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A novel conotoxin, qc16a, with a unique cysteine framework and folding

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Abstract

A novel conotoxin, qc16a, was identified from the venom of vermivorous *Conus quercinus*. qc16a has only 11 amino acid residues, DCQPCGHNVCC, with a unique cysteine pattern. Its disulfide connectivity was determined to be I–IV, II–III. The NMR structure shows that qc16a adopts a ribbon conformation with a simple beta-turn motif formed by residues Gly6, His7 and Asn8. qc16a causes depression symptom in mice when injected intracranially. Point mutation results showed that Asp1, His7 and Asn8 are all essential for the activity of qc16a. Electrophysiologically, qc16a has no strong effect on the whole-cell currents of neurons and the currents of *Drosophila* Shaker channels, human BK channels and Nav1.7 channels. Its specific target still remains to be identified.

Keywords

conotoxin; *Conus quercinus*; disulfide connectivity; mutation; NMR; electrophysiology

1. Introduction

Conotoxins are highly diverse peptide neurotoxins from marine cone snails. Each species of cone snail produces 50–200 different conotoxin components in its venom. From the 500–700 species of the *Conus* genus, it is estimated that there are overall about 50,000 different conotoxins (Terlau and Olivera 2004). These conotoxins could be considered as a natural peptide library with striking structural and functional diversity.

The structural diversity of conotoxins is exhibited at different levels. In addition to the highly diverse sequences, conotoxins also have a large array of post-translational

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modifications, and highly different cysteine frameworks and disulfide linkages (Olivera 2006). Among many the intriguing features of conotoxins, the cysteine patterns are of special interest, because they are relatively conserved within the conotoxin family and largely define the three-dimensional structures of conotoxins (Halai and Craik 2009). Up to now, 21 cysteine frameworks have been identified (Kaas, Westermann et al. 2008).

For a peptide with four Cys residues, there are three possible disulfide linkages, the so-called globular (C1-C3, C2-C4), ribbon (C1-C4, C2-C3), and beads linkages (C1-C2, C3-C4) (Halai and Craik 2009). Only the globular and ribbon linkages have been found in conotoxins (Table 1). The relationship between the cysteine pattern and disulfide linkage is not consistent, even for the conotoxins with only four cysteine residues, as some conotoxins with the same cysteine pattern adopt different disulfide connectivity, such as χ -MrIA vs. α -ImI (Gehrmann, Daly et al. 1999; Sharpe, Gehrmann et al. 2001).

In this paper, we report a novel mini-conotoxin, qc16a, identified in the venom of *C. quercinus*, with a cysteine framework of C-C-CC cross-linked between C1-C4 and C2-C3. We describe the purification, chemical synthesis, disulfide connectivity, NMR structure, functional assays and mutational study of this conotoxin. These results expand our understanding of the diversity of conotoxins.

2. Materials and methods

2.1 Materials

Specimens of *Conus quercinus* were collected from South China Sea and stored at -80°C until used. PepmapTM C18 reverse-phase analytical column (4.6 mm $\times$ 250 mm) was obtained from Applied Biosystems (Foster City, CA, USA). ZORBAX 300SB-C18 semi-preparative column (9.2 mm $\times$ 250mm) from Agilent Technologies (Santa Clara, CA, USA). Reagents for Edman sequencing and Solid phase chemical synthesis were purchased from Applied Biosystems. Trifluoroacetic acid and acetonitrile for HPLC from Merck (Darmstadt, Germany). Reduction and alkylation reagents, i.e., DTT, Iodoacetamide, 4-vinylpiperidine(4-VP) and N-Ethylmaleimide(NEM), were purchased from Sigma. Other reagents were analytical grade.

2.2 HPLC Purification, Edman sequencing and Mass spectrometry

Crude venom was extracted from the venom ducts of *C. quercinus* with 30% acetonitrile (ACN) in H₂O containing 0.1% trifluoroacetic acid (TFA), lyophilized, and applied onto a PepmapTM C18 reverse-phase analytical column. The components were eluted with the following gradients: 0–5 min, Buffer A; 5–10 min, 0–10% Buffer B; 10–80 min, 10–70% Buffer B. Buffer A is 0.1% TFA in H₂O, and Buffer B is 0.1% TFA in ACN. The flow rate was 0.5 mL/min.

The molecular weights of native and synthetic conotoxins were analyzed on an API2000 ESI-Q-trap Mass spectrometer (Applied Biosystems, Foster city, CA, USA). Samples were loaded on Bionbrene pretreated glass filters and sequenced by Edman degradation on an ABI Procise 491 protein sequencer (Applied Biosystems, Foster city, CA, USA).

2.3 Disulfide connectivity determination

About 20 μg purified qc16a was dissolved in 100 μL Buffer A, and mixed with 100 μL 20 mM TCEP in 0.17 M sodium citrate, pH 3.0. The mixture was kept at 37°C for 5 min, then immediately injected onto a Pepmap C18 reverse phase column. The partially reduced intermediate was identified by mass spectrometry, and was pooled and alkylated immediately in dark at room temperature with overdose N-ethylmaleimide (NEM). The

alkylated intermediate was purified, and then fully reduced with DTT and further alkylated with 4-vinylpiperidine (4-VP). The purified double labeled final product was confirmed by mass spectrometry and sequenced with the Edman degradation method.

2.3 Chemical synthesis, oxidative folding, and HPLC coelution

Linear peptides of conotoxin qc16a and its four mutants, [H7A]qc16a, [Δ D, H7A]qc16a, [N8A]qc16a, and [Δ D, N8A]qc16a, were synthesized on a 433A peptide synthesizer (Applied Biosystems) using standard Fmoc chemistry. Peptides were grown on preloaded HMP resins. Orthogonal protection was used on cysteines: Cys2 and Cys11 were protected as the stable Cys(S-acetamidomethyl, AcM), whereas Cys5 and Cys10 were protected as the acid-labile Cys(S-trityl, Trt). After the completion of synthesis, linear peptides were cleaved from the resins by 3hrs treatment of 87% TFA plus scavengers including 5% EDT (ethanedithiol), 3% TES(triethylsilane), 3% thioanisole and 2% H₂O. All side chain protection groups were removed during cleavage step except for AcM groups at Cys2 and Cys11. The linear peptides were precipitated in ice-cool anhydrous ethyl-ether, and fully washed by ethyl-ether to remove scavengers and the cleaved protection groups. Air dried linear peptides were dissolved in 5% acetic acid, and purified on a Semi-preparative C18 column. Folding of the linear peptides was achieved by a two-step oxidation method. Firstly, the linear peptides were air-oxidized in 50 mM NH₄HCO₃, pH 8.2, overnight at room temperature to form the disulfide bond between Cys5 and Cys10. After being purified, the peptides were further oxidized with 1 mg/mL iodine at room temperature for 1 hr to form the disulfide bond between Cys2 and Cys11. After the completion of iodine oxidation, the reactions were quenched by overdose Vitamin C as indicated by a color switch of the solution from light yellow to transparent.

2.4 NMR spectroscopy and structure calculation

For NMR experiments, 2.5 mg qc16a was dissolved in 500 μ L 90% H₂O/10% D₂O or 99.99% D₂O at pH 3.5. All NMR experiments were carried out on a Varian Unity Inova 600-MHz spectrometer equipped with three RF channels and a triple resonance z-axis pulse-field gradient probe. The 2D TOCSY spectra were acquired with a mixing time of 75 ms, while NOESY spectra were acquired with mixing times of 200 ms, 300 ms and 350ms. Both the Watergate approach and the pre-saturation scheme were employed for water suppression. All spectra were recorded both in H₂O and D₂O with 512 t1 increments and 2048 complex data points. Signals were averaged over 32 transients. All NMR data were processed and analyzed using the NMRPipe/NMRDraw software and the Sparky program (Delaglio, Grzesiek et al. 1995; Nilges, Macias et al. 1997). Linear prediction in the t1 dimension was used before the Fourier transformation. Assignments of the proton resonances were achieved using TOCSY and NOESY spectra. All proton chemical shifts were referenced to H₂O. Proton-proton distance restraints were derived primarily from the NOESY spectrum recorded with a mixing time of 150 ms in both H₂O and D₂O. The structure calculations were performed according to the standard ARIA/CNS protocol (Brunger, Adams et al. 1998; Brunger 2007; Rieping, Habeck et al. 2007). Ramachandran plot analysis was performed using the PROCHECK program (Laskowski R A, MacArthur M W et al. 1993).

2.5 Bioassay

Peptide solutions in normal saline solution at a concentration of 4 μ g/ μ L were used as stock solutions. Two-week-old Kunming mice were intracranially injected with 20 μ L of toxin solutions at different concentrations to assess biological responses and potency. Normal saline solution was used as negative control.

2.6 Two electrode voltage clamp

Stage V–VI oocytes were injected with cRNA encoding *Drosophila* Shaker or mammalian slo1 (BK channel) subunits. Whole cell currents were recorded 1–3 days after injection under two electrode voltage clamp mode using an OC-725 amplifier (Warner Instrument Corporation, Hamden, CT, USA), which is driven by the Pulse+PulseFit software package (HEKA Elektronik, Lambrecht, Germany). The intracellular electrodes were filled with 1 M KCl and had a resistance between 0.3 and 1.0 M Ω . The bath solution was normal ND96 containing (in mM) 115 NaCl, 2.5 KCl, 1.8 CaCl₂, 10 HEPES, pH 7.2. Toxins dissolved in ND96 solution were perfused (for concentrations lower than 100 μ M) onto the oocytes, or directly puffed onto the surface of oocytes (for concentration higher than 100 μ M). ND96 solution similarly administrated by perfusing or puffing onto the surface of oocytes was used as a negative control. Current signals were low pass filtered at 2 kHz, digitized at a sampling frequency of 10 kHz, and leak-subtracted by a P/4 protocol.

2.7 Patch clamp

HEK 293 cells stably expressing rat BK channel were maintained in 5% CO₂ incubator at 37°C. HEK293 cells expressing Na_v1.7 channels were a generous gift from Dr. Stephen Waxman's laboratory, Yale University. Primary neurons dissociated from neonatal rat hippocampus or prefrontal cortex were provided by Dr. Mark Yeckel, Yale University. Patch pipettes were pulled from borosilicate glass tubes (Kimble Chase, Vineland, New Jersey, USA) to give a resistance of 1.5 – 3.0 M Ω when filled with the pipette solution. For recording Na_v1.7 currents, the extracellular buffer contained (in mM): 120 NaCl, 20 TEA-Cl, 5 CsCl, 1 MgCl₂, 10 Glucose, 10 HEPES, 0.1 CdCl₂, 1 CaCl₂; the intracellular solution contained (in mM): 140 CsCl, 1 MgCl₂, 1 EGTA, 5 MgATP, 10 HEPES. For the neuron whole-cell current recording, the extracellular buffer contained (in mM): 155 NaCl, 5 KCl, 1 EGTA, 2 CaCl₂, 10 HEPES, pH 7.4; the intracellular solution contained (in mM): 20 KCl, 140 KAsp, 2 MgCl₂, 1 EGTA, 10 HEPES, pH 7.4. The toxin effects on the currents of Na_v1.7 or neurons were examined by bath application at a final concentration of 2 μ M. Whole cell currents were recorded through a EPC9 amplifier driven by Pulse+Pulsefit software (HEKA Elektronik, Lambrecht, Germany), digitized at a rate of 10 kHz and low-pass filtered at 2 kHz. A P/4 protocol was used for leak subtraction.

3. Results

3.1 Identification of qc16a

Preliminarily fractionated crude venom of *C. quercinus* was separated on a Pepmap C18 analytical column (Figure 1A). The peak marked with an asterisk containing target compound was further purified to homogeneous (Figure 1B) as indicated by mass spectrometry (Figure 1C). Direct sequencing of this conotoxin gave a sequence of DCQPCGHNVCC if all blank cycles were presumably designated as Cys. Theoretical molecular weight of the sequence is 1174.3, which is in very good consistence with the observed one. Besides, the Mw of carboxylamidomethylated derivative of the compound shifted to 1406.8 (data not shown), suggesting that four cysteine residues exist in its sequence, therefore confirmed the Edman sequencing result. Following the nomenclature of Olivera and Cruz (Olivera and Cruz 2001), this conotoxin was named qc16a.

3.2 Disulfide connectivity of qc16a

To determine the disulfide connectivity of qc16a, the partial reduction strategy was applied (Han, Wang et al. 2006). The partially reduced intermediate of qc16a (Figure 2A), as indicated by a +2 dalton molecular weight shift comparing with native qc16a (data not shown), was sequentially alkylated with NEM and 4-VP as described in Methods and purified. The double labeled qc16a was sequenced by Edman degradation. In the

sequencing cycles, two newly generated peaks were eluted just before PTH-Pro at the 2nd and 11th cycles, suggesting the presence of PTH-PE-Cys at the corresponding cycles, while another two novel peaks positioning between PTH-Pro and PTH-Met appeared at the 5th and 10th cycles, indicating the presence of PTH-NEM-Cys. As shown in Figure 2D, yields for PTH-NEM-Cys at the 5th and 10th cycles and PTH-PE-Cys at the 2nd and 11th cycles were especially higher comparing with those of their surrounding cycles.. These results clearly indicated that qc16a has the ribbon disulfide linkage of Cys2-Cys11 and Cys5-Cys10.

To confirm the disulfide linkage, we further examined HPLC profiles of native and synthetic qc16a. First, qc16a was regioselectively synthesized and folded as described in Method (Figure 2A, B and C). The completeness of each oxidative folding step was checked with mass spectrometry. The synthetic qc16a co-eluted with native peptide on HPLC (Figure 2D, E and F), which shows that the native qc16a has the same disulfide folding as the synthetic peptide, i.e. Cys5-Cys10 and Cys2-Cys11.

3.2 NMR structure of qc16a

The solution structure of qc16a was determined with NMR methods. Quality of the determined structure is assessed with RMSD (Root Mean Square Deviation) values calculated from the coordinates of backbone and heavy atoms in secondary structures, which are 0.32 Å and 0.96 Å, respectively. The quality evaluation by PROCHECK program indicates that all the backbone dihedral angles of qc16a residues are within the allowed regions of the Ramachandran plot, in which 60.7% residues fall into the most favored region (Table 2). The coordinates for the family of 20 structures and NMR constraints have been deposited in SMSDep (small molecule structure deposition system) with BMRB entry 20128.

Due to the ribbon disulfide linkage, qc16a unsurprisingly adopts a β -turn conformation formed of Gly6, His7 and Asn8. This structure shows some resemblance to χ -MrIA which has also a ribbon disulfide linkage (Sharpe, Gehrmann et al. 2001), while being clearly different from the conformation of conotoxins with globular disulfide connectivity, such as α -conotoxins (Figure 3A). One could consider that the structure of qc16a and χ -MrIA is mainly composed of two spatial loops, the lower loop being composed of the second and third Cys residues and the residues in between, while the upper loop being composed of residues between the two disulfide bonds (viewed with the orientation shown in Figure 3A). Because of the different Cys positioning in qc16a and χ -MrIA sequences, the conformation of the upper loop differs considerably. The functionally critical residues for χ -MrIA are all in the lower loop (Sharpe, Palant et al. 2003) (Figure 3B). Two residues of the corresponding loop of qc16a, His7 and Asn8, as well as the N-terminal Asp1, were chosen to be mutated and found to be important for the activity of qc16a, too (see below).

3.3 Bioassay of qc16a and its mutants

To assess the effect of qc16a on mice, two-week-old mice were injected intracranially with qc16a. When 10 μ g qc16a was injected into a mouse, no obvious symptom was observed. When the dose was increased to 20 μ g/mouse, the tested mice became depressed and reluctant to move when being pushed. With a dose of 40 μ g qc16a/mouse, the tested mice turned into coma status with the eyes closed and body crouched. But the muscle strength was still observed, suggesting that the tested mice were not in sleeping status. All the tested mice gradually recovered from the inhibitory impacts of qc16a in 30 min.

Mutation of His7 to Ala completely abolished the activity of qc16a. The mutant [H7A]qc16a had no any obvious effect when injected into mice. This indicates that His7 is probably

critical for the interaction of qc16a with its target. Further deletion of Asp1 did not cause any more effect, as [Δ D1, H7A]qc16a had no effect on mice, either.

Unexpectedly, the mutant [N8A]qc16a exhibited excitatory effect on mice. When injected intracranially with 40 μ g [N8A]qc16a, most of the tested P14 mice first trembled, then burst into seizure-like symptoms 15 min later. The seizure symptom lasted for more than 10 min before the mice died 30 min after injection. One of the four tested mice remained upright with tremor upon injection, then suddenly died at about 5 min. The strikingly different effects of qc16a and [N8A]qc16a showed that Asn8 is a very influential residue, mutation of which either changed the effect of qc16a on its target, or even changed the working target of qc16a. In any case, Asn8 most probably participates into the interaction of qc16a with its target.

Surprisingly, further deletion of Asp1 caused total loss of activity, as [Δ D1,N8A]qc16a caused no any effect on mice. This indicates that Asp1 is also required for the function of [N8A]qc16a.

From the mutation results above, it is plausible to speculate that His7 may be the most critical residue for the interaction of qc16a with its target, i.e. the primary binding site, while Asp1 is probably the secondary binding site. Once qc16a binds to the target with the presence of His7, Asp1 is required for qc16a to fully function (compare the activity of [N8A]qc16a and [Δ D1,N8A]qc16a). If the primary binding site is mutated, the toxin is not able to interact with its target even with the presence of Asp1 (see the lose of activity for [H7A]qc16a).

3.4 Electrophysiology studies

In an effort to identify the target of qc16a, electrophysiological studies were carried out. Either qc16a or [N8A]qc16a showed no obvious effects on the currents of Na ν 1.7 channels (n=4), the whole cell current of neurons from hippocampus and prefrontal cortex (n=5), and the whole cell currents of hypocretin/orexin containing neurons (n=2–3).

Only when puffed onto oocytes at 1 mM concentration did qc16a have an effect. It caused an immediate 25% inhibition of Shaker channel current (Figure 4A and B), but an increase of BK channel current (Figure 4C and D). No effect of [N8A]qc16a was observed on the same channels. Because these effects of qc16a were observed only at a very high concentration, Shaker channels and BK channels are unlikely to be physiological targets of qc16a.

4. Discussions

The cysteine pattern of qc16a, C-C-CC, is quite unique among conotoxins. So far, only one native toxin, CalTx from *C. californicus*, has been reported to have this cysteine framework, but its disulfide connectivity was not determined (Bernaldez, Lopez et al. 2011). Furthermore, qc16a and CalTx share low sequence similarity except for the Cys pattern. It is still not clear whether they belong to the same family and whether they have the same physiological function.

By comparing the structure of qc16a and MrIA (Figure 3B and C), we could see that the active site of MrIA is only located at the lower part of the molecule, while both ends of qc16a molecule are important for its function. Thus they may have totally different molecular targets and mechanisms of action, although they all have the ribbon disulfide linkage. This is not surprising given that their molecular shape is quite different (Figure 3B

and C). Further study of the toxin-target interaction at the molecular level would be needed to give more precise information about their mechanisms of action.

In summary, we have described the identification, disulfide connectivity determination, three-dimensional structure, bioassay and mutational study of a novel conotoxin, qc16a. It is the second conotoxin reported to have the unique cysteine pattern C-C-CC, and the first one whose disulfide linkage pattern and three-dimensional structure have been determined. This toxin, when intracranially injected, causes depression symptoms in mice, but its physiological target is still unknown. The identification of this conotoxin enriches our understanding of the diversity of conotoxin, and paves the way for further studies.

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A

B

C

Figure 1.

The identification of qc16a and its disulfide connectivity determination. (A) The crude venom of *C. quercinus* was separated on a C18 HPLC column with the gradient described in Methods. The peak marked with an asterisk contains qc16a. (B) qc16a was further purified to homogeneity on the same column with the same gradient. (C) Products of qc16a partial reduction were separated on a C18 HPLC column with flow rate of 0.8 mL/min and a gradient of 10–40% Buffer B in 0–30 min. The partially reduced intermediate was identified with mass spectrometry.

Figure 2.

The folding of synthetic qc16a and its coelution with native qc16a. The synthetic linear peptide of qc16a (panel A, determined Mw. 1320.0) was air-oxidized (panel B, determined Mw. 1318.0), and then iodine-oxidized (panel C, determined Mw. 1173.5), as described in Methods. These peptides were purified on a Zorbax Semi-preparation C18 column using a gradient from 5–30% buffer B over 30 min at a flowrate of 1.5 mL/min. The elution of native qc16a (D), synthetic qc16a (E) and an equal mixture of both (F) were run on a Pepmap analytical C18 column at a flow rate of 0.8 mL/min and monitored at 214 nm, using a gradient of 10–30% Buffer B in 0–35 min.

Figure 3. NMR structure of qc16a. (A) Backbone representation of qc16a in comparison with χ -MrIA and α -ImI. The peptide chain of each toxin is rainbow colored with N-terminus in blue and C-terminus in red. The disulfide bonds are shown in yellow ball-and-stick representation. (B) The molecular surface of qc16a and χ -MrIA is shown with the same orientation as in panel A. The surface is shown in slate except for the disulfide bonds in yellow. The side chains of functionally important residues are shown and the corresponding surface is highlighted in dark blue. (C) The molecular surface of qc16a and χ -MrIA is shown rotated by 180° from panel B.

Figure 4.

The activity of qc16a on Shaker channels (A, B) and BK channels (C, D) expressed in *Xenopus* oocytes. (A) Representative current traces of Shaker channel in the absence or presence of high concentration qc16a. Depolarizations to +20 mV were given from a holding potential of –80 mV. (B) Time course of the effect of qc16a on the current of Shaker channels at +20 mV. (C) Representative current traces of BK channels in the absence or presence of a high concentration qc16a. Depolarizations to +80 mV were given from a holding potential of –80 mV. (D) Time course of the effect of qc16a on BK channel currents.

Table 1

Representative conotoxins with 4 Cys residues.

Conotoxin	Sequence	S-S linkage	Superfamily	Targets	Reference
qc16a	DCQP CGHNVCC	1-4, 2-3	unknown	BK and Shaker channel at high concentration	This work
CaITx	NCPAG CRSQGCCM	unknown	unknown	HVA Ca ²⁺ channels	(Bernalde z, Lopez et al. 2011)
χ-Mr1A	NGV CCGYKLCHOC	1-4, 2-3	T	Noradrenaline transporter	(Sharpe, Gehrmann et al. 2001)
α-1ml	G CCSDPRCAWRC*	1-3, 2-4	A	α7	(Gehrmann, Daly et al. 1999)
ρ-TIA	FNW RCC LIPACRRNHKK FC*	1-3, 2-4	A	α1-adrenoreceptor	(Sharpe, Gehrmann et al. 2001)
tx5a	γ CC γDGB CC gTrAAO	1-4, 2-3	T	Unknown	(Rigby, Lucas-Meunier et al. 1999)
α1-ts14a	DGCP HPVPGMHPCMTNTC	1-3, 2-4	A	Unknown	(Peng, Ye et al. 2010)
Lt16.1	RTGEDFLEE CMGGCAFDCCCK	unknown	M	Unknown	(Pi, Liu et al. 2006)

* C-terminal amidation; gTr: glycosylation of Threonine; γ: gamma-carboxylated glutamate; O: hydroxyproline.

Table 2

Structure statistics of qc16a from the NMR structural analysis

Quantity	Value
Total unambiguous distance restraints	55
Intra residual	32
Sequential ($ i - j = 1$)	12
Medium ($2 \leq i - j \leq 4$)	6
Long range ($ i - j \geq 5$)	5
Dihedral angles	6
RMSD from the average atomic coordinates (Å)	
Backbone (N, C $^{\alpha}$, C')	
2 nd structure	0.32±0.11
All residues	0.58±0.17
Heavy atoms	
2 nd structure	0.96±0.16
All residues	1.19±0.20
Deviations from idealized covalent geometry	
Bond (Å)	0.0041 ± 0.0002
Angles (°)	0.57 ± 0.04
Improper (°)	1.66 ± 0.34
Ramachandran analysis (%)	
Residues in most favored regions	60.7%
Residues in additional allowed regions	21.4%
Residues in generously allowed regions	17.9%
Residues in disallowed regions	0%

Table 3

Biological assays of qc16a and its mutants

Sample	Dosage ($\mu\text{g}/\text{mouse}$)	Symptoms
qc16a	<10	reluctant to move, recovered in 2–3 min (n=3).
	20	coma or depressed state with head lowered and eyes closed, recovered in 2–5 min (n=5).
	40	coma state, crouched, eyes closed. Muscle tone remained. Recovered in 30 min to 3 hr (n=5).
[H7A]qc16a	40	no effect (n=4)
[Δ D,H7A]qc16a	40	no effect (n=2)
[N8A]qc16a	20	weakness, gradually recovering in 15 min (n=4)
	40	trembling, then seizure, died in 30 min (n=3); One mouse became quiet with slight tremor, then suddenly died at 5 min (n=1).
[Δ D,N8A]qc16a	40	no effect (n=2)