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Identification of novel juvenile-hormone signaling activators *via* high-throughput screening with a chemical library

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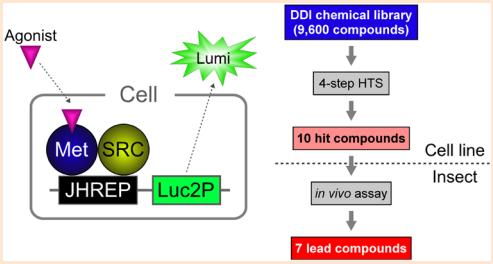
Supplementary material

Juvenile hormone (JH) is an insect-specific hormone that regulates molting and metamorphosis. Hence, JH signaling inhibitors (JHSIs) and activators (JHSAs) can be used as effective insect growth regulators (IGRs) for pest management. In our previous study, we established a high-throughput screening (HTS) system for exploration of novel JHSIs and JHSAs using a *Bombyx mori* cell line (BmN_JF&AR cells) and succeeded in identifying novel JHSIs from a chemical library. Here, we searched for novel JHSAs using this system. The four-step HTS yielded 10 compounds as candidate JHSAs; some of these compounds showed novel basic structures, whereas the others were composed of a 4-phenoxyphenoxymethyl skeleton, the basic structure of several existing JH analogs (pyriproxyfen and fenoxycarb). Topical application of seven compounds to *B. mori* larvae significantly prolonged the larval period, suggesting that the identified JHSAs may be promising IGRs targeting the JH signaling pathway.



Keywords: juvenile hormone, juvenile hormone signaling activator, high-throughput screening, insect growth regulator, pest management.

Introduction

Juvenile hormone (JH), which contains a sesquiterpenoid carbon skeleton, is an insect-specific hormone that suppresses precocious metamorphosis in the larva and nymph of holometabolous and hemimetabolous insects, respectively.[1)] Hence, JH signaling inhibitors (JHSIs) and activators (JHSAs) have applications as insect growth regulators (IGRs) in the fields of agriculture and sanitation.[2–4)] For example, JHSAs are efficacious in controlling small and short-lived insects by impairing their metamorphosis and reducing proliferation. Indeed, pyriproxyfen, a JH analog, has been often utilized for the management of the sweet potato (cotton) whitefly (*Bemisia tabaci*) in agricultural fields.[5)] Pyriproxyfen also inhibits adult emergence and sterilizes adult female mosquitoes, which are known to carry many viral and parasitic pathogens, thereby preventing the spread of infections, such as malaria.[6)]

Previously, it was considered that it is difficult for JH analogs to induce acquired resistance and that JH analogs are safe for the ambient environment.[7)] However, in laboratory selection experiments, researchers have established strains showing high resistance to JH analogs (pyriproxyfen and methoprene) in the housefly (*Musca domestica*) and fruit fly (*Drosophila melanogaster*).[8,9)] In the agricultural field, resistance to pyriproxyfen in *B. tabaci* has appeared in many countries.[5)] Very recently, resistance to pyriproxyfen reached noticeable levels in field populations of the yellow fever mosquito (*Aedes aegypti*) in California.[10)] Moreover, pyriproxyfen was reported to induce reproductive impairment in male and female zebrafish (*Danio rerio*) at the gene expression and histopathological levels.[11)] Therefore, further studies are needed to identify JHSA compounds with a novel basic structure in order to overcome these limitations.

Based on earlier studies of the JH signaling pathway in target cells of the silkworm (*Bombyx mori*),[12–17)] a model lepidopteran insect, we previously developed a high-throughput screening (HTS) system using a *B. mori* cell line to evaluate JHSI and JHSA activities of test compounds (BmN_JF&AR cells).[18)] Ad-

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ditionally, we identified novel JHSIs from a chemical library.[18] Accordingly, in this study, we aimed to identify novel JHSA compounds from the chemical library using BmN_JF&AR cells and to evaluate the JHSA activity of the best HTS-performing compounds using bioassays on *B. mori* larvae.

Materials and methods

1. Chemicals

JH I was purchased from SciTech (Prague, Czech Republic). All compounds used for the HTS were supplied by the Drug Discovery Institute (DDI) of The University of Tokyo; approximately 220,000 compounds were included in the chemical library (https://www.ddi.u-tokyo.ac.jp/en/). All chemicals used in this study were commercial products, and their structures and purities were confirmed using liquid chromatography-mass spectrometry (LC-MS) and/or nuclear magnetic resonance.

2. HTS using BmN_JF&AR cells

BmN_JF&AR cells were maintained at 25°C in a culture medium [IPL-41 medium (Thermo Fisher Scientific, Waltham, MA) containing 10% fetal bovine serum (HyClone, South Logan, UT, USA) and 100 μg/mL hygromycin (InvivoGen, San Diego, CA, USA)]. We used a core library from the DDI containing 9,600 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 dimethyl sulfoxide (DMSO) were dispensed into 384- or 96-well plates using the POD Automation Platform (Labcyte, San Jose, CA, USA). The final concentrations of the compounds were 5 μM in the first and second screenings, 5, 1, 0.5, 0.1, and 0.05 μM in third screening, and 5, 0.5, and 0.05 μM in fourth screening; the concentration of DMSO was adjusted to 0.5% in all screenings. The layouts of the plates are shown in Supplemental Fig. S1. In the first screenings, 10 μL culture medium containing BmN_JF&AR cells (final density: 5×10^4 cells/well) and 10 μL culture medium were added to 384-well plates using a Multidrop Combi (Thermo Fisher Scientific) and incubated at 25°C for 20 hr. The treated cells were analyzed using a Dual-Luciferase Reporter 1000 Assay System (Promega, Madison, WI, USA) and a microplate reader (PHERAstar Plus; BMG Labtech, Ortenberg, Germany) according to the manufacturer's instructions. As the number of test compounds was small, the second screening was performed by hand using the following method:

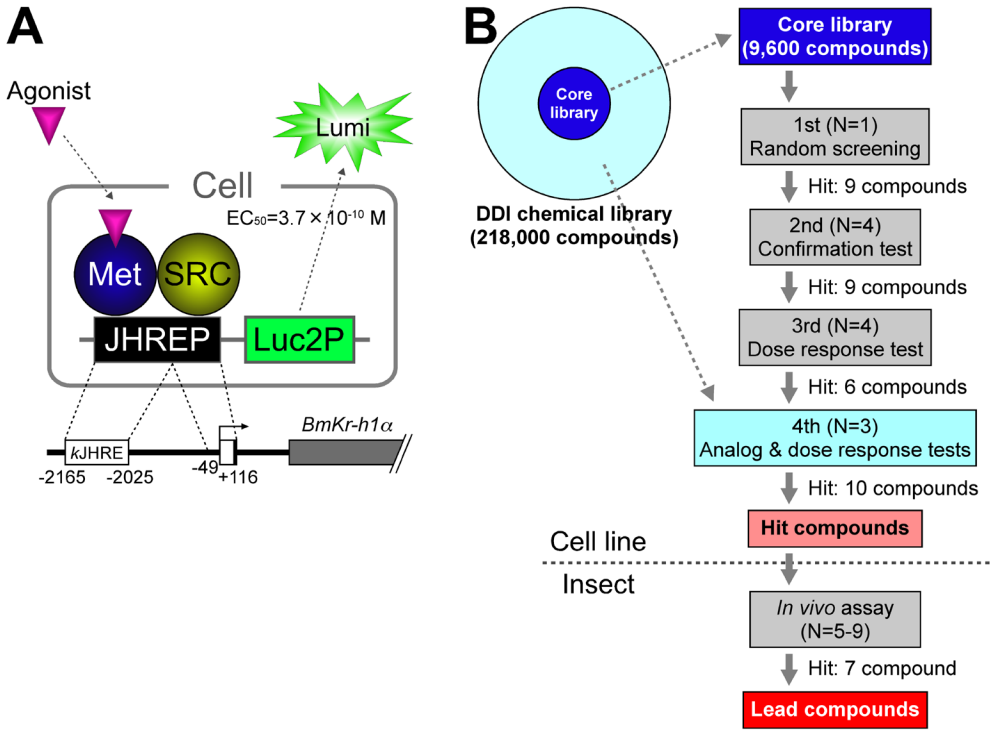

Fig. 1. JHSA screening system and scheme of high-throughput screening (HTS). (A) Schematic JHSA assay using BmN_JF&AR cells. Increased reporter activity observed by adding a test compound indicated that the compound had JHSA activity.[18] Met, JH receptor (methoprene tolerant); SRC, steroid receptor coactivator; JHREP, JH response element (*k*JHRE) and the basal promoter region of α isoform of *B. mori Krüppel homolog 1* (*BmKr-h1α*); Luc2P, luciferase reporter gene containing the first degradation sequence (PEST); Lumi, luminescence. The median effective concentration (EC_{50}) of JH I was 3.7×10^{-10} M.[18] (B) Flow chart of HTS to identify JHSAs from the DDI chemical library using BmN_JF&AR cells. The DDI chemical library possessed 218,000 compounds in total, and the core library (9600 compounds) was composed of structurally diverse compounds for random screening. The core library was used from the first through third screenings, and analogs of the fourth screening were selected from another approximately 210,000 compounds based on the chemical structure of compounds screened in the third screening. The hit compounds selected by the four-step HTS using BmN_JF&AR cells were evaluated by topical application in *B. mori* larvae using in *vivo* assays. Adapted from Kayukawa *et al.*[18]

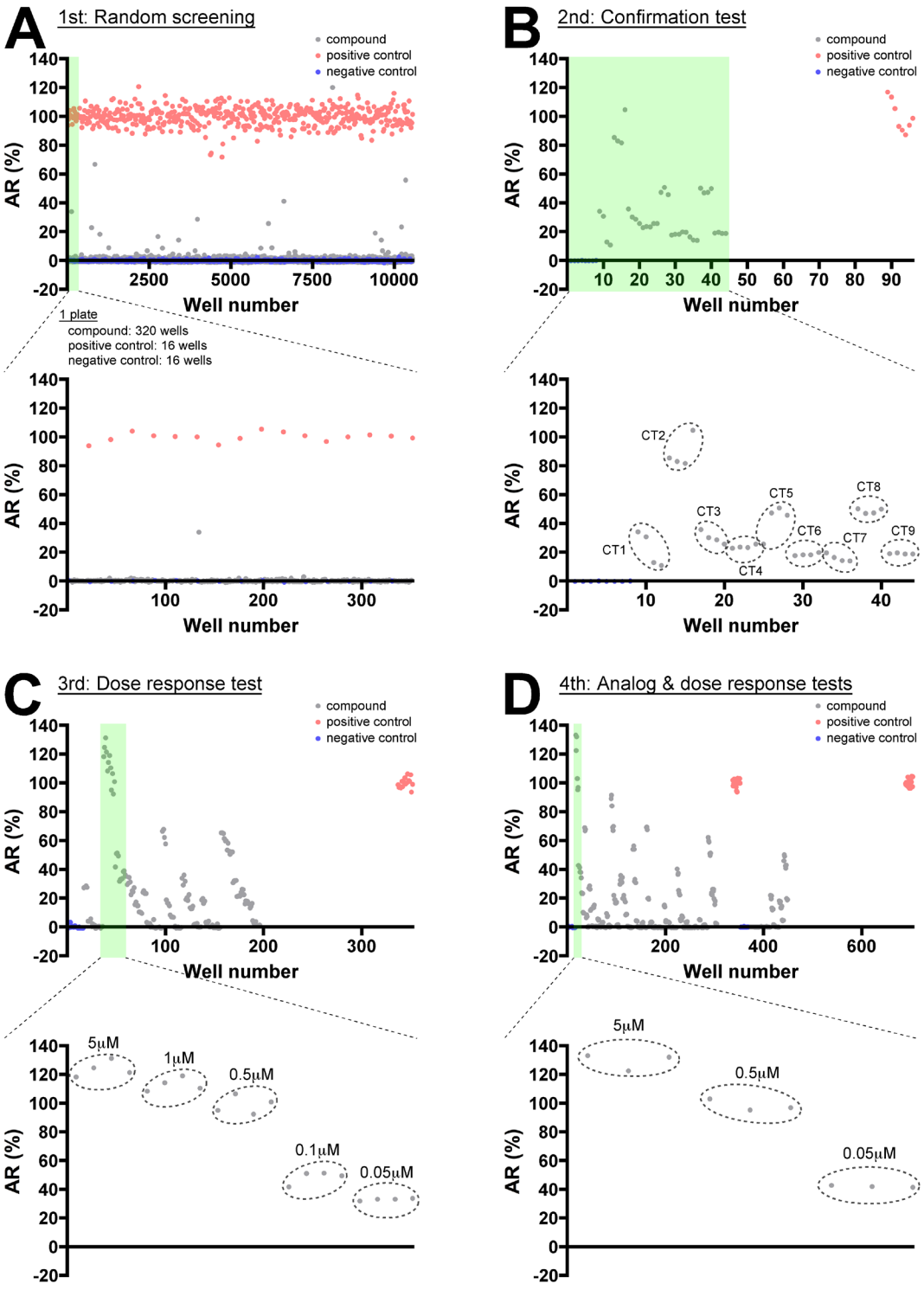

Fig. 2. Scatter plots of HTS. JHSA activities were examined using the Luciferase reporter assay system in BmN_JF&AR cells. The JHSA activities of compounds in BmN_JF&AR cells were determined using the activation rate (AR [%]), where AR 0% and 100% indicated no activity and maximum JHSA activity, respectively. Gray dots are wells containing a test compound. The red and blue dots indicate positive (JH I, 10 nM) and negative (only DMSO) controls, respectively. Plate layouts are described in Supplemental Fig. S1. (A) The core library (9600 compounds, 5 μM) was screened in the first screening (n=1). The green shadow indicates a plate consisting of 320 compounds, with 16 positive and 16 negative control wells, and the lower plot shows an enlarged image of a plate as an example. (B) The activities of the compounds obtained by the first screening were confirmed in the second screening (confirmation test, n=4). CT, compounds in the confirmation test. (C) The dose–dependent responses of compounds selected by the second screening were examined in the third screening (n=4). The green shadow focuses on a compound, and the dose-dependent response is indicated below. (D) Analogs of the selected compounds in the third screening were evaluated in the fourth screening (analog and dose-response tests, n=3).

100 μL culture medium containing BmN_JF&AR cells (final density: 1.5×10^5 cells/well) and 100 μL culture medium were added to 96-well plates, and the reporter activities were measured as described previously.[12] The reason for scaling up the assay volume in this screening was to ensure accuracy in manual experiment. The third and fourth screenings were carried out using the same method as the first screening.

The JH activation rate (AR [%]) was calculated using the following equation: AR (%)$=100\times([\text{sampleFL}-\text{mean}_\text{n}\text{FL}]/[\text{mean}_\text{p}\text{FL}-\text{mean}_\text{n}\text{FL}])$, where mean_p is the mean of Fluc luminescence (FL) in the positive control (10 nM JH I), and mean_n is the mean of the negative control (only DMSO).[19] To evaluate the accuracy of the screenings, the Z′ factor values for each plate were calculated using the following equation: $Z=1-(3\text{SD}_\text{n}\ \text{FL}+3\text{SD}_\text{p}\ \text{FL})/(\text{mean}_\text{p}\text{FL}-\text{mean}_\text{n}\ \text{FL})$, where SD_n is the standard deviation of the negative control, and SD_p is the standard deviation of the positive control.[20]

3. Experimental animals and bioassays

B. mori (Kinsyu×Showa strain) was reared on an artificial diet (Nosan Corp, Yokohama, Japan) at 25°C under a 12-hr light/dark cycle. For *in vivo* assays, 2 μL of a hit compound (10 mM, in DMSO) selected during the fourth screening was topically applied to the dorsal epidermis on day 0 fifth (final) instar larvae. The larval period until spinning was then measured and used as an index of JHSA activity *in vivo*.

Results and discussion

1. HTS of JHSAs

To identify JHSAs from a chemical library, we utilized BmN_JF&AR cells, which had been established previously.[18] If Fluc luminescence was enhanced when the cells were treated with a test compound, we concluded that the compound possessed JHSA activity (Fig. 1A). Using these cells, we performed HTS using a four-step hit validation assay (Fig. 1B). According to the dose-response to JH I in BmN_JF&AR cells,[18] we used 10 nM JH I in DMSO and DMSO alone as positive and negative controls, respectively. The plate layout used for each screening is shown in Supplemental Fig. S1.

The positive and negative controls showed stable results in all screening assays (Fig. 2), and the performance was qualitatively assessed by Z′ factor analysis between the positive and negative controls. An HTS plate with a Z′ factor of less than 0.5 is generally judged to be inaccurate.[20] The average Z′ factor values of the first to fourth screenings were 0.79±0.05, 0.67, 0.87, and 0.91±0.01 (Table 1), respectively, indicating that our screening was a highly quantitative and reproducible assay.

First, we carried out random screening with a core library consisting of structurally diverse compounds (9600 compounds, $n=1$; Figs. 1B and 2A). The screening identified nine hit compounds that activated Fluc luminescence, with an activation rate (AR) of at least 23% at a compound concentration of 5 μM (Fig. 2A). The activities of these hit compounds were re-evaluated in confirmation tests during the second screening ($n=4$; Fig. 2B). Sufficient reproducibility was observed for all compounds, and all compounds were then applied to the next screening step (Fig. 2B).

Next, the nine compounds from the second screening were applied to dose–response assays ($n=4$; Fig. 2C). As shown in Fig. 2C, activation of Fluc luminescence decreased with diminishing concentrations of the test compounds. This screening yielded six hit compounds with an AR of greater than 10% at 1 μM. For the fourth screening ($n=4$), analogs of six hit compounds in the third screening were selected from another ~210,000 compounds in the DDI chemical library, yielding 35 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 extracted as having ARs of greater than 15% at 0.5 μM (Fig. 2D). Eventually, 10 compounds were identified as candidate JHSAs that activated the reporter activities in BmN_JF&AR cells (Fig. 2D). Structures and purities of the 10 compounds were confirmed using LC-MS and/or NMR (Supplemental Fig. S2).

2. Characteristics of the screened compounds

We generated heat maps to characterize the compounds used in the fourth screening (Fig. 3A). These compounds were assigned analog and dose–response test (ADT) numbers, as detailed in Fig. 3A. In the fourth screening, we obtained some additional analogs that showed JHSA activities higher than those of the original compounds identified in the third screening; these analogs included the ADT2, ADT3, and ADT4 groups (Fig. 3A). Of all the identified JHSAs (Fig. 3A), only the JHSAs selected in the fourth screening were extracted, and the characteristics and chemical structures of these compounds are summarized in Fig. 3B.

These hit compounds could be divided into four groups based on their characteristic structures: i) 4-phenoxyphenoxymethyl group (JHSA1–3, blue shading), ii) 4-(morpholin-4-ylsulfonyl)phenoxymethyl group (JHSA4–6, light blue shading), iii) 5-[(tetrahydro-1,4-oxazepin-4(5*H*)-yl)sulfonyl]benzimidazol-2-yl group (JHSA7 and JHSA8, purple shading), and iv) oth-

Table 1. Z′ factor values of HTS using BmN_JF&AR cells

Screening name	Number of plates	Plate type	Z′ factor[a]
First screening (random screening)	30	384 well	0.79±0.05
Second screening (confirmation test)	1	96 well	0.67
Third screening (dose–response test)	1	384 well	0.87
Fourth screening (analog & dose–response test)	2	384 well	0.91±0.01

[a] Data represent means ± S.D.

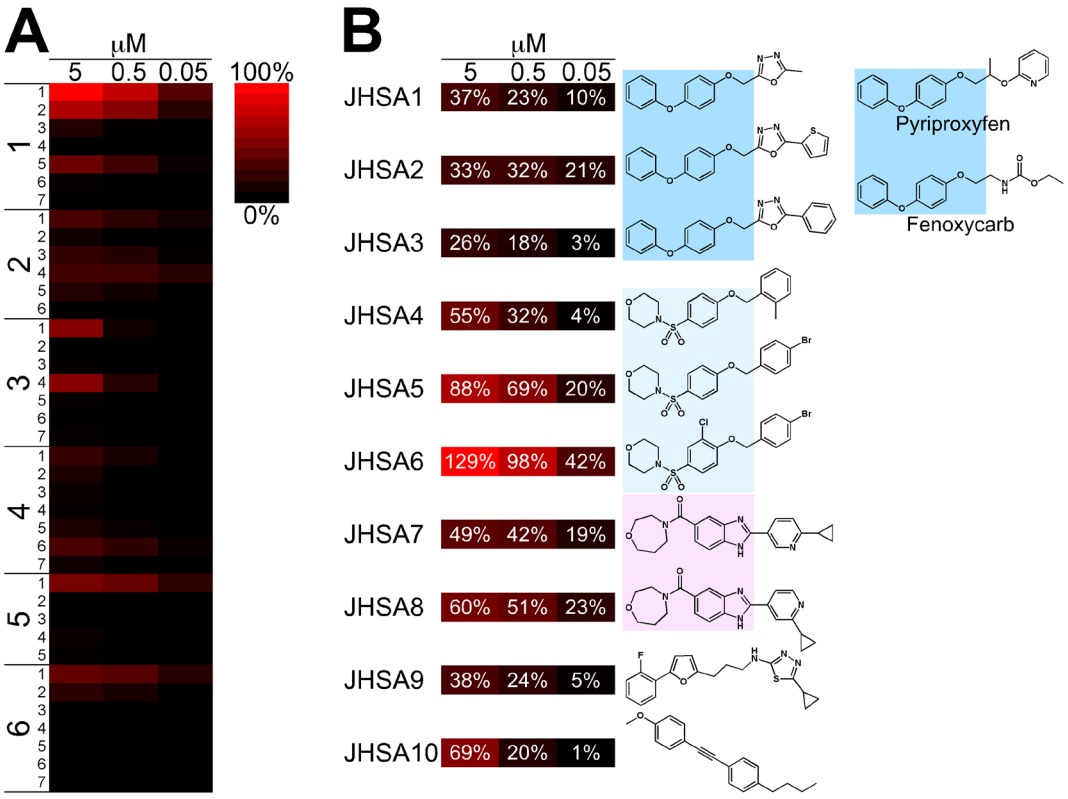

Fig. 3. Hit compounds from HTS using BmN_JF&AR cells. (A) The heat map represents the results of the fourth screening. Compounds in the fourth screening were assigned numbers (analog and dose-response test [ADT] no., ADTX-Y). X numbers (large and left of the heat map column) showed the compound groups, and Y numbers (small and left of the heat map column) show each analog in the compound group; the top line for each group (ADTX-1) shows the compound hits identified during the third screening, and the subsequent lines (ADTX-2, -3, -4, *etc.*) show analogs of ADTX-1. Heat map columns indicate means of AR (%) in fourth screening, and red (100%) and black (0%) areas indicate JHSA activities of 10 nM JH I (positive control) and DMSO alone (negative control), respectively. (B) Summary of JHSA hitting using the 4-step cell-based HTS. Heat maps of the JHSAs, which met the criteria in fourth screening (≥15% at 0.5 μM), were extracted from (A). The JHSAs were renamed JHSA, followed by a number, and the chemical structures are shown. 4-Phenoxyphenoxymethyl (light blue shading) is the basic structure of pyriproxyfen and fenoxycarb, two known JH agonists. Light blue and purple shading indicate 4-(morpholin-4-ylsulfonyl)phenoxymethyl (JHSA4–6) and 5-[(tetrahydro-1,4-oxazepin-4(5*H*)-yl)sulfonyl]benzimidazol-2-yl, respectively.

ers (JHSA9 and JHSA10; Fig. 3B). 4-Phenoxyphenoxymethyl is the basic structure of the practical JH analogs pyriproxyfen and fenoxycarb,[3] and its analogs were obtained by our screening, supporting the high accuracy of our screening. The JHSA activities of the compounds in the 4-(morpholin-4-ylsulfonyl)-phenoxymethyl group were the highest among the four groups in BmN_JF&AR cells, and the chemical skeleton from this group was relatively similar to that of 4-phenoxyphenoxymethyl (Fig. 3B).

Interestingly, JHSA1, -2, -3, -7, -8, and -9 were identified as JHSIs in our previous study;[18] that is, these compounds can either inhibit or activate JH signaling in the presence or absence of JH, respectively. This pharmaceutical activity is generally known to be a characteristic property of a partial agonist. Regarding compounds related to JH, this partial agonist activity has been only reported previously for ethyl 4-[2-(*t*-butylcarbonyloxy)butoxy]benzoate compounds.[21–25]

3. *JHSA activity* in vivo

The 10 JHSAs obtained in our cell line-based screening were topically applied to final instar larvae on day 0 of *B. mori*, and the larval period until spinning was observed as an index of JHSA activity *in vivo*. In final instar larvae of *B. mori*, JH in the hemolymph disappears completely until the spinning stage,[26,27] and topical application of JH analogs inhibits spinning, resulting in extension of the larval stage.[28] Therefore, if a given JHSA is suspected of having JH-like activity *in vivo*, application of that JHSA should inhibit metamorphosis and prolong the larval period. The larval period was significantly prolonged by treatment with JHSA1–4, JHSA6, JHSA9, and JHSA10 compared with treatment by DMSO alone (control; Table 2). In particular, JHSA6 increased the larval period by 2 weeks, and JHSA1, -2, -3, and -10 increased this period by 3 to 10 days (Table 2).

JHSA activities in BmN_JF&AR cells did not completely correlate with those of bioassays using *B. mori* (Fig. 3B and Table 2). For example, all 4-phenoxyphenoxymethyl compounds (JHSA1–3), which exhibited low JHSA activities in the cells, possessed high activities *in vivo*, and no activity was observed for JHSA5 and JHSA8, which showed relatively high activities in cells. In general, insecticide efficiency depends not only on intrinsic activ-

Table 2. JHSA activities of JHSAs compounds in *B. mori* larvae

Compound name	*N*	Days until spinning[a)]
Control (DMSO treatment)	8	6.38±0.52
JHSA1	8	11.63±0.74***
JHSA2	9	15.89±1.69***
JHSA3	7	10.00±0.00***
JHSA4	7	7.43±0.53**
JHSA5	7	7.00±0.82
JHSA6	8	20.38±8.14***
JHSA7	7	6.86±0.69
JHSA8	7	7.14±0.90
JHSA9	7	7.14±0.69*
JHSA10	5	9.20±1.10***

[a)] Data represent means ± S.D. Data were analyzed using Student's *t*-tests (***$P<0.001$; **$P<0.01$; *$P<0.05$; not indicated, $P>0.05$).

ity but also cuticular permeability and detoxification activities; thus, it is essential for the maximum effect to be absolutely applied to the target molecule. Recent studies have demonstrated that insecticide resistance may be caused by reductions in insecticide uptake related to decreased cuticular permeability and enhanced detoxification activities *via* quantitative or qualitative changes in detoxifying enzymes.[29–31] Taken together, our findings suggest that the JHSAs that were selected had JH-like activity in the cells; however, in some cases, this activity was likely blocked *in vivo* due to reduced permeability and/or detoxification. Future synthetic development to improve cuticular permeability and detoxification could yield improved JHSA activity *in vivo*.

In conclusion, we succeeded in identifying several JHSAs with a novel basic structure. These JHSAs may be useful as seed compounds to overcome insecticide resistance and facilitate ambient safety and maintenance of the ambient environment.

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Author contributions

T. K., K. Y., and T. O. designed the study; T. K., K. F., and K. Y. performed the experiments; T. K., K. F., K. Y., and T. O. analyzed the data; and T. K. and K. F. wrote the manuscript.

Competing interests

The authors declare no competing interests.

Electronic supplementary materials

The online version of this article contains supplementary materials (Supplemental Figs. S1 and S2), which are available at http://www.jstage.jst.go.jp/browse/jpestics/.

References

1) L. M. Riddiford: Cellular and molecular actions of Juvenile hormone I. General considerations and premetamorphic actions. *Adv. Insect Physiol.* **24**, 213–274 (1994).
2) G. B. Staal: Insect growth regulators with juvenile hormone activity. *Annu. Rev. Entomol.* **20**, 417–460 (1975).
3) C. Minakuchi and L. M. Riddiford: Insect juvenile hormone action as a potential target of pest management. *J. Pestic. Sci.* **31**, 77–84 (2006).
4) M. Jindra and L. Bittova: The juvenile hormone receptor as a target of juvenoid "insect growth regulators". *Arch. Insect Biochem. Physiol.* **103**, e21615 (2019).
5) A. R. Horowitz, M. Ghanim, E. Roditakis, R. Nauen and I. Ishaaya: Insecticide resistance and its management in *Bemisia tabaci* species. *J. Pestic. Sci.* **93**, 893–910 (2020).
6) S. Y. Ohba, K. Ohashi, E. Pujiyati, Y. Higa, H. Kawada, N. Mito and M. Takagi: The Effect of Pyriproxyfen as a "Population Growth Regulator" against Aedes albopictus under Semi-Field Conditions. *PLoS One* **8**, e67045 (2013).
7) C. M. Williams: Third-generation pesticides. *Sci. Am.* **217**, 13–17 (1967).
8) L. Zhang, K. Harada and T. Shono: Genetic analysis of Pyriproxyfen resistance in the housefly, Musca domestica L. *Appl. Entomol. Zool.* **32**, 217–226 (1997).
9) T. G. Wilson and J. Thurston: Genetic variation for methoprene resistance in *Drosophila melanogaster. J. Insect Physiol.* **34**, 305–308 (1988).
10) T. Su, J. Thieme, T. Lura, M. L. Cheng and M. Q. Brown: Susceptibility profile of Aedes aegypti L. (Diptera: Culicidae) from Montclair, California, to commonly used pesticides, with note on resistance to pyriproxyfen. *J. Med. Entomol.* **56**, 1047–1054 (2019).
11) K. Maharajan, S. Muthulakshmi, C. Karthik, B. Nataraj, K. Nambirajan, D. Hemalatha, S. Jiji, K. Kadirvelu, K. C. Liu and M. Ramesh: Pyriproxyfen induced impairment of reproductive endocrine homeostasis and gonadal histopathology in zebrafish (*Danio rerio*) by altered expression of hypothalamus-pituitary-gonadal (HPG) axis genes. *Sci. Total Environ.* **735**, 139496 (2020).
12) T. Kayukawa, C. Minakuchi, T. Namiki, T. Togawa, M. Yoshiyama, M. Kamimura, K. Mita, S. Imanishi, M. Kiuchi, Y. Ishikawa and T. Shinoda: Transcriptional regulation of juvenile hormone-mediated induction of Krüppel homolog 1, a repressor of insect metamorphosis. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 11729–11734 (2012).
13) T. Kayukawa, K. Tateishi and T. Shinoda: Establishment of a versatile cell line for juvenile hormone signaling analysis in *Tribolium castaneum. Sci. Rep.* **3**, 1570 (2013).
14) T. Kayukawa, M. Murata, I. Kobayashi, D. Muramatsu, C. Okada, K. Uchino, H. Sezutsu, M. Kiuchi, T. Tamura, K. Hiruma, Y. Ishikawa and T. Shinoda: Hormonal regulation and developmental role of Krüppel homolog 1, a repressor of metamorphosis, in the silkworm *Bombyx mori. Dev. Biol.* **388**, 48–56 (2014).
15) T. Kayukawa and T. Shinoda: Functional characterization of two paralogous JH receptors, methoprene-tolerant 1 and 2 in the silkworm, *Bombyx mori* (Lepidoptera: Bombycidae). *Appl. Entomol. Zool.* **50**, 383–391 (2015).
16) T. Kayukawa, K. Nagamine, Y. Ito, Y. Nishita, Y. Ishikawa and T. Shinoda: Krüppel homolog 1 inhibits insect metamorphosis *via* direct transcriptional repression of *Broad-Complex*, a pupal specifier gene. *J. Biol. Chem.* **291**, 1751–1762 (2016).

17) T. Kayukawa, A. Jouraku, Y. Ito and T. Shinoda: Molecular mechanism underlying juvenile hormone-mediated repression of precocious larval-adult metamorphosis. *Proc. Natl. Acad. Sci. U.S.A.* **114**, 1057–1062 (2017).
18) T. Kayukawa, K. Furuta, K. Nagamine, T. Shinoda, K. Yonesu and T. Okabe: Identification of a juvenile-hormone signaling inhibitor *via* high-throughput screening of a chemical library. *Sci. Rep.* **10**, 18413 (2020).
19) S. Ishii, K. Fukui, S. Yokoshima, K. Kumagai, Y. Beniyama, T. Kodama, T. Fukuyama, T. Okabe, T. Nagano, H. Kojima and T. Yano: High-throughput screening of small molecule inhibitors of the Streptococcus quorum-sensing signal pathway. *Sci. Rep.* **7**, 4029 (2017).
20) J. H. Zhang, T. D. Chung and K. R. Oldenburg: A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J. Biomol. Screen.* **4**, 67–73 (1999).
21) G. B. Stall: Anti juvenile hormone agents. *Annu. Rev. Entomol.* **31**, 391–429 (1986).
22) L. M. Riddiford, C. R. Roseland, S. Thalberg and A. T. Curtis: Action of two juvenile hormone antagonists on lepidopteran epidermis. *J. Insect Physiol.* **29**, 281–286 (1983).
23) K. Furuta, K. Ashibe, H. Shirahashi, N. Fujita, H. Yamashita, N. Yamada and E. Kuwano: Synthesis and anti-juvenile hormone activity of ethyl 4-(2-benzylalkyloxy)benzoates and their enantiomers. *J. Pestic. Sci.* **32**, 99–105 (2007).
24) N. Fujita, K. Furuta, K. Ashibe, S. Yoshida, N. Yamada, T. Shiotsuki, M. Kiuchi and E. Kuwano: Juvenile hormone activity of optically active ethyl 4-(2-benzylalkyloxy)benzoates inducing precocious metamorphosis. *J. Pestic. Sci.* **33**, 383–386 (2008).
25) N. Yamada, K. Maeda, M. Masumoto, Y. Inagaki and K. Furuta: Anti-juvenile hormone activity of ethyl 4-[(7-substituted 1,4-benzodioxan-6-yl)methyl]benzoates and their effect on the juvenile hormone titer in the hemolymph of the silkworm, *Bombyx mori. J. Pestic. Sci.* **41**, 38–43 (2016).
26) S. Sakurai and S. Niimi: Development changes in juvenile hormone and juvenile hormone acid titers in the hemolymph and *in-vitro* juvenile hormone synthesis by corpora allata of the silkworm, *Bombyx mori. J. Insect Physiol.* **43**, 875–884 (1997).
27) K. Furuta, A. Ichikawa, M. Murata, E. Kuwano, T. Shinoda and T. Shiotsuki: Determination by LC-MS of juvenile hormone titers in hemolymph of the silkworm, *Bombyx mori. Biosci. Biotechnol. Biochem.* **77**, 988–991 (2013).
28) Z. Kajiura, K. Kadono-Okuda and O. Yamashita: Induction of dauer larvae by a juvenile hormone analogue and their response to ecdysteroids in the silkworm, *Bombyx mori. J. Seric. Sci. Jpn.* **56**, 398–406 (1987).
29) G. A. Yahouédo, F. Chandre, M. Rossignol, C. Ginibre, V. Balabanidou, N. G. A. Mendez, O. Pigeon, J. Vontas and S. Cornelie: Contributions of cuticle permeability and enzyme detoxification to pyrethroid resistance in the major malaria vector *Anopheles gambiae. Sci. Rep.* **7**, 11091 (2017).
30) V. Balabanidou, L. Grigoraki and J. Vontas: Insect cuticle: A critical determinant of insecticide resistance. *Curr. Opin. Insect Sci.* **27**, 68–74 (2018).
31) C. T. Zimmer, W. T. Garrood, K. S. Singh, E. Randall, B. Lueke, O. Gutbrod, S. Matthiesen, M. Kohler, R. Nauen, T. G. E. Davies and C. Bass: Neofunctionalization of duplicated P450 genes drives the evolution of insecticide resistance in the brown planthopper. *Curr. Biol.* **28**, 268–274.e5 (2018).