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# Cloning, expression and functional characterization of a D-superfamily conotoxin Lt28.1 with previously undescribed cysteine pattern

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ABSTRACT

As a class of peptides with 10 cysteine residues (-C-CC-C-CC-C-C-C-C-), D-superfamily conotoxins (D-conotoxins) can specifically act on nicotinic acetylcholine receptors (nAChRs). According to the conserved signal peptides of D-conotoxins, seven D-conotoxin precursor sequences with a previously undescribed Cys arrangement (-C-C-C-CC-C-C-C-C-C-) were identified by PCR-RACE methodology in the present study. The alignment of sequences revealed that signal peptide regions were same as D-VxXXA from *Conus vexillum,* and their mature peptides were almost different from the D-conotoxins. Analyses of the evolutionary tree demonstrated that they had low homology to those reported conotoxins with 10 cysteine residues (less than 35%) and lied in a separate branch in the evolutionary tree. Furthermore, a previously undescribed D-superfamily conotoxin Lt28.1 was further expressed in *Pichia pastoris* and then functionally characterized. The results showed that the recombinant Lt28.1 targeted α9α10 nAChRs but not other nAChRs subtypes. These findings defined a new branch of D-superfamily and expanded our knowledge of targets and potential application of D-conotoxins.

## 1. Introduction

As bioactive peptides secreted by *Conus* venom gland, conotoxins can specifically act on various ion channels ($Na^+$,$K^+$, $Ca2^+$) and membrane receptors (nAChRs, 5-HT3R, NMDAR and G-protein-coupled receptors) [1–4]. Some conotoxins have been used as neuropharmacological tools, several conotoxins have entered into clinical trials, and even one conotoxin was approved by FDA for the treatment of pain in 2004 [5]. According to their different signal peptide sequences, cysteine patterns and functional targets, conotoxins can be categorized into a total of 30 superfamilies [6], such as A-, M-, O-, P-, S-, T-, I-, D-, J-superfamily [7,8]. The D-superfamily conotoxins are a type of longer peptides and possess 10 cysteine residues (-C-CC-C-CC-C-C-C-C-), which was first found from *Conus vexillum* [9]. To date, only 22 D-superfamily conotoxins have been identified. Moreover, a few of these D-superfamily conotoxins have been identified as antagonists of nicotinic acetylcholine receptors (nAChRs) with selectivity for α7, α3β4 and α9α10 subtypes (α3β2 and α4β2) [9–12]. Due to their highly concentrated disulfide bonds, there is no report about the chemical synthesis or expression of D-superfamily conotoxins.

In the present study, seven previously undescribed D-superfamily conotoxins were cloned from *Conus litteratus* by 3′-RACE using the conservative sequence of signal peptide of D-superfamily conotoxins (VxXXA, VxXXB) [9,10]. Interestingly, these conotoxins exhibited a novel cysteine framework (-C-C-C-CC-C-C-C-C-C-) and were different from those previously reported D-superfamily conotoxins (-C-CC-C-CC-C-C-C-C-) [9–12] and other superfamily conotoxins. In addition, 5′-RACE results showed that their signal peptide sequences were completely identical with those of D-superfamily conotoxins, such as VxXXA and VxXXB. The phylogenetic tree revealed that the above-mentioned D-superfamily conotoxins lied in a separate branch. In order to assess their functions, a D-superfamily conotoxin Lt28.1 was expressed in yeast system and then functionally characterized. The results showed that Lt28.1 was successfully expressed in yeast *Pichia pastoris* (*P. pastoris*). Moreover, the recombinant Lt28.1 could inhibit α9α10 nAChRs with an $IC_{50}$ of 3 μM and had no obvious activity on α3β4, α4β4, α2β2, α2β4, α7 and α6α3β2β3 subtypes. These findings defined a previously undescribed branch of D-superfamily and expanded our knowledge of targets and potential application of D-superfamily conotoxins.

## 2. Materials and methods

### 2.1. Plasmids, strains and reagents

The yeast *P. pastoris* expression plasmid pPIC9, harboring the promoter of the methanol-inducible *P. pastoris* alcohol oxidase 1 (AOX1) gene, the α-mating factor prepro secretion signal from *Saccharomyces cerevisiae* and a HIS4 auxotrophic selection marker, was purchased from

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**Table 1**
The precursor sequence of previously undescribed D-superfamily conotoxins.

| Contoxin | Precursor Sequence | Accession number |
|---|---|---|
| **Lt28.1** | MPKLEMMLLVLLILPLCYIDAVGPPPPWNMEDEIIEHWQK<br>**LHCHEISDLTPWILCSPEPLCGGKGCCAQEVCDCSGPACTCPPCL** | HM003926 |
| **Lt28.2** | MPKLEMMLLVLLILPLCYIDAVGPPPPWNMEDEIIEHWQK<br>**LHCHEISD*P*TPWILCSPEPLCGGKGCCAQEVCDCSGP*V*CTCPPCL** | HM003927 |
| **Lt28.3** | MPKLEMMLLVLLILPLCYIDAVGP***L***PPWNMEDEIIEHWQK<br>**LHC*Y*EISDLTPWILCSPEPLCGGKGCCAQEVCDCSGPACTCPPCL** | HM003928 |
| **Lt28.4** | MPKLEMMLLVLLILPLCYIDAVGPPPPWNMEDEIIEHWQK<br>**LHCHEISDLTPWILCSPEPLCGGKGCCAQEVCDCSG*T*ACTCPPCL** | HM003929 |
| **Lt28.5** | MPKLEMMLLVLLILPLCYIDAVGPPPPWNMEDEIIEHWQK<br>**LHCHEISDLTPWILCSPEPLCGGKGCCAQ*G*VCDCSGPACTCPPCL** | HM003930 |
| **Lt28.6** | MPKLEMMLLVLLILPLCYIDAVGPPPPWNMEDEIIEHWQK<br>**LHCHEISDLTPWILCSPEPLCGGKGCCAQEVCDCSGP*V*CTCPPCL** | HM003931 |
| **Lt28.7** | MPKLEMMLLVLLILPLCYIDAVGPPPPWNMEDEIIEHWQ***E***<br>**LHCHEISDLTPWILCSPEPLCGGKGCCAQEVCDCSGP*V*CTCPPCL** | HM003932 |

The signal peptide sequence is indicated by underlining, and the mature peptide sequence is shown in bold style. The cysteine residues are indicated by shading. The mutant amino acids of Lt28.2, Lt28.3, Lt28.4, Lt28.5, Lt28.6 and Lt28.7 are shown in bold and italic.

Invitrogen (San Diego, CA, USA). The *P. pastoris* GS115 (his4) host strain was supplied by Invitrogen.

Moreover, 3′-Full RACE Core Set Ver.2.0 kit, 5′-Full RACE kit and LA Taq were purchased from TaKaRa Co. (Dalian, China). T4 DNA ligase, DH5α, the pGEM-T Easy Vector system and gel purification kit were obtained from Tiangen Biotech (Beijing, China). The other reagents were of analytical grade from SABC (Sino-American Biotechnology Company, Luoyang, China).

### 2.2. Cloning of previously undescribed framework XXVIII conotoxins

*C. litteratus* were collected from an area around the Hainan Island located in the South China Sea. The venom duct was dissected from the snails and immediately frozen in liquid nitrogen. Subsequently, 100 mg frozen venom duct tissue was homogenized, and total RNA from each *Conus* species was extracted using TRIzol reagent(Invitrogen, Carlsbad, CA, USA).

The previously undescribed conotoxins were cloned by 3′-RACE as previously described [13]. In the present study, amplifications were performed using the primers as follows. The forward primer F1 (5′-GCTGCTGGTTCTGCTGATTTT-3′) was designed based on the N-terminus of the signal sequence of αD-conotoxins [9], while the reverse 3′-RACE outer primer R1 (5′-TACCGTCGTTCCACTAGTGATT-3′) was a shorter version of the 3′-RACE adaptor primer without the poly (dT) tail. Briefly, PCR amplification was carried out with 30 cycles at a melting temperature of 94 °C for 30 s, an annealing temperature of 55 °C for 30 s, and an extension temperature of 72 °C for 50 s. Amplifications were analyzed by gel electrophoresis, and the a-tailed purified products of the expected size were ligated into the T-tailed plasmid vector pGEM-T (Tiangen Biotech). Subsequently, the ligation products were transformed into competent cells of DH5α. Plasmids containing inserts of approximately 300–700 bp in size were sequenced, and the predicted protein sequences were analyzed with software Seqtools (http://www.bio-soft.net/sms).

The gene-specific primer D1 (5′-TGCGCACTCTCCGTTCTCTT-3′) was designed and synthesized based on the cDNA sequence from the 3′-untranslated region (UTR) of the D-conotoxins obtained by 3′-RACE. The 5′-RACE outer primer (5′-CATGGCTACATGCTGACAGCCTA-3′) was included in the 5′-RACE kit. The experiment was conducted as previously described [13]. Briefly, a prominent PCR band of 500 bp was separated and purified. The purified product was ligated into a pGEM-T Easy vector (Tiangen Biotech) for sequencing. The signal peptide sequences of seven previously undescribed D-conotoxins were predicted using the SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP).

### 2.3. Sequence analysis

Database searches were performed with the BLASTN algorithm (http://www.ncbi.nlm. nih.gov/BLAST/) [14]. The predicted protein sequences were analyzed with software Seqtools (http://www.bio-soft.net/sms). Multiple alignments of protein sequences were obtained from CLUSTALX1.83. The NJ method by MEGA 4.0 was used in the phylogenetic analysis of the previously undescribed D-conotoxin sequences and the reported typical sequences with 10 cysteines in the NCBI database [15,16]. Bootstrap values were estimated from 1000 replicates. Here, cDNA sequences containing 10 cysteines were selected, including 22 D-conotoxins, 23 S-superfamily conotoxins [17], di19A[18] and the D-conotoxin sequences from this work.

Table 1 shows the D-conotoxin sequences, which were deposited in the Genbank nucleotide sequence database. Initially, the seven previously undescribed D-conotoxins were named as Lt15.2, Lt15.3, Lt15.6, Lt15.9, Lt15.7, Lt15.5 and Lt15.6c, respectively. To date, the toxin disulfide framework identifier XV or 15 has been used. Since these above-mentioned seven D-conotoxins contained a previously undescribed framework, and a total of 27 cysteine scaffolds have been reported, we proposed that the framework of these previously undescribed D-conotoxins from *C. litteratus* was XXVIII, and Lt15.2, Lt15.3, Lt15.6, Lt15.9, Lt15.6a, Lt15.5a and Lt15.6c were accordingly renamed as Lt28.1, Lt28.2, Lt28.3, Lt28.4, Lt28.5, Lt28.6 and Lt28.7, respectively.

### 2.4. Cloning, expression, purification and identification of recombinant lt28.1 in P. pastoris

The mature Lt28.1 coding sequence was amplified by PCR using forward primer Lt28.1F1 (5′-ccgCTCGAGAAAAGACTTCATTGTCA TGAAATTTCAGAT-3′), Lt28.1F2 (5′-ccgCTCGAGAAAAGAGAGG CTGAAGCTCTTCATTGTCATGA AATTTCAGAT-3′) and reverse primer Lt28.1R (5′- ccgGAATTCTTAGTGATGGTGATGGTGATGTAGACA TGGCGGGCATGTACAA-3′). PCR reactions were performed using Prime STAR DNA polymerase (TaKaRa BIOTECH Co., Dalian, China). Briefly, after a denaturation step at 94 °C for 4 min, amplifications were carried out with 30 cycles at a melting temperature of 94 °C for 30 s, an annealing temperature of 60 °C for 30 s, and an extension temperature of 72 °C for 1 min. Finally, an extra extension step was performed at 72 °C for 10 min. Amplifications were cloned into the XhoI and EcoRI sites of *P. pastoris* expression vector pPIC9 to generate the Lt28.1 expression vector (pPIC9-Lt28.1). The sequence of Lt28.1 was verified through sequencing. Recombinant Lt28.1 was expressed in *P. pastoris* following the scale-up expression protocol in shaker flasks as described in the *P. pastoris* expression manual (Invitrogen Life Technologies). The pPIC9-