Review

Reporter gene assays for screening and identification of novel molting hormone- and juvenile hormone-like chemicals

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A reporter gene assay (RGA) is used to investigate the activity of synthetic chemicals mimicking the molting hormones (MHs) and juvenile hormones (JHs) of insects, so-called insect growth regulators (IGRs). The MH receptor, a heterodimer of the ecdysone receptor (EcR) and ultraspiracle (USP), and the JH receptor Methoprene-tolerant (Met) are ligand-dependent transcription factors. Ligand-bound EcR-USP and Met bind to specific cis-acting DNA elements, referred to as the ecdysone-responsive element (EcRE) and the JH-responsive element (JHRE), respectively, in order to transactivate target genes. Insect hormone-induced transactivation systems have been reconstituted by the introduction of reporter genes under the control of EcRE and JHRE, or two-hybrid reporter genes, into insect, mammalian, and yeast cells expressing receptor proteins. RGA is easy to use and convenient for examining the MH- and JH-like activities of synthetic chemicals and is suitable for the high-throughput screening of novel structural classes of chemicals targeting EcR-USP and Met.

Keywords: reporter gene assay, ecdysone receptor, juvenile hormone receptor, agonist, insect growth regulator.

Introduction

Insect species that belong to a large class of Arthropoda utilize unique endocrine systems consisting of two peripheral hormones, molting hormones (MHs) and juvenile hormones (JHs). These hormones are important for regulating the growth and development of insects. The steroid 20-hydroxyecdysone (20E) and sesquiterpenoid JH III are a representative MH and JH, respectively. Synthetic nonsteroidal ecdysone agonists and juvenile hormone agonists (JHAs) have recently been developed as insecticides and categorized as insect growth regulators (IGR).1,2)

Since these synthetic insect hormones interfere with insect-specific hormone responses, they are considered to be safer for mammals than other classes of insecticides.1) Nonsteroidal ecdysone agonists exhibit insect-selective toxicity for certain taxonomic orders. These IGRs may be ideal compounds for pest control.1,3,4)

The activities of synthetic MHs and JHs were previously analyzed in the whole body using classical in vivo bioassay methods.5–11) Experimental procedures to examine hormone-dependent morphological changes and the stimulation/inhibition of proliferation using cultured insect cell lines have also been developed as in vitro bioassays.12–18) The measurement of ligand-receptor binding affinity using insect cell extracts or in vitro translated receptor proteins is another procedure for detecting natural and synthetic hormones.19–28) In the 1990s, genes encoding the ecdysone receptor (EcR) and its partner protein ultraspiracle (USP), a homolog of mammalian retinoid X receptor (RXR), and the JH receptor Methoprene-tolerant (Met) were initially identified in the fruit fly Drosophila melanogaster.29–32) EcR-USP and Met are ligand-dependent transcription factors belonging to the nuclear receptor (NR) superfamily and the basic helix-loop-helix Per/Arnt/Sim (bHLH-PAS) family, respectively (Fig. 1a and Fig. 4a). The genes encoding EcR-USP and Met are conserved among various insect species and have
been functionally characterized.\textsuperscript{33–35)} The reporter gene assay (RGA) is a versatile experimental method for monitoring gene expression associated with signal transduction cascades in response to intra- and extracellular stimuli. RGA has been employed to elucidate the molecular mechanisms of signal transduction via EcR-USP and Met, and it has also played important roles in the development of high-throughput screening systems for IGRs with MH- and JH-like activities. In this review, we summarized the application of RGA to the study of IGRs.

1. RGA

The term reporter gene refers to a gene encoding a protein product with unique and readily measurable enzymatic activity or that is distinguishable from a large amount of various proteins expressed in cells.\textsuperscript{36–38)} The following reporter genes are frequently and commonly used: genes for bacterial chloramphenicol acetyltransferase (CAT), \(\beta\)-galactosidase (\(\beta\)-gal), and \(\beta\)-glucuronidase (GUS); luciferase from bacteria, firefly, or \textit{Renilla}; green fluorescent protein (GFP) of jellyfish; and the secretable form of alkaline phosphatase (SEAP) derived from the human placenta.\textsuperscript{36–40)} These reporter gene products have been characterized in detail, and their advantages and disadvantages as well as detection methods have been reviewed elsewhere.\textsuperscript{36,39,40)}

Reporter genes are placed downstream of promoters that function in host cells. RGA is a versatile experimental method for examining various cellular events. The reporter gene may be used as a marker of gene transfer or in promoter analysis, the identification of cis- and trans-acting DNA elements of genes of interest, the spatiotemporal imaging of gene expression patterns, the characterization of receptors and their ligands, and analyses of signaling pathways.\textsuperscript{41)}

2. Molecular mechanisms underlying the activation of EcR-USP by MHs

The RGA procedure has been applied in many studies to investigate the molecular mechanisms underlying signal transduction initiated by MHs. Riddihough \textit{et al.} reported that the transcription of the \textit{D. melanogaster} heat shock protein 27 (\textit{hsp27}) gene was induced by ecdysone as well as heat shock. A deletion analysis of the \textit{hsp27} promoter identified ecdysone-specific cis-acting DNA elements, referred to as the ecdysone-responsive element (EcRE), with a putative palindrome structure (Fig. 1b).\textsuperscript{42,43)} A gene for the MH receptor EcR was cloned as the first member of the NR superfamily in \textit{D. melanogaster}. The EcR protein was shown to bind to active MH 20E as well as DNA with high specificity at EcRE.\textsuperscript{29)} MH-bound EcR forms a heterodimer with its partner protein, USP, on EcRE.\textsuperscript{30,31,44)} Furthermore, the steroid receptor coactivator (SRC), Taiman of \textit{D. melanogaster} (DmTai), was identified as an important component in the transduction of MH signals to downstream effectors.\textsuperscript{45)}

EcREs with distinct sequences were subsequently identified in the regulatory region of \textit{D. melanogaster} \textit{Fbp1}, \textit{Eip28}/29, and \textit{Aedes aegypti} vitellogenin (Vg) genes.\textsuperscript{46–49)} Previous studies also indicated that EcR-USP induced reporter gene expression \textit{via} asymmetric RE, a direct repeat of the core sequence (half site) in African green monkey CV-1 cells.\textsuperscript{50,51)} The heterodimeriza-
Table 1. *In vitro* RGA systems for the screening of EcR-USP ligands

| Host cells | Organism and cell lines | Transfected receptor genes | EcRE | Reporter gene | Types of test compounds | Ref. |
|------------|-------------------------|----------------------------|------|--------------|------------------------|------|
| Insect cells* | *Drosophila melanogaster* Kc | — | Dm hsp27 promoter | Luc | 20E, α-ecdysone | 55) |
| D. melanogaster Kc | — | Dm hsp27 promoter | Luc | 3,5-di-tert-butyl-4-hydroxy-N-isobutyl-benzamide (DTBHIB) | 56) |
| D. melanogaster Kc | — | Dm hsp27 promoter | Luc | Diacylhydrazine (DAH) compounds | 57) |
| D. melanogaster Schneider 2 (S2) | — | Dm hsp27 | lacZ | Curcubitacins | 134) |
| D. melanogaster Schneider SL2 | — | Dm hsp27 | EcRE | Insect and plant ecdysteroids | 139) |
| *Spodoptera frugiperda* Sf9 | — | Dm hsp27 | EcRE | 20E, ponasterone A (pon A), chromafenozide | 67) |
| *D. melanogaster* Kc | — | Dm hsp27 | EcRE | Plant compounds | 58) |
| *Bombyx mori* Bm5 | — | Dm hsp27 | CAT | DAH library | 67) |
| *S. frugiperda* Sf9 | CjEcR | Dm hsp27 | CAT | RG-102240 | 68) |
| Lymantria dispar (Ld652Y) | CjEcR MnRXR | GAL4 RE (two-hybrid) | CAT | RG-102240 | 68) |
| *B. mori* Bm5 | — | Dm hsp27 | GFP | DAHs | 70) |
| *D. melanogaster* S2 | — | Dm hsp27 | EcRE | Chromafenozide | 69) |
| *B. mori* Bm5 | — | Dm hsp27 | EcRE | Chromafenozide | 69) |
| *Anthonomus grandis* BRL-AG-3A | — | Dm hsp27 | EcRE | Methoxyfenozide | 70) |
| *BRL-AG-3C* *Leptinotarsa decemlineata* BCRL-Lepd-SL1 | — | Dm hsp27 | EcRE | DAHs, acylaminoketone (AAK) analogs, tetrahydroquinoline (THQ) compounds, piperidiamine analogs | 80) |
| *D. melanogaster* S2 | — | Dm hsp27 | EcRE | DAHs, AAK analogs, THQs | 71) |
| *B. mori* Bm5 | — | Dm hsp27 | EcRE | DAHs, AAK analogs, THQs | 71) |
| *S. littoralis* SL2, SLj2b | — | Dm hsp27 | EcRE | DAHs, AAK analogs, THQs | 71) |
| *S. frugiperda* Sf9 | — | Dm hsp27 | EcRE | Non-steroidal compounds | 78) |
| *L. decemlineata* BCRL-Lepd-SL1 | — | Dm hsp27 | EcRE | DAHs | 77) |
| *D. melanogaster* S2 | — | Dm hsp27 | EcRE | Ecdysteroids | 135) |
| *B. mori* Bm5 | — | Dm hsp27 | EcRE | Ecdysteroids | 135) |
| *S. frugiperda* Sf9 | — | Dm hsp27 | EcRE | N-tert-butylphenyl thenoylhydrazide compounds | 140) |
| *D. melanogaster* S2 | — | Dm hsp27 | EcRE | Compounds registered in Maybridge database | 79) |
| *B. mori* Bm5 | — | Dm hsp27 | EcRE | Compounds registered in Maybridge database | 79) |
| Mammalian cells | *Cricetulus griseus* CHO | VgEcR | E/GRE | lacZ | Phytochemicals | 90) |
| CHO | HsRXR | | | | | |
| *Mus musculus* NIH 3T3 | AaEcR | GAL4 RE (two-hybrid) | Luc | THQs | 3) |
| NIH 3T3 | MnRXR | | | | | |
Table 1. Continued

| Host cells          | Organism and cell lines | Transfected receptor genes | EcRE       | Reporter gene | Types of test compounds | Ref. |
|---------------------|-------------------------|-----------------------------|------------|---------------|-------------------------|------|
| Mammalian cells     | *Homo sapiens*          | BmEcR                       | Dm hsp27 EcRE | β-gal         | AAKs                   | 72(  |
| HEK293              |                         | BmEcR                       | Dm hsp27 EcRE | β-gal         | AAKs                   | 60(  |
| *H. sapiens*        |                         | CIEcR                       | GAL4 RE (two-hybrid) | Luc          | DAHs, THQs             | 61(  |
| HEK293              |                         | DmEcR                       | GAL4 RE (two-hybrid) | Luc          | THQs                   | 4(   |
| *C. griseus*        | CHO                     | CIEcR                       | GAL4 RE (two-hybrid) | Luc          | Natural and semi-synthetic ecdysteroids (hydroxylation) | 62(  |
| *M. musculus*       | NIH 3T3                 | AaEcR                       | E/GRE       | Luc          | Natural and semi-synthetic ecdysteroids (alkylation) | 63(  |
| NIH 3T3             |                         | CIIEcR                      | GAL4 RE (two-hybrid) | Luc          | Natural and semi-synthetic ecdysteroids (alkylation) | 63(  |
| *M. musculus*       | NIH 3T3                 | VgEcR                       | E/GRE       | Luc          | Natural and semi-synthetic ecdysteroids (alkylation) | 63(  |
| NIH 3T3             |                         | HsRXR                       | GAL4 RE (two-hybrid) | Luc          | Steroidal ecdysterones, DAHs | 64(  |
| *H. sapiens*        | HEK293                  | BmaEcR                      | Dm hsp27 EcRE | β-gal         | Nonsteroidal ecdysone agonists | 85(  |
| *S. cerevisiae*     |                         | CIEcR                       | Dm hsp27 EcRE | β-gal         | Ecdysteroids, nonsteroidal ecdysone agonists | 84(  |
| *S. cerevisiae*     |                         | DmEcR                       | IR0         | β-gal         | Ecdysteroids, DAHs, THQ | 87(  |

Abbreviations: *Aa*: *Aedes aegypti*; *Ama*: *Amblyomma americanum*; *Ba*: *Bemisia argentifolii*; *Bm*: *Bombyx mori*; *Bma*: *Brugia malayi*; *Ce*: *Choristoneura fumiferana*; *Cs*: *Chilo suppressalis*; *Dm*: *Drosophila melanogaster*; *E/GRE*: ecdysone/glucocorticoid responsive element (hybrid RE); *Hs*: *Homo sapiens*; *Lm*: *Locusta migratoria*; *Ms*: *Manduca sexta*; *Nc*: *Nephotettix cincticeps*; *Tc*: *Tribolium castaneum*; *Tm*: *Tenebrio molitor*; *Mm*: *Mus musculus*; CHO; Chinese hamster ovary; GAL4 RE: DNA element for binding of the GAL4 DNA binding domain (DBD); VgEcR: hybrid of *D. melanogaster* EcR carrying modified DBD and VP16 AD that recognizes an E/GRE. *Reporter gene assays in insect cells expressing endogenous EcR and USP. Only the reporter plasmid was introduced.
and induce reporter gene expression in host cells. VP16, respectively (and vice versa fused to Gal4-DBD and transcriptional activation domain (AD) of Gal4 or USP). Two-hybrid-based RGA for EcR-USP. EcR and USP (LBD or full length) by recruiting RNA pol II general transcription factors of host cells. (b) Two-hybrid-based RGA for EcR-USP. EcR and USP (LBD or full length) fused to Gal4-DBD and transcriptional activation domain (AD) of Gal4 or VP16, respectively (and vice versa), interact in an MH-dependent manner and induce reporter gene expression in host cells.

Fig. 2. RGA for EcR-USP. (a) Schematic summary of RGA for EcR-USP. RGA using a reporter gene under the control of EcRE, in which multiple copies of EcRE are integrated upstream of the promoter (EcRE×n). Ligand-bound EcR and USP form a heterodimer on EcRE. The interaction of transcriptional coactivator SRC enhances reporter gene expression by recruiting RNA pol II general transcription factors of host cells. (b) Two-hybrid-based RGA for EcR-USP. EcR and USP (LBD or full length) fused to Gal4-DBD and transcriptional activation domain (AD) of Gal4 or VP16, respectively (and vice versa), interact in an MH-dependent manner and induce reporter gene expression in host cells.

3. RGA for detecting Synthetic ecdysone agonists by surveying gene expression mediated by EcR-USP

The identification of EcR and USP on DR-RE (Fig. 1c) was confirmed using a biochemical approach, the electrophoretic mobility shift assay (EMSA).

The activation mechanism of EcR-USP revealed in D. melanogaster was conserved in other insect species (Fig. 1d). The heterodimerization of ligand-bound EcR and USP enables the transactivation of two-hybrid reporter genes.

Pioneering studies using RGA on insect cell lines were conducted by Mikitani, who introduced a Luc reporter plasmid connected to a Dm hsp27 promoter into D. melanogaster Kc cells that were highly sensitive to ecdysteroids. The ecdysteroid-dependent expression of luciferase by endogenous EcR-USP was reproducibly observed. This procedure was very sensitive, rapid, and simple, suggesting that RGA of insect cells is a valuable tool in the search for new ecdysteroid compounds.

The findings obtained using RGA correlated with the effects of synthetic ecdysteroid compounds on ligand-binding affinity, efficacy toward cellular morphological changes, and larvicidal activity. To date, a number of RGA systems for EcR-USP have been developed in several cell lines from insect species in distinct phylogenetic orders.

RHS849, a diacylhydrazine (DAH)-type lead compound exhibiting MH-like activity, was identified as the first nonsteroidal ecdysone agonist. DAHs exhibited in vivo toxicity in a lepidopteran insect–selective manner, which correlated with the transactivation activity observed in RGA. α-Acylaminoketone and its analogs were identified as novel types of nonsteroidal ecdysone agonists with potent selectivity on lepidopteran EcR-USP, while tetrahydroquinoline (THQ) compounds were shown to strongly activate dipteran EcR-USP. The insect order-selective activity of nonsteroidal ecdysone agonists was associated with the divergence of the primary sequence of the ligand-binding domain (LBD) of EcR. Differences in the three-dimensional (3D) structure of the ligand-binding pocket affected the binding potency of nonsteroidal ecdysone agonists; however, the binding mode of natural ecdysteroids was similar among different phylogenetic orders of insects.

To discover new classes of ecdysone agonists for non-lepidopteran insect pests, RGA was established in coleopteran cell lines from Leptinotarsa decemlineata and Anthonomus grandis, and various nonsteroidal ecdysone agonists were tested by methods combined with (quantitative) structure–activity relationship ((Q) SAR) studies and/or the 3D-modeling of the ligand-binding pocket and ligand docking studies (virtual screening). Similarly, the agonist/antagonist activities for dipter-
an and lepidopteran EcR-USP of several compounds identified by ligand-based virtual screening were confirmed by RGA. Approaches combining (QS)AR and RGA were also used to examine the ligand potencies of semi-synthetic ecdysteroids for EcR-USP of various insect species.

In mammalian cell-based RGA, EcR-USP derived from several different orders of insects was expressed to examine the MH-like activity of test compounds. RGA was also utilized to compare the species-selective in vivo toxicities and in vitro transactivation activities of synthetic compounds in the lepidopteran insects *B. mori* and *Spodoptera littoralis.*

4. Reconstitution of MH-induced transactivation systems in yeast

RGA has been established in the yeast *Saccharomyces cerevisiae* as another heterologous host system. *S. cerevisiae* is the simplest eukaryote possessing highly conserved gene expression mechanisms of higher eukaryotes. Previous studies reported that the ligand–dependent transactivation activity of *D. melanogaster* EcR-USP via *hsp27* EcRE was not confirmed, whereas the EcR protein expressed in yeast was capable of binding to ecdysteroid ligands with the co-expression of USP. Tran et al. showed that the expression of mouse SRC GRIP1 was required to reconstitute the ligand-dependent transactivation of EcR-USP from *Choristoneura fumiferana* and *Aedes aegypti.* The ligand-induced heterodimerization of the LBDs of EcR and USP from *D. melanogaster* was also demonstrated by a two-hybrid method and EMSA in yeast. Based on these findings, we recently established a new yeast RGA system for detecting MHs. EcR and USP derived from three insect species belonging to different taxonomic orders—the dipteran *D. melanogaster,* lepidopteran *Chilo suppressalis,* and coleopteran *L. decemlineata*—were expressed in yeast in conjunction with the SRC DmTai (Fig. 3). The ligand-induced transactivation in arthropods

5. Molecular mechanisms underlying JH-dependent transactivation in arthropods

JH receptor Met proteins are members of the bHLH-PAS family that function as ligand-dependent transcription factors as well as EcR-USP (Fig. 4a). The Met gene was originally cloned as a gene that complemented the methoprene resistance of a mutant fly. The Met protein was shown to bind to JH III with high affinity. The GAL4-DBD-Met fusion protein directed to GAL4 RE transactivated the Luc reporter in a JH/JHA-dependent manner. Met homologs have since been identified in various insects, such as holo-, hemi-, and ametabolous species. The paralogous gene germ cell–expressed (Gce), which is conserved in *Drosophila* species, is also involved in signal transduction for the exertion of JH effects. Met-Met and Met-Gce dimer complexes are formed as inactive states in the absence of JHs. Upon ligand binding to the PAS-B domain of Met and Gce, Met-Met and Met-Gce complexes are rapidly dissociated. Met/Gce proteins then form a heterodimer with other bHLH-PAS protein SRCs, such as DmTai, *A. aegypti* FISC, and *Tribolium castaneum* TeSRC. The Met-SRC and Gce-SRC heterodimer complex binds to a specific DNA element (JHRE) in a JH-dependent manner and activates the transcription of early JH-inducible genes, such as Krüppel homolog 1 (*Kr-h1*) and early trypsin (*ET*) (Fig. 4b). Previous studies...
on the expression of anti-metamorphic gene Kr-h1 identified a 13-nucleotide motif containing an E-box (CAC GTG) as JHRE, which was essential for the binding of Met-SRC to mediate the effects of JH. This motif is highly conserved in the Kr-h1 regulatory region of a wide range of insect species (Fig. 4c). In A. aegypti, Met and Cycle (Cyc) have been shown to form a heterodimer on response elements containing an imperfect E-box-like (CACGCG) sequence in female mosquitos. The Met-Cyc complex induces the JH-dependent expression of the Kr-h1 gene, as well as the Met-FISC heterodimer. JH-regulated gene expression is shared in microcrustacea Daphnia pulex and D. magna, in which Met and SRC form a heterodimer in response to various juvenoids. Although Met and SRC play essential roles in normal embryogenesis, the expression of D. pulex Kr-h1 is not regulated by the JH-Met signaling cascade, as it is in insects.

6. RGA systems for detecting JHs and JHAs

Table 2 summarizes RGAs previously established in studies on JH receptors. The strategy of constructing reporter plasmids is similar to that of constructing RGAs for EcR-USP: DNA fragments containing JHRE identified in genes such as Kr-h1, juvenile hormone esterase (jhe), and early trypsin (ET) were integrated upstream of basal promoters (Table 2).
Ligand-bound Met heterodimerizes with SRC and binds to JHRE in order to transactivate reporter gene expression (Fig. 5a). The JH-dependent expression of reporter genes by Met and/or Gce has been measured in various insect cell lines (Table 2). Although two-hybrid-based RGAs have been used frequently to examine JH-dependent heterodimer formation between Met and SRC in heterologous host systems (Fig. 5b), insect Kr-h1 JHRE-regulated transactivation was successfully reconstituted using mammalian cells in three studies (Table 2). Some of these RGAs indicated dose-dependent responses against various juvenoids, such as JH III, methyl farnesoate, methoprene, pyriproxyfen, and fenoxycarb. RGAs for Met from Daphnia species also have been established. D. pulex Met exhibited dose-dependent responses against several juvenoids via T. castaneum Kr-h1 JHRE, while the Kr-h1 homolog of D. pulex was not regulated by JH-Met signaling. This finding suggests that the DNA-binding properties of Met proteins are conserved in insects and daphnids. RGA for Met derived from non-insect species will be useful.

### Table 2. RGA systems for arthropod JHRs

| Host cells   | Organisms and cell lines | Transfected receptor genes | Response element | Reporter gene | Ref. |
|--------------|--------------------------|----------------------------|------------------|---------------|------|
| Insect cells | C. fumiferana CF-203*    | —                          | Cfjhe JHRE       | Luc           | 114  |
|              | D. melanogaster S2       | DmMet                      | GAL4 RE (one-hybrid) | Luc           | 24   |
|              | D. melanogaster L57      | AaMet-AaFISC               | GAL4 RE (two-hybrid) | Luc           | 102  |
|              | D. melanogaster L57*     | —                          | AaET JHRE       | Luc           | 102  |
|              | B. mori NIAS-Bm-aF3*     | —                          | BmKr-h1 JHRE   | Luc           | 105  |
|              | Tribolium castaneum Tc81*| —                          | TcKr-h1 JHRE   | Luc           | 106  |
|              | D. melanogaster S2       | TcMet-TcSRC                | TcKr-h1 JHRE   | Luc           | 106  |
|              | Aedes aegypti Agr2*      | —                          | Synthetic AaMFBS | Luc           | 104  |
|              | Aedes aegypti Agr2*      | —                          | AaKr-h1 JHRE   | Luc           | 107  |
|              | D. melanogaster Kc*      | —                          | DmKr-h1 JHRE   | Luc           | 115  |
|              | D. melanogaster S2       | BmMet-BmSRC                | BmKr-h1 JHRE   | Luc           | 116  |
|              | D. melanogaster S2       | DmMet/Gce-DmTai            | AaET JHRE       | Luc           | 27   |
|              | D. melanogaster S2*      | —                          | AaET JHRE       | Luc           | 28   |
| Mammalian cells | M. musculus         | TcMet-TcSRC                | GAL4 RE (two-hybrid) | Luc           | 103  |
| NIH 3T3              | H. sapiens HEK293       | BmMet2-BmSRC               | GAL4 RE (two-hybrid) | Luc           | 105  |
|              | H. sapiens HEK293*      | BmMet2-BmSRC               | BmKr-h1 JHRE   | Luc           | 105  |
|              | H. sapiens HEK293*      | TcMet-TcSRC                | GAL4 RE (two-hybrid) | Luc           | 106  |
|              | C. griseus CHO          | DapmaMet-DapmaSRC          | GAL4 RE (two-hybrid) | Luc           | 109  |
|              | DapuMet-DappuSRC        | TcMet-TcSRC                | GAL4 RE (two-hybrid) | Luc           | 120  |
|              | H. sapiens HEK293*      | BmMet2-BmSRC               | BmKr-h1 JHRE   | Luc           | 116  |
|              | H. sapiens HEK293*      | BmMet1-BmSRC               | GAL4 RE (two-hybrid) | Luc           | 116  |
|              | C. griseus CHO          | DapmaMet-DapmaSRC          | GAL4 RE (two-hybrid) | Luc           | 119  |
|              | DapuMet-DappuSRC        | TcMet-TcSRC                | GAL4 RE (two-hybrid) | Luc           | 120  |
|              | C. griseus CHO          | DapuMet-DappuSRC           | TcKr-h1 JHRE   | Luc           | 118  |
|              | H. sapiens HEK293T      | DmMet-DmTai                | GAL4 RE (two-hybrid) | Luc           | 28   |
|              | C. griseus CHO          | DapuMet-DappuSRC           | GAL4 RE (two-hybrid) | Luc           | 117  |
| Yeast         | S. cerevisiae           | AaMet-AaCYC                | GAL4 RE (two-hybrid) | β-gal         | 122  |
|              | S. cerevisiae           | AaMet-AaFISC               | GAL4 RE (two-hybrid) | β-gal         | 123  |

Abbreviations: JHR: juvenile hormone receptor; Aa: Aedes aegypti; Bm: Bombyx mori; Cf: Choristoneura fumiferana; Dm: Drosophila melanogaster; Tc: Tribolium castaneum; Dapma: Daphnia magna; Dappu: Daphnia pulex; jhe: juvenile hormone esterase; ET: early trypsin; MFBS: Met-FISC binding site. * Reporter gene assays in insect cells expressing endogenous JHRs and a transcriptional coactivator. Only the reporter plasmid was introduced.
nists, derivatives of imidazothiadiazole,\textsuperscript{124} 1-phenyl-4-cyano- 5-aminopyrazoles,\textsuperscript{125} heptacyclic pyrazolamide,\textsuperscript{126} methylene lactams,\textsuperscript{127} oxadiazolines,\textsuperscript{128} and imidazoles\textsuperscript{129} have been lined up by (Q)SAR studies and/or virtual screening methods. These compounds have potential as candidates of new classes of EcR-targeting insecticides. An \textit{in silico} screening method was also used to obtain novel bioactive juvenoids.\textsuperscript{130} Previous studies have reported several thousand synthetic compounds with JH-like effects, and chemically divergent compounds act as juvenoids.\textsuperscript{131–133} Therefore, a novel class of JHAs, ideally insect-selective juvenoids, may be obtained in the future. Furthermore, RGA can confirm the antagonist activity of chemicals of interest.\textsuperscript{58,78,79,90,122,123,134,135} In comparison with agonist screening, however, the general cytotoxicity of test chemicals that may repress reporter gene expression in a receptor-independent manner should be carefully considered in antagonist assays.\textsuperscript{80} RGA using a specifically engineered reporter gene construct to positively select for a decrease in receptor activity may allow for the effective screening of antagonists from a large pool of chemical compounds. RGA systems are suitable and advantageous for the high-throughput screening of chemicals that affect the activity of EcR and Met.

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