Identification of a juvenile-hormone signaling inhibitor via high-throughput screening of a chemical library

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Insecticide resistance has recently become a serious problem in the agricultural field. Development of insecticides with new mechanisms of action is essential to overcome this limitation. Juvenile hormone (JH) is an insect-specific hormone that plays key roles in maintaining the larval stage of insects. Hence, JH signaling pathway is considered a suitable target in the development of novel insecticides; however, only a few JH signaling inhibitors (JHSIs) have been reported, and no practical JHSIs have been developed. Here, we established a high-throughput screening (HTS) system for exploration of novel JHSIs using a Bombyx mori cell line (BmN_JF&AR cells) and carried out a large-scale screening in this cell line using a chemical library. The four-step HTS yielded 69 compounds as candidate JHSIs. Topical application of JHSI48 to B. mori larvae caused precocious metamorphosis. In ex vivo culture of the epidermis, JHSI48 suppressed the expression of the Krüppel homolog 1 gene, which is directly activated by JH-liganded receptor. Moreover, JHSI48 caused a parallel rightward shift in the JH response curve, suggesting that JHSI48 possesses a competitive antagonist-like activity. Thus, large-scale HTS using chemical libraries may have applications in development of future insecticides targeting the JH signaling pathway.

In current pest control, integrated pest management (IPM) is a promising eco-friendly approach that combines different control methods, rather than using only chemical insecticides, to suppress pest populations to lower levels than would cause economic damage. However, chemical insecticides still play a pivotal role in modern agriculture pest control and are indispensable, even in the IPM system. Recently, resistance to existing insecticides has become a major concern, and the number of usable insecticides has decreased. Thus, the development of insecticides with novel mechanisms of action is essential for the maintenance of stable agricultural production.

Insect growth regulators (IGRs), which inhibit insect-specific development, are a class of environmentally friendly insecticides compatible with the concept of IPM. Juvenile hormone (JH) is an insect-specific hormone that suppresses precocious metamorphosis during the larval stage, and which has different chemical structures in different insects. Hence, JH signaling pathway may be a suitable target for developing novel IGRs with selectivity to target pests and safety to the ambient environment. Indeed, JH signaling activators (JHSAs), such as JH agonists (pyriproxyfen and fenoxycarb), which suppress larval-pupal and nymph-adult metamorphosis in holometabolous and hemimetabolous insects, respectively, are often used against agricultural and sanitary insect pests. JHSAs are efficacious in controlling small and short-lived insects, such as thrips and aphids, by impairing their metamorphosis and reducing the number of next generations. However, the application of JHSAs to the larvae of relatively large and long-lived insects, such as moths and beetles, may elongate the larval stage, thereby causing the damage to spread. In contrast, JH signaling inhibitors (JHSIs) induce precocious metamorphosis and are expected to reduce feeding-related damage to agricultural products and subsequent generations. Accordingly, JHSIs are theoretically superior to JHSAs as they have applications in the control of both long- and short-lived insect pests; however, only a few JHSIs and some inhibitors of JH biosynthesis have been reported, while none have been practically developed to date.

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The molecular mechanisms of the JH signaling pathway in target cells have been elucidated in recent studies. Briefly, JH that has entered target cells is received by a JH receptor, methoprene-tolerant protein (Met). Met proteins are a family of basic helix-loop-helix Per-ARNT-Sim (bHLH-PAS) transcription factors. Then, JH-ligated Met forms a heterodimer with steroid receptor coactivator protein (SRC), which also belongs to the family of bHLH-PAS transcription factors. The JH/Met/SRC complex activates the Krüppel homolog 1 gene (Kr-h1), a C2H2 zinc-finger type transcription factor, via the JH response element upstream of Kr-h1 (JHRE). Then, Kr-h1 blocks the expression of the pupal and adult specifier genes, broad-complex (BR-C) and ecdysone-induced protein 93F (E93), thereby preventing larva from precocious pupal and adult development. Based on this information, we can develop a high-throughput screening (HTS) system to conveniently evaluate the activation and inhibition activities of chemical compounds and subsequently explore novel seed compounds for JHSAs and JHSIs.

The silkworm (Bombyx mori) is a model lepidopteran insect with many available research tools, including genome information, transgenic methods, and cell lines. In this study, we aimed to develop a practical JHSI by establishing a JH screening system for HTS using a B. mori cell line, in which the JH signaling pathway had been characterized. Then, we carried out large-scale screening using this HTS system and succeeded in finding a novel JHSI.

Results and discussions

Establishment of a JH screening system. A previous study reported the identification of inhibitors of Met/SCR complex formation from plant compounds using a yeast two-hybrid system. Treatment of mosquitoes with these inhibitors caused defects in ovary development, whereas no effects were observed in larval development. Here, we propose a screening system using an insect cell line to explore novel JHSAs and JHSIs. Because the Kr-h1 transcript has been reported to be induced by JH in most insect cell lines, the intrinsic factors involved in JH signaling, such as Met and SRC, are thought to be sufficiently expressed in these cell lines. In the presence of targeting insect cells, JHSA and JHSI activities were evaluated by introduction of a JH response element (JHRE)-reporter into the cells (the JHRE screening system). In this study, we established a JHRE screening system using a B. mori cell line as model lepidopteran insect.

First, we constructed a reporter vector for stable cell lines in the JHRE screening system (Fig. 1A). To reduce the background of the luc2 reporter in the JHRE reporter plasmid, as described previously (pGL4.14–2165 to –2305 and –49 to +116), the luc2 reporter gene was swapped for a luc2P reporter gene containing the first 2165 bp of Met/SCR complex formation from plant compounds using a yeast two-hybrid system. Moreover, this construct contained a hRlucP reference gene, which was continuously driven by the BmA3 promoter, as a reference reporter to evaluate the cytotoxicity of compounds (Fig. 1A).

This plasmid was transfected into B. mori cells (BmN cells), and the cells were selected by hygromycin for establishment of the stable line (BmN_JHRE-Fluc and A3-Rluc, BmN_JF&AR). Dose-dependent increases in Fluc luminescence by a test compound indicates that the compound possesses JHSA activity. Meanwhile, in the JHSI assay (right), if Fluc luminescence was reduced when the cells were simultaneously treated with JH and a test compound, the compound possesses JHSI activity. False-positive results were caused by cytotoxicity of the compound and could be excluded by measurement of Rluc luminescence. In this study, we focused on exploration of JHSIs using BmN_JF&AR cells.

HTS of JHSIs. To identify JHSIs from a chemical library, we performed HTS using a four-step hit validation assay in BmN_JF&AR cells (Fig. 1D). We used 1 nM JH I in JHSI screening based on the dose–response to JH I in BmN_JF&AR cells (Fig. 1B). The plate layout used for each screening is shown in Supplementary Fig. S1. JHSI activity was calculated by the inhibition rate (InH (%)), which was evaluated according to whether a test compound inhibits the reporter activity of 1 nM JH I. Therefore, positive and negative controls were set as dithiothreitol (DTT) and DMSO alone, respectively. Positive and negative controls yielded consistent results in all screenings (Fig. 2B). The performance was qualitatively assessed by Z’ factor analysis as an indication of the positive and negative controls. The average Z’ factor values of the first to fourth screenings were 0.81 ± 0.03, 0.83 ± 0.06, 0.86 ± 0.02, and 0.90 ± 0.02 (Supplementary Table S1), respectively, indicating that our screening was a highly qualitative and reproducible assay.

First, we carried out random screening with a core library consisting of structurally diverse compounds (9600 compounds, n = 1), provided by the Drug Discovery Initiative (DDI, The University of Tokyo) (Figs. 1D, 2A). The screening yielded 120 hit compounds that inhibited 1 nM JH I activity by an inhibition rate (InH) of at least 26% at a compound concentration of 5 μM (Fig. 2A). The activities of selected compounds were re-evaluated in confirmation tests during the second screening (n = 4) (Fig. 2B). Overall, the results of the first screening were highly reproducible. We excluded compounds that inhibited JH I activity at less than InH 20% or weakened cell adherence to the plate, resulting in 75 hit compounds (Fig. 2B).

Next, dose–response assays (n = 4) narrowed the hit compounds of the second screening (Fig. 2C). For example, as shown in the inset in Fig. 2C, inhibition of 1 nM JH I activity was decreased by treatment with the compounds in a concentration-dependent manner. This screening yielded 47 hit compounds with an InH of greater than 10% at 1 nM. For the fourth screening (n = 4), analogs of 47 hit compounds in the third screening were extracted from another ~210,000 compounds in the DDI chemical library, yielding 279 compounds. This identified approximately six analogs per hit compound in the third screening. These analogs were evaluated at...
three concentrations (0.05, 0.5, and 5 μM) and were selected as having InHs of greater than 15% at 0.5 μM and −10% at 0.05 μM (Fig. 2D). Ultimately, 69 compounds were identified as candidate JHSIs that inhibited the reporter activities of 1 nM JHI in BmN_JF&AR cells (Fig. 2D).

Characteristics of the screened compounds. To characterize the compounds used in the fourth screening, we generated heat maps (Fig. 3A). Compounds of the fourth screening were assigned ADT numbers (ADT: analog and dose–response test), the detail for which are presented in the Fig. 3A legend. In the fourth screening, many additional analogs were observed with higher JHSI activity compared to those of the original compounds identified in the third screening, were obtained by the fourth screening, including the ADT8, ADT9, and ADT13 groups (Fig. 3A). In approximately half of the ADT groups, including ADT2, ADT7, and ADT10, the activities of the analogs were converted into JHSA activities by exchanging with slightly different functional groups (Fig. 3A). For example, ADT16-3 and ADT16-5 exhibited JHSA activities, whereas ADT16-1, ADT16-2, and ADT16-4 had JHSI activities (Fig. 3B). Interestingly, ADT5 compounds had a 4-phenoxyphenoxymethyl skeleton, similar to that of the practical JH analogs pyriproxyfen and fenoxycarb⁷, and exhibited JHSI activities (Fig. 3C).

JHSI activity in vitro, vivo, and ex vivo. Only JHSIs selected by the fourth screening were extracted from Fig. 3A and are summarized in Fig. 4A. Their chemical structures are shown in Supplementary Table S2. With regard to the cytotoxicity of the JHSIs, the highest InH (%) of Rluc luminescence was 42% for 5 μM JHSI27, while the others were all less than 20% (Supplementary Fig. S2), suggesting that no false-positive results were obtained based on our criterion (> InH 50%).

All JHSIs were topically applied to third instar larvae on day 0 of B. mori, and precocious metamorphosis was observed as an index of JH activity in vivo (n = 10). Only JHSI48 \(N\)-[2-(4-tert-butyl-2-chlorophenoxy)
 Establishment of a stable cell line. The BmN cell line (KATAKURA) derived from the ovaries of *B. mori* was maintained at 25 °C in IPL-41 medium (Gibco, Invitrogen) containing 10% fetal bovine serum (HyClone). BmN cells were seeded at a density of 1.5 x 10^5 cells/well in 96-well plates 1 day before transfection. The cells were transfected with pGL4.14_JHREP-luc2P and BmA3P-hRlucP using FugeneHD (Promega). The cells were incubated for 6 h after transfection and selected with IPL-41 medium containing 100 μg/mL hygromycin (InvivoGen) for 3 months, and the stable cell line (BmN_JF&AR) was then established.

Methods

Construction of plasmids. To reduce the background of the luc2 reporter and evaluate the cytotoxicity of compounds, the reporter plasmids pGL4.14_− 2165 to − 2025 and − 49 to + 116 were reconstructed as follows. To reduce the luc2 background, a luc2P reporter gene containing PEST sequence (Promega) was swapped for the luc2 in pGL4.14_− 2165 to − 2025 and − 49 to + 116 and a pGL4.27 plasmid (Promega), respectively, using KpnI and II sites was amplified from the plasmid pBacA3GAL4/3 × P3DsRed27 using BmA3P_F and BmA3P_R primers (Supplementary Table S2). The amplified fragment was digested with KpnI and BglII, and inserted into the pGL4.80 plasmid (Promega). The digested luc2P and pGL4.14_− 2165 to − 2025 and − 49 to + 116 were ligated using a Ligation high (Toyobo) (pGL4.14_JHREP-luc2P). As a reference reporter to evaluate cytotoxicity, the BmA3 promoter region anchored with KpnI and BglII sites was amplified from the plasmid pBacA3GAL4/3 × P3DsRed27 using BmA3P_F and BmA3P_R primers (Supplementary Table S2). The amplified fragment was digested with KpnI and BglII, and inserted into the pGL4.80 plasmid (Toyobo) (pGL4.80_BmA3P-hRlucP). The BmA3P-hRlucP region in pGL4.80_BmA3P-hRlucP was amplified using BmA3P-hRlucP_F and BmA3P-hRlucP_R primers (Supplementary Table S2) and inserted into the SalI site of pGL4.14_JHREP-luc2P (pGL4.14_JHREP-luc2P and BmA3P-hRlucP).

Construction of plasmids. To reduce the background of the luc2 reporter and evaluate the cytotoxicity of compounds, the reporter plasmids pGL4.14_− 2165 to − 2025 and − 49 to + 116 were reconstructed as follows. To reduce the luc2 background, a luc2P reporter gene containing PEST sequence (Promega) was swapped for the luc2 in pGL4.14_− 2165 to − 2025 and − 49 to + 116 and a pGL4.27 plasmid (Promega), respectively, using KpnI and II sites was amplified from the plasmid pBacA3GAL4/3 × P3DsRed27 using BmA3P_F and BmA3P_R primers (Supplementary Table S2). The amplified fragment was digested with KpnI and BglII, and inserted into the pGL4.80 plasmid (Toyobo) (pGL4.80_BmA3P-hRlucP). The BmA3P-hRlucP region in pGL4.80_BmA3P-hRlucP was amplified using BmA3P-hRlucP_F and BmA3P-hRlucP_R primers (Supplementary Table S2) and inserted into the SalI site of pGL4.14_JHREP-luc2P (pGL4.14_JHREP-luc2P and BmA3P-hRlucP).

Conclusion

We established a screening system for exploration of JHSIs and found JHSI48 as a novel JHSI, which were utilized as a seed of future insecticides targeting the JH signaling pathway. Future synthetic development to improve activity could yield a practical JHSI with applications in pest management. For example, treatment of young larvae with this compound induces precocious metamorphosis and is expected to reduce feeding-related damage to agricultural products and subsequent generations. Additionally, since JH has different chemical structures in different insects, this approach may afford species specificity depending on the specific synthetic development.

Chemicals. JH I was purchased from SciTech. All compounds used for the HTS were supplied by the DDI of The University of Tokyo; approximately 220,000 compounds were included in the chemical library (https://
 HTS using BmN_JF&AR cells. We used a core library from the DDI containing 9600 diverse compounds for a random screening (first screening). Analogs of compounds selected by the third screening were extracted from another ~ 210,000 compounds in the DDI chemical library and were used in the fourth screening. Compounds in DMSO were dispensed into 384- or 96-well plates using the POD Automation Platform (Labcyte). The layouts of the plates are shown in Supplementary Fig. S1. In the first, third, and fourth screenings, 10 μL...
medium containing BmN_JF&AR cells (final density: $5 \times 10^4$ cells/well) and 10 μL of 2 nM JH I medium (final concentration: 1 nM JH I) were added to 384-well plates using a Multidrop Combi (Thermo) and incubated at 25 °C for 20 h. The treated cells were analyzed using a Dual-Luciferase Reporter 1000 Assay System (Promega) and a microplate reader (PHERAstar Plus; BMG Labtech) according to the manufacturer’s instructions. In the second screening, 100 μL medium containing BmN_JF&AR cells (final density: $1.5 \times 10^5$ cells/well) and 100 μL of 2 nM JH I medium (final concentration: 1 nM JH I) were added to 96-well plates, and the reporter activities were measured as described previously.

The inhibition rate (InH %) was calculated using the following equation: InH (%) = 100 \times (1 – [sample – meanp]/[meann – meanp]), where meanp is the mean of Fluc or Rluc luminescence in the positive control (only DMSO), and meann is the mean of the negative control (JH I in DMSO). To evaluate the accuracy of the screenings, the Z' factor values for each plate were calculated using the following equation: $Z' = 1 – (3SD_p + 3SD_n)/(mean_n – mean_p)$, where SDp is the standard deviation of the positive control, and SDn is the standard deviation of the negative control. False-positive activity caused by the cytotoxicity of the compound was monitored by determining the InH (%) of Rluc luminescence, and compounds with greater than InH 50% were excluded from the hit compound list.

**Figure 4.** JHSI48 inhibited the JH signaling pathway in vivo. (A) Heat map of JHSIs extracted from the fourth screening. The JHSIs were renamed JHSI, followed by a number. The arrowhead indicates JHSI48 [N-[2-[(tert-butyl-2-chlorophenoxy)ethyl]-1H-1,2,4-triazole]. (B) Chemical structure of JHSI48. (C) Dose–response of JHSI48 in BmN_JF&AR cells. BmN_JF&AR cells were treated with 1 nM JH I and different concentrations of JHSI48, and the inhibition rate (InH %) was determined 20 h after treatment. Data represent means ± SD (n = 3). (D) Effects of JHSI48 treatment on the phenotype of B. mori. Third instar larvae were treated with 0.5 μL DMSO (control) or JHAT48 (50 mM in DMSO) topically on day 0, and the phenotypes were observed. (E) Phenotypes of cocoons and pupae treated with JHSI48. Third instar larvae were treated with 0.5 μL DMSO (control) or JHSI48 (50 mM in DMSO) topically on day 0, and the phenotypes were observed. (F) Dose–response curve of JHSI48 ex vivo. Integuments on day 5 of the fifth instar larvae were cultured in the presence of 10 nM JH I and different concentrations of JHSI48, and BmKr-h1 expression was monitored by qPCR. Data represent means ± SD (n = 10). (G) Pharmacological analysis of JHSI48. BmN_JF&AR cells were treated with different concentrations of JH I (0, $10^{-10}$, $10^{-9}$, $10^{-8}$, $10^{-7}$, $10^{-6}$ M) and JHSI48 (0, 0.5, 5 μM), after which, reporter activity was examined. Grey, 0 μM; light red, 0.5 μM; Red, 5 μM JHSI48.
Experimental animals and bioassays. *B. mori* (Kinsyu × Showa strain) was reared on an artificial diet (Nosan Corp) at 25 °C under a 12-h light/dark cycle. In vivo assays, 0.5 μL hit compounds in DMSO (10 mM), which were selected by the fourth screening, were topically applied to the dorsal epidermis on day 0 of the third instar larvae, and precocious metamorphosis was observed as an index of JH antagonist activity.

Dissection and tissue culture. Dorsal abdominal integuments on day 5 of the fifth instar larvae were dissected in phosphate-buffered saline (137 mM NaCl, 8 mM NaH2PO4, 2.7 mM KCl, and 1.5 mM KH2PO4, pH 7.4) and then cultured in Grace’s insect medium (Gibco, Invitrogen) at 25 °C, as previously described. The integuments were incubated in medium containing 10 nM JH I and different concentrations of JHSIs for 4 h.

Identification of competitive and non-competitive antagonist. To verify whether JHSIs are competitive or non-competitive antagonists, pharmacological assays via dose–response testing in the presence of a maximal effect caused by non-competitive antagonist, the luminescence of only 10–6 M JH I was compared to 0, 10–10, 10–9, 10–8, 10–7, and 10–6 M, and JHSIs were tested at 0, 0.5, and 5 μM. To monitor the reduction in the maximal effect caused by non-competitive antagonist, the luminescence of only 10–6 M JH I was compared to that of 0.5 μM of JHSIs using Student’s t tests. p < 0.001 was regarded as non-competitive antagonist.

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Authors’ contributions
T.K., T.S., K.Y., and T.O. designed the study; T.K., K.F., K.N., and K.Y. performed the experiments; T.K., K.F., K.N., and K.Y. analyzed the data; and T.K., K.F., and T.S. wrote the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
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