; Alewood, P. F.; Lewis, R. J. Comparative Venomics Reveals the Complex Prey Capture Strategy of the Piscivorous Conus Snail Conus catus. J. Proteome Res. 2015, 14, 4372–4381. (36) Triggel, D. J. Drug targets in the voltage-gated calcium channel family: why some are and some are not. Drug Dev. Technol. 2003, 1, 719–733.

(37) Lirazan, M. B.; Hooper, D.; Corpuz, G. P.; Ramilo, C. A.; Bandyopadhyay, P.; Cruz, L. J.; Olivera, B. M. The spasmodic peptide defines a new conotoxin superfamily. Biochemistry 2000, 39, 1583–1588.

(38) Zhangsuns, D.; Luo, S.; Wu, Y.; Zhu, X.; Hu, Y.; Xie, L. Novel O-superfamily conotoxins identified by cDNA cloning from three vermicorous Conus species. Chem. Biol. Drug Des. 2006, 68, 256–265.

(39) Craig, A. G.; Jimenez, E. C.; Dykert, J.; Nielsen, D. B.; Gulyas, J.; Abogadie, F. C.; Porter, J.; Rivier, J. E.; Cruz, L. J.; Olivera, B. M.; McIntosh, J. M. A Novel Post-translational Modification Involving Bromination of Tryptophan. J. Biol. Chem. 1997, 272, 4689–4698.

(40) Bayrhuber, M.; Vijayan, V.; Ferber, M.; Graf, R.; Konukkutu, J.; Imperial, J.; Garrett, J. E.; Olivera, B. M.; Terlau, H.; Zweckstetter, M.; Becker, S. Conkunitzin-SI is the First Member of a New Kunitz-type Neurotoxin Family. J. Biol. Chem. 2005, 280, 23766–23770.

(41) Robinson, S. D.; Safavi-Hemami, H.; McIntosh, L. D.; Purcell, A. W.; Norton, R. S.; Papenfuss, A. T. Diversity of conotoxin gene superfamilies in the venomous snail, Conus victorius. PLoS One 2014, 9, e87648.

(42) Hu, H.; Bandyopadhyay, P. K.; Olivera, B. M.; Yandell, M. Elucidation of the molecular envenomation strategy of the cone snail Conus geographus through transcriptome sequencing of its venom duct. BMC Genomics 2012, 13, 284.

(43) Philip, N. S.; Carpenter, L. L.; Price, L. H. The α6/β2/β3 Nicotinic Acetylcholine Receptor Attenuates Nicotine-Induced Conditioned Place Preference in Mice. Mar. Drugs 2012, 10, 685–698.

(44) Panorska, A.; D’Sa, M. J.; Collewijn, H.; Osipova, T.; van de Water, D.; Lough, T. M.; Wiersma, D.; Reuten, J. K.; de Bruijn, A. C.; Bisschop, P. J. Relative Antibody Affinity for α7 and α3β2 Nicotinic Acetylcholine Receptors Induced by the Two-Site Binding Peptide Alzet. J. Pharmacol. Exp. Ther. 2012, 342, 578–585.

(45) Ferrero, M. A.; Moccia, A.; Versini, R.; Vona, A. J.; Pissato, R. M.; Pizzolato, G.; Loguercio, D.; Di Bella, G.; de Vos, M. J.; Borron, S. W. Super-antagonists for the Nicotinic Acetylcholine Receptor: Effect on Clinical Breast Cancer Cells. J. Oncol. 2013, 2013, 289628.

(46) Panorska, A.; D’Sa, M. J.; Collewijn, H.; Osipova, T.; van de Water, D.; Lough, T. M.; Wiersma, D.; Reuten, J. K.; de Bruijn, A. C.; Bisschop, P. J. Relative Antibody Affinity for α7 and α3β2 Nicotinic Acetylcholine Receptors Induced by the Two-Site Binding Peptide Alzet. J. Pharmacol. Exp. Ther. 2012, 342, 578–585.

(47) Philip, N. S.; Carpenter, L. L.; Price, L. H. The nicotinic acetylcholine receptor as a target for antidepressant drug development. Sci. World J. 2012, 2012, 104105.

(48) Panorska, A.; D’Sa, M. J.; Collewijn, H.; Osipova, T.; van de Water, D.; Lough, T. M.; Wiersma, D.; Reuten, J. K.; de Bruijn, A. C.; Bisschop, P. J. Relative Antibody Affinity for α7 and α3β2 Nicotinic Acetylcholine Receptors Induced by the Two-Site Binding Peptide Alzet. J. Pharmacol. Exp. Ther. 2012, 342, 578–585.
(53) de Lera Ruiz, M.; Kraus, R. L. Voltage-Gated Sodium Channels: Structure, Function, Pharmacology, and Clinical Indications. J. Med. Chem. 2015, 58, 7093–7118.
(54) Bagal, S. K.; Marron, B. E.; Owen, R. M.; Storer, R. I.; Swain, N. A. Voltage-gated sodium channels as drug discovery targets. Channels 2015, 9, 360–366.
(55) Wulff, H.; Castle, N. A.; Pardo, L. A. Voltage-gated potassium channels as therapeutic targets. Nat. Rev. Drug Discovery 2009, 8, 982–1001.
(56) Piascik, M. T.; Perez, D. M. Alpha1-adrenergic receptors: new insights and directions. J. Pharmacol. Exp. Ther. 2001, 298, 403–410.
(57) Chen, H.-S. V.; Lipton, S. A. The chemical biology of clinically tolerated NMDA receptor antagonists. J. Neurochem. 2006, 97, 1611–1626.
(58) Kemp, J. A.; McKernan, R. M. NM1DA receptor pathways as drug targets. Nat. Neurosci. 2002, 5, 1039–1042.
(59) Rogawski, M. A. Revisiting AMPA receptors as an antiepileptic drug target. Epilepsia Curr. 2011, 11, 56–63.
(60) Zhu, X.; Bi, J.; Yu, J.; Li, X.; Zhang, Y.; Zhangsun, D.; Luo, S. Recombinant Expression and Characterization of α-Conotoxin LvIA in Escherichia coli. Mar. Drugs 2016, 14, 11.
(61) Xia, Z.; Chen, Y.; Zhu, Y.; Wang, F.; Xu, X.; Zhan, J. Recombinant omega-conotoxin MVIIA possesses strong analgesic activity. BioDrugs 2006, 20, 275–281.
(62) Luo, S.; Zhangsun, D.; Harvey, P. J.; Kaas, Q.; Wu, Y.; Zhu, X.; Hu, Y.; Li, X.; Tsetlin, V. I.; Christensen, S.; Romero, H. K.; McIntyre, M.; Dowell, C.; Baxter, J. C.; Elmslie, K. S.; Craik, D. J.; McIntosh, J. M.; Cloning, synthesis, and characterization of αO-conotoxin GeXIVA, a potent α9α10 nicotinic acetylcholine receptor antagonist. Proc. Natl. Acad. Sci. U.S.A. 2015, 112, E4026–E4035.
(63) Armishaw, C. J. Synthetic α-Conotoxin Mutants as Probes for Studying Nicotinic Acetylcholine Receptors and in the Development of Novel Drug Leads. Toxins 2010, 2, 1471–1499.
(64) Banerjee, J.; Gyanda, R.; Chang, Y.-P.; Armishaw, C. J. The Chemical Synthesis of α-Conotoxins and Structurally Modified Analogs with Enhanced Biological Stability. Peptide Modifications to Increase Metabolic Stability and Activity, Methods in Molecular Biology, Humana Press, 2013; Vol. 1081, pp 13–34.
(65) Clark, R. J.; Fischer, H.; Nevin, S. T.; Adams, D. J.; Craik, D. J. The Synthesis, Structural Characterization, and Receptor Specificity of the α-Conotoxin Vc1.1. J. Biol. Chem. 2006, 281, 23254–23263.