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GluCl a target of indole alkaloid okaramines: a 25 year enigma solved

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In 1989, indole alkaloid okaramines isolated from the fermentation products of *Penicillium simplicissimum* were shown to be insecticidal, yet the mechanism of their toxicity to insects remains unknown. We therefore examined the action of okaramine B on silkworm larval neurons using patch-clamp electrophysiology. Okaramine B induced inward currents which reversed close to the chloride equilibrium potential and were blocked by fipronil. Thus it was tested on the silkworm RDL (resistant-to-dieldrin) γ-aminobutyric-acid-gated chloride channel (GABACl) and a silkworm L-glutamate-gated chloride channel (GluCl) expressed in *Xenopus laevis* oocytes. Okaramine B activated GluCl, but not RDL. GluCl activation by okaramines correlated with their insecticidal activity, offering a solution to a long-standing enigma concerning their insecticidal actions. Also, unlike ivermectin, okaramine B was inactive at 10 μM on human α1β2γ2 GABACl and α1β glycine-gated chloride channels and provides a new lead for the development of safe insect control chemicals.

Okaramines are indole alkaloids isolated from *Penicillium simplicissimum* and shown to be toxic to larvae of the silkworm (*Bombyx mori*)[1]. Okaramines A and B possess a six-membered diketopiperazine ring and an eight-membered azocine ring in addition to two indole rings (Fig. 1). Okaramine B has an additional four-membered azetidine ring and shows higher insecticidal activity than okaramine A (Fig. 1)[1]. Subsequently, okaramines C[2], D–F[3], G[4], J–M[5] and N-R[6] were isolated from *Penicillium simplicissimum* and okaramines H and I[7] were described from *Aspergillus aculeatus*. Structure-activity studies showed the importance of the azetidine and azocine rings to okaramine insecticidal activity[8].

The unique and complex structures of okaramines inspired the total chemical synthesis of okaramines C[9], J[10] and N[11], but the mechanism of their insecticidal activity has remained elusive. An okaramine treated silkworm dies rapidly and since insect control chemicals targeting ligand-gated ion channels (LGICs) are fast-acting, we employed patch-clamp electrophysiology to investigate okaramine B actions on native LGICs. It activated native silkworm ligand-gated chloride channels and thus we tested it on the recombinant silkworm RDL (resistance to dieldrin) γ-aminobutyric-acid-gated chloride channel (GABACl) and an L-glutamate-gated chloride channel (GluCl); both are expressed abundantly in the insect central nervous system. We report for the first time that okaramine B selectively activates GluCl, thereby offering an explanation for its insecticidal activity and introducing new lead chemistry targeting a ligand-gated ion channel only found in invertebrates.

Results

Effects of okaramine B on silkworm larval neurons. We adopted okaramine B[1] as a representative with which to study okaramine insecticidal actions as it shows the highest toxicity to insects. The indole alkaloid was applied via U-tube on to cultured silkworm larval neurons for 2 s at a holding potential of −60 mV, resulting in a transient inward current (Fig. 2a). To determine whether such an inward current is cationic or anionic, the effects of the nicotinic receptor antagonist mecamylamine[12] and the ligand-gated chloride channel antagonist fipronil[13,14] were investigated on the okaramine B-induced current. Mecamylamine (1 μM) scarcely influenced the okaramine-induced current (n = 3, Fig. 2a), whereas fipronil (10 μM) markedly reduced the current (n = 3, Fig. 2b), when bath-applied for 1 min prior to co-application with 1 μM okaramine B, indicating a possible action on ligand-gated chloride channels. To confirm this, the okaramine-induced currents were measured at various membrane potentials (Fig. 2c); currents reversed at −7.0 mV, close to the chloride equilibrium potential (E_{Cl}) of −8.8 mV

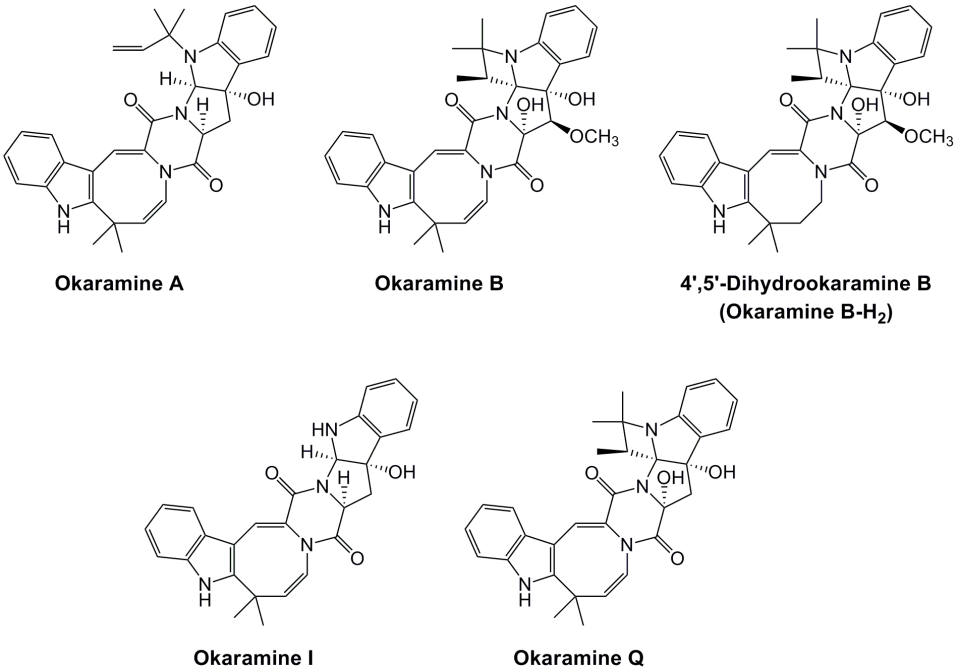

Figure 1 | **Structures of okaramines A, B, 4′,5′-dihydrookaramine B (okaramine B-H_2), I and Q.**

(Fig. 2d). Changing the extracellular chloride ion concentration from 156 mM to 56 mM also shifted the reversal potential to +18.2 mV, a value similar to that predicted (+17.1 mV) by the shift in E_{Cl} (Fig. 2d).

Effects of okaramine B on ligand-gated Cl channels expressed in *Xenopus laevis* oocytes. The RDL (resistant to dieldrin) γ-aminobutyric-acid-gated chloride channel (GABACl)[15] and L-glutamate-gated chloride channel (GluCl)[16] are cys-loop, ligand-gated chloride channels abundantly expressed in the nervous system of insects. We investigated the actions of okaramine B on the silkworm RDL GABACl and GluCl expressed separately in *Xenopus laevis* oocytes using two-electrode voltage-clamp electrophysiology. When tested alone, 10 μM okaramine B was inactive on the membrane current in oocytes expressing RDL (Fig. 2e). Also, it had no significant effect on the GABA-induced RDL response when applied at 10 μM for 1 min prior to co-application with 30 μM GABA (Fig. 2f). By contrast, okaramine B did evoke inward currents concentration-dependently in oocytes expressing GluCl (n = 4, Fig. 2g).

GluCl actions and insect toxicity of okaramines. To compare the actions of a series of okaramines on native and recombinant GluCls, we determined the two activities in pEC_{50} ($=-\log EC_{50}$) for okaramines A, B, 4′,5′-dihydrookaramine B (okaramine B-H_2), I and Q (Supplementary Table S1) from the concentration-response curves (Fig. 3a, b) where the peak current amplitude of the okaramine-induced response was normalized by that of 100 μM L-glutamate-induced response (see Supplementary Fig. S1 for L-glutamate- and okaramine B-induced currents recorded in the same neuron). For both activities, okaramine B was most potent, whereas okaramine I was ineffective, hence it's EC_{50} could not be determined. Okaramines A and B-H_2 followed okaramine B and okaramine Q was the second least active ahead of okaramine I. Overall, the chloride-current inducing activity showed a high correlation with the GluCl activating activity ($r^2 = 0.964$) (Fig. 3c).

We also determined the insecticidal activity in pLD_{50} for the five okaramines on the silkworm larvae (Table S1). The insecticidal activity of these compounds showed a high correlation with the GluCl activating activity ($r^2 = 0.936$) as well as the chloride-current inducing activity on the larval neurons ($r^2 = 0.914$) (Fig. 3c).

Actions of okaramine B on human ligand-gated chloride channels. Finally, we investigated the effects of okaramine B on human α1β2γ2 GABACl and α1β glycine-gated chloride channel (GlyCl), both of which are major ligand-gated chloride channels expressed in human brain. Okaramine B tested at 10 μM had no effect on the α1β2γ2 GABACl, whereas ivermectin, a macrocyclic lactone known to act on GluCl, activated the GABACl at the same concentration (Fig. 4a). Also, okaramine B hardly affected the peak response amplitude to 30 μM GABA of the α1β2γ2 GABACl (Fig. 4b). When tested at 10 μM, okaramine B, unlike ivermectin, failed to activate the human α1β GlyCl (Fig. 4c), nor did it show allosteric modulatory actions (Fig. 4d).

Discussion

Our studies provide the first insight into a mechanism for the insecticidal action of okaramines. The okaramine-induced currents on silkworm larval neurons reversed at the chloride equilibrium potential, pointing to interactions with ligand-gated chloride channels. They were blocked by fipronil which can block both GABACls and GluCls of invertebrates[13,17]. GABACls and GluCls are the major ligand-gated chloride channels expressed in the nervous system of insects. GABACls play a central role in fast inhibitory neurotransmission and the RDL subunit is a major component of native GABACls[15]. GluCls are generated from a single gene and underlie the control of locomotion, feeding, and sensory input in insects[16]. Okaramine B was neither an activator, nor a blocker of RDL, excluding this channel from the primary site of action. By contrast, okaramines activated GluCl with EC_{50} similar to that determined in the native larval neuron, suggesting a significant contribution of the GluCl-activating action to the chloride current induction in the silkworm larval neurons.

Okaramine B was more potent than okaramines A and I on silkworm GluCl, which likely results from the four-membered azetidine ring. Also, a comparison of the activity of okaramines A and I indicates an important role for the methoxy group in okaramine activity. In addition, the higher activity of okaramine B compared to okaramine B-H_2 suggests interactions with GluCl of the eight-membered azocine ring.

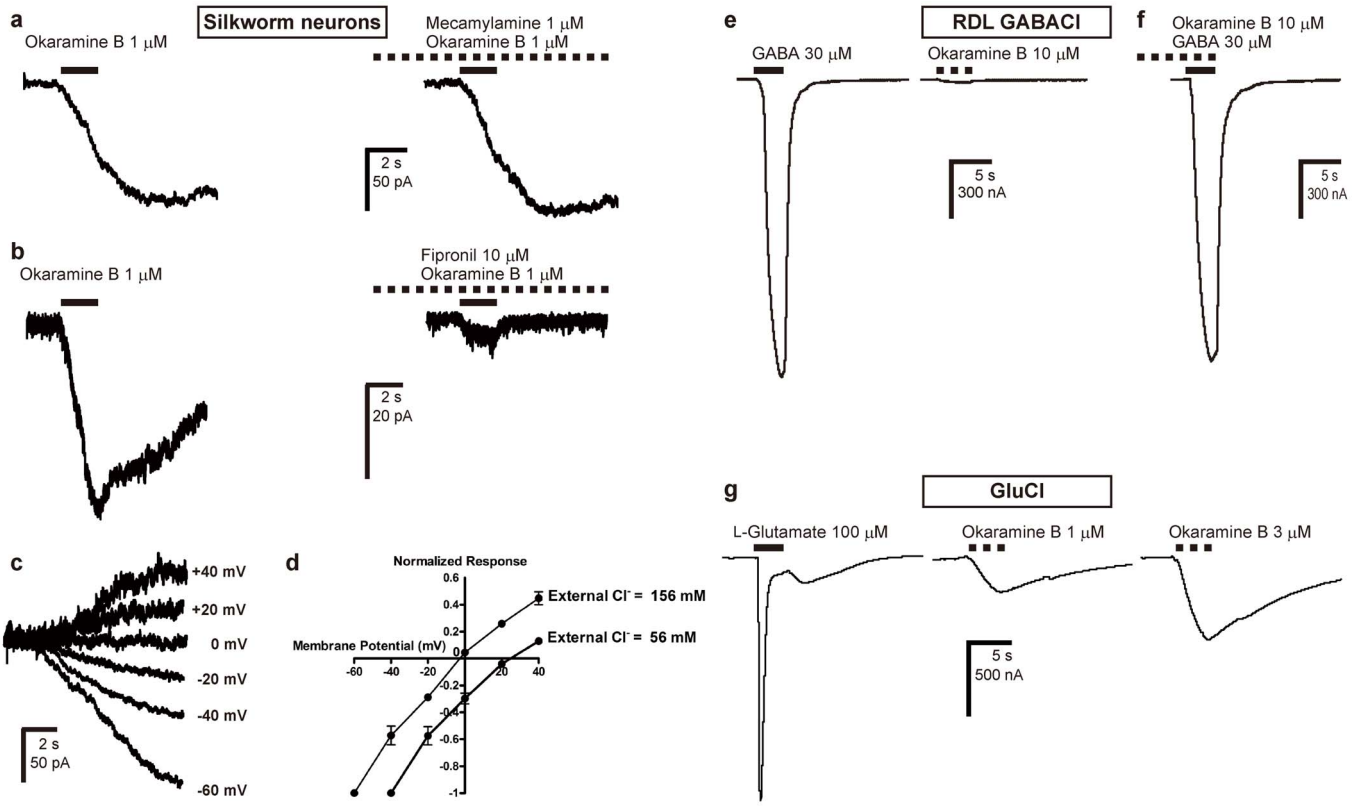

Figure 2 | Action of okaramine B on the membrane currents of silkworm larval neurons as measured with whole-cell patch-clamp electrophysiology (a–d) and on silkworm larval RDL γ-aminobutyric-acid-gated chloride channel (GABACl) (e, f) and L-glutamate-gated chloride channel (GluCl) (g) expressed in *Xenopus laevis* oocytes as measured with two-electrode voltage-clamp electrophysiology. Okaramine B was applied via U-tube, whereas antagonists were bath-applied to the neurons. (a) Inward current induced by okaramine B at 1 μM and the effect of nicotinic receptor antagonist mecamylamine (1 μM) applied for 1 min prior to co-application with 1 μM okaramine B. (b) Inward current induced by okaramine B at 1 μM and the effect of ligand-gated chloride channel blocker fipronil (10 μM) applied for 1 min prior to co-application with 1 μM okaramine B. (c) Inward current induced by 1 μM okaramine B at various holding potentials. (d) Current-voltage relationship at two different extracellular chloride concentrations. Each plot indicates the mean ± standard error of repeated experiments (n = 3). (e) Five min after recording the response to 30 μM GABA of RDL, 10 μM okaramine was bath-applied. (f) Okaramine B was bath-applied at 10 μM for 1 min prior to co-application with 30 μM GABA. Okaramine B had a minimal impact on the GABA response RDL. (g) Five min after recording the response to 100 μM L-glutamate of GluCl, 1 and 3 μM okaramine B was bath-applied with at 5-min interval.

Besides RDLs and GluCls, histamine-gated chloride channels (HisCls) and proton-sensitive chloride channels (pHCls) are other LGICs expressed in insects[18]. HisCls are fipronil-insensitive and mainly involved in neurotransmission in the eye[19–22]. pHCls are also fipronil-insensitive and expressed in the central nervous system and, less abundantly, in the hindgut[23]. From our studies on okaramine B-induced chloride currents in silkworm larval neurons, we cannot exclude the possibility of some actions on HisCls and/or pHCls. However, the absence of any response to histamine in the silkworm larval neurons (Supplementary Fig. S2) and the ability of okaramine B to induce fipronil-sensitive currents (Fig. 2b) in neurons points to actions on GluCls. A strong correlation between okaramine insecticidal activity and GluCl actions (Fig. 3c) indicates that interactions with GluCl are important in okaramine insecticidal actions.

Okaramine B scarcely affected human α1β2γ2 GABACl and α1β GlyCl, suggesting a selectivity for insects over human ligand-gated anion channels (Fig. 4), although we cannot rule out actions of okaramine at concentrations higher than those tested. Thus, okaramines may serve as leads for a new generation of compounds for use in crop protection.

Methods

Okaramines. Okaramines A, B and Q were isolated from the okara-based fermentation products of *P. simplicissimum*, whereas okaramine I was obtained from the products of *A. aculeatus*. Okaramine B-H_2 was prepared by catalytic reduction of okaramine B as reported previously[8]. Fipronil and mecamylamine as well as salts used for electrophysiology were purchased from Sigma Aldrich (St. Louis, MO, USA). Okaramines were dissolved at either 10 or 100 mM in dimethyl sulfoxide as stock solutions and stored at −20°C until use.

***Bombyx mori* larval neurons.** The mushroom body neurons of the silkworm larvae were prepared as described previously[24]. In brief, the mushroom body was dissected from the 4th instar larvae of *Bombyx mori* and the sheath was removed manually in a Ca^{2+}-free buffer consisting of 135 mM NaCl, 3 mM KCl, 4 mM $MgCl_2$, 10 mM glucose and 10 mM HEPES (pH 7.3, adjusted with NaOH), supplemented with 50 units ml^{-1} penicillin and 50 μg ml^{-1} streptomycin. After treating with 1.0 mg mL^{-1} collagenase (Type IA, Sigma Aldrich Japan) in the Ca-free buffer, the mushroom bodies were transferred to a Ca^{2+}-supplemented saline solution containing 135 mM NaCl, 3 mM KCl, 4 mM $MgCl_2$, 5 mM $CaCl_2$, 10 mM glucose, 10 mM trehalose and 10 mM HEPES (pH 7.3, adjusted with NaOH) supplemented with 10% fetal bovine serum and 50 units ml^{-1} penicillin and 50 μg ml^{-1} streptomycin. The neurons were dissociated and seeded on cover slips coated with poly-D-lysine. After washing with the Ca^{2+}-supplemented saline solution, the neurons were incubated for 12–24 hours at 25°C in the same solution before electrophysiology.

Patch-clamp electrophysiology. Dissociated silkworm larval neurons on cover slips were superfused at a flow rate of about 5 ml min^{-1} with an extracellular bath solution consisting of 135 mM NaCl, 3 mM KCl, 5 mM $CaCl_2$, 4 mM $MgCl_2$, 10 mM glucose and 10 mM HEPES (pH 7.3, adjusted with NaOH). The whole-cell configuration was established using a patch pipette filled with an internal solution consisting of 100 mM KCl, 1 mM $CaCl_2$, 4 mM $MgCl_2$, 20 mM sodium pyruvate, 10 mM EGTA and 10 mM HEPES (pH 7.3, adjusted with Tris). Only borosilicate glass pipettes with a resistance of 5–6 MΩ were used for current recordings. The membrane currents were recorded at a holding potential of −60 mV using an Axopatch 200B amplifier

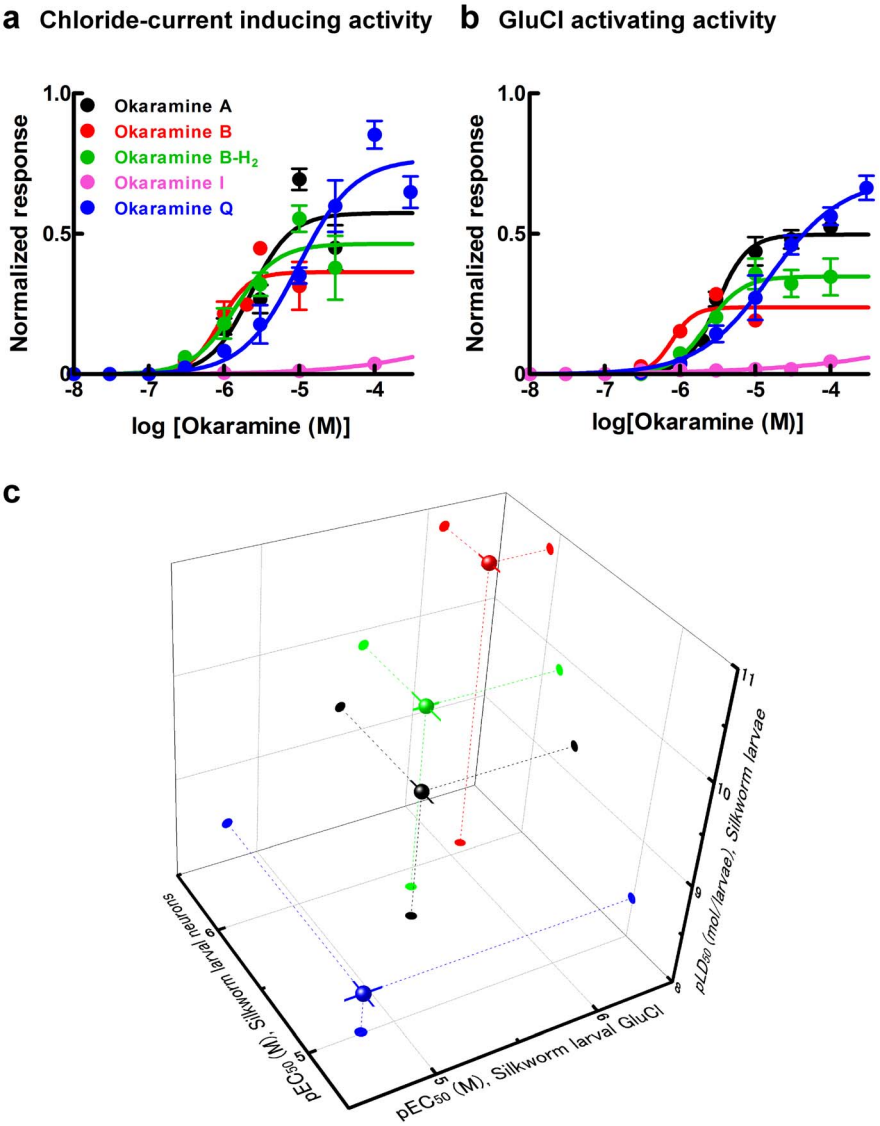

Figure 3 | Concentration-response relationships of okaramines A, B, 4′,5′-dihydrookaramine B (okaramine B-H_2), I and Q for the Cl^- current inducing activity on the silkworm neurons and the GluCl activating activity, and correlations of these two activities with the insecticidal activity on the silkworm larvae. (a) Concentration-response relationship for the chloride-current inducing activity on the silkworm larval neuron. (b) Concentration-response relationship for the GluCl activating activity. Each plot indicates the mean ± standard error of the mean of repeated experiments (n = 4). (c) 3D plot for the relationship of the GluCl activating activity (X axis), and the Cl^- current inducing activity on the larval neuron (Y axis) with the insecticidal activity (n = 3) (Z axis). Each sphere plot in (c) is projected to X-Y, X-Z and Y-Z planes to show its position in each plane.

(Molecular Devices, Sunnyvale, CA, USA) by using Clampex 9 software (Molecular Devices) and the analog current data was digitized at sampling frequency of 10 kHz using a Digidata 1322A data acquisition system (Molecular Devices). The digitized current data was analyzed off-line using Clampfit 9 software (Molecular Devices). Okaramines were applied using a U-tube, while antagonists were bath-applied. The reversal potential for okaramine-induced currents was determined by subtracting an experimentally-measured liquid junction potential from a zero current potential. When changing the chloride ion concentration, the chloride ions were replaced with equimolar amounts of isethionate ions.

cDNAs of ligand-gated chloride channels. The entire cDNAs of the RDL (resistant to dieldrin) GABACl (Accession no. AB847423) and GluCl (Accession no. KC342244) was amplified by PCR from the cDNA library of the mushroom body of the 4th instar larvae of *B. mori* (strain P-50) using KOD Plus polymerase (Toyobo, Osaka, Japan) with BmRDL forward (5′-CGGGGTACCATGAGCGGCGCCAA-GCCCCGCACC-3′) and BmRDL reverse (5′-CAGGGATCCCTATTTATCTTC-TTCCAGAAGAACC-3′) primers for RDL and BmGluCl forward (5′-CGGGGT-ACCATGGAATTCCCTCGGCGGCCATGT-3′) and BmGluCl reverse (CAGGGATCCTCACCAGTAAGCCAAATTGAAAATG) primers for GluCl according to the following cycle reaction: 94°C for 2 min; 30 cycles of 94°C for 15 s, 56°C for 30 s and 68°C for 2 min. The cDNA was cut with *Kpn*I and *Bam*HI and cloned into the same restriction sites of the pcDNA3.1 (+) vector (Life Technologies). The cDNA sequences were confirmed by means of a 3100 Genetic Analyzer (Life Technologies).

cDNAs of the α1 (Accession no. NM_000806.5), β2 (Accession no. NM_021911.2) and γ2 (Accession no. NM_000816.3) human GABACl subunits were synthesized by Life Technologies, whereas those of α1 (Accession no. BC074980.2) and β (Accession no. BC032635.1) human GlyCl subunits were purchased from Thermo Fisher Scientific (Waltham, MA, USA). These cDNAs were cloned into the pcDNA3.1 (+) vector in a similar way to the cDNAs of *Bombyx* GABACl and GluCl.

***Xenopus laevis* oocytes preparation and functional expression of ligand-gated chloride channels.** The oocytes were dissected from female frogs that were anesthetized with tricaine according to the U. K. Animals (Scientific Procedures) Act, 1986. The oocytes were treated with collagenase (Type IA, Sigma Aldrich Japan) in a Ca-free standard oocyte saline (Ca^2-free SOS) consisting of 100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$ and 5 mM HEPES (pH 7.6). After collagenase treatment, the follicle layers were removed manually using forceps in SOS consisting of 100 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM HEPES 5.0 (pH 7.6).

cRNA for the chloride channel subunits was prepared by *in vitro* transcription with mMESSAGE mMACHINE T7 kit (Life Technologies) and dissolved in nuclease-free water at a concentration of 1 μg μL^{-1}. The oocytes were cytoplasmically injected with 50 nL of cRNA solution. The oocytes were then incubated at 18°C in SOS supplemented with penicillin (100 units mL^{-1}), streptomycin (100 μg mL^{-1}), gentamycin

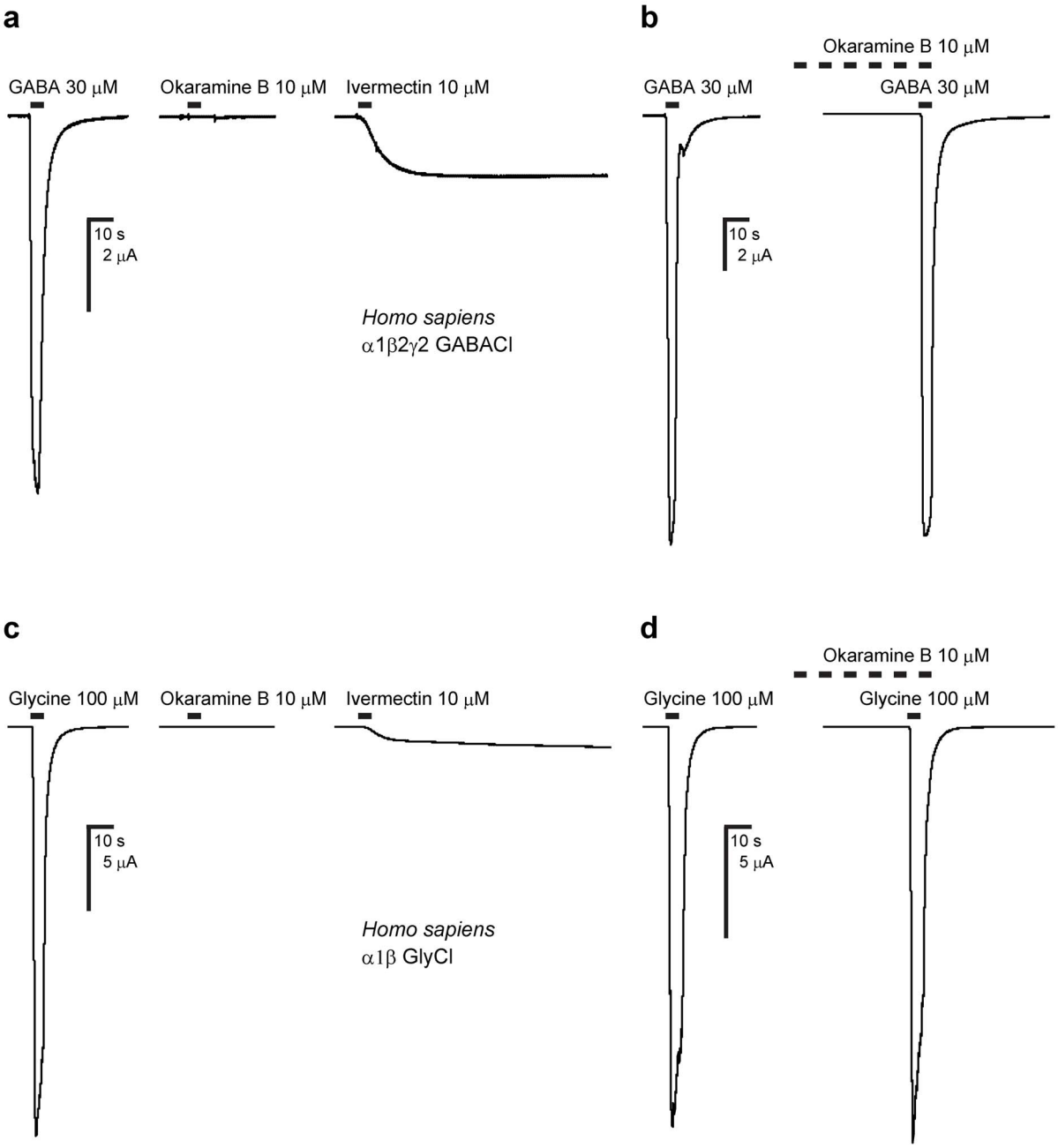

Figure 4 | Actions of okaramine B on human α1β2γ2 GABACl and α1β glycine-gated chloride channel (GlyCl) expressed in *Xenopus laevis* oocytes. (a) Three minutes after recording a response to 30 μM GABA, 10 μM okaramine B was bath-applied to oocytes expressing the human α1β2γ2 GABACl. After 3 min wash, 10 μM ivermectin was bath-applied. (b) Okaramine B (10 μM) was pre-applied for 1 min prior to co-application with 30 μM GABA to the α1β2γ2 GABACl. (c) Three minutes after recording a response to 100 μM glycine, 10 μM okaramine B was bath-applied to oocytes expressing the human α1β GlyCl. After 3 min wash, 10 μM ivermectin was bath-applied. (d) Okaramine B (10 μM) was pre-applied for 1 min prior to co-application with 100 μM glycine to oocytes expressing the α1β GlyCl. All the results were reproducible (n = 4).

(20 μg mL^{-1}) and sodium pyruvate (2.5 mM). Electrophysiology was conducted one day after cRNA injection.

Two-electrode voltage-clamp electrophysiology. *Xenopus* oocytes injected with cRNA encoding the ligand-gated chloride channels were secured in a recording chamber and superfused with SOS at 20–23°C at a flow rate of 7–10 mL min^{-1} as described previously[25,26]. The currents were recorded with a GeneClamp 500B amplifier and Clampex 8 software (Molecular Devices) at a holding potential of −80 mV and were digitized at sampling frequency of 1 kHz using a Digidata 1200 interface (Molecular Devises) and stored in a PC for subsequent analyses. The digitized data was analyzed with Clampfit 9 software.

Concentration-response data analyses. The peak amplitude of the okaramines response of the silkworm larval neurons and *Xenopus* oocytes expressing GluCl was normalized by dividing each response by the maximum current amplitude of the response to 100 μM L-glutamate recorded 5 min prior to okaramine application. The normalized data were fitted by nonlinear regression analysis using Prism 4 software (GraphPad Software, La Jolla, CA, USA) to estimate the EC_{50} (Concentration (M) giving half the maximum response) and I_{max} (Normalized maximum response) as described previously[25,26].

Insecticidal activity. Larvae of *B. mori* were purchased from Ehime-Sanshu Co. (Nishi-Uwa Gun, Ehime, Japan). Okaramines were dissolved in DMSO and 5 μl of the sample solution was injected into the 5th instar larvae (Body weight ca 1 g) of *B. mori*. Twenty-four hours after injection, the number of dead larvae was counted to evaluate their insecticidal activity. Injection of 5 μl DMSO had no toxicity. Ten larvae were used for each dose and the dose-toxicity relationship was analyzed to determine LD_{50} (mol/larvae) by non-regression analysis using Prism 4 software as described for the neural activity. Experiments were repeated (n = 3).

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Author contributions

S.F. mainly contributed to elucidate the target of okaramines. Y.N. and Y.M. investigated the actions of okaramines on the silkworm larval neuron and the GluCl expressed in *Xenopus* oocytes. K.K. and H.H. isolated the okaramines from the fungal products and measured their insecticidal activity. M.I. assisted in gene cloning and electrophysiology. K.M. designed the experiments. S.F., K.K., H.H. and K.M. wrote the manuscript.

Additional information

Accessions numbers of cDNA sequences: RDL GABACl subunit, AB847423; GluCl, KC342244; human α1 GABACl subunit, NM_000806.5, human β2 GABACl subunit, NM_021911.2; human γ2 GABACl subunit, NM_000816.3; human α1 GlyCl subunit, BC074980.2; human β GlyCl subunit, BC032635.1.

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