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Quantitative evaluation of the molting hormone activity in coleopteran cells established from the Colorado potato beetle, *Leptinotarsa decemlineata*

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ABSTRACT

A novel reporter gene assay system using BCIRL-Lepd-SL1, a cell line from the coleopteran Colorado potato beetle (*Leptinotarsa decemlineata*), was established. Cells were transiently transfected with the reporter plasmid that was composed of a firefly luciferase gene with upstream ecdysone response elements, and an internal control plasmid that constitutively produces *Renilla reniformis* luciferase. Transfected cells were incubated with various molting hormone agonists, and the activity of these agonists was quantitatively determined by measuring luminescence emission. Transcription-inducing activity for ecdysone, 20-hydroxyecdysone and ponasterone A in terms of EC_{50} (50% effective concentration) were determined to be 1 μM (pEC_{50} = 5.99), 68 nM (pEC_{50} = 7.17) and 1.3 nM (pEC_{50} = 8.88), respectively. Among tested diacylhydrazine (DAH)-type compounds, 11 compounds were active (pEC_{50} = 3.56 ~ 6.41), but two compounds were inactive. The EC_{50} values were linearly correlated to their binding affinity except for one compound. While several ecdysone reporter systems were developed before that employ dipteran and lepidopteron cell lines, the assay system described here is only the second one that employs a coleopteran cell line. This reporter system will allow screening in high-throughput format for new and more potent molting accelerating compounds that specifically target coleopteran pests.

1. Introduction

Insect molting is strictly regulated by a molting hormone (20-hydroxyecdysone; 20E), and the molecular mechanism of 20E has been well studied. 20E binds to the ecdysone receptor (EcR) and transactivates genes regulating the molting process in complex with the heterodimeric partner protein, ultraspiracle (USP). Both EcR and USP belong to the superfamily of nuclear receptors (NRs) [1,2], that typically consist of 5 different domains: A/B (transcription-activation function), C (DNA binding), D (hinge), E (ligand binding, transcription-activation function) and F (very short and without reported function in EcR). Among NRs, EcR is categorized in the subgroup NR1, and USP, an ortholog of the mammalian retinoid X receptor (RXR), belongs to NR2 [1–3]. To date, multiple orthologs of EcR and USP (RXR) have been identified not only in insects but also in other arthropods [1,2,4]. The EcR/USP (RXR) heterodimer binds to ecdysone response elements (EcREs) in the promoter region of genes related to the molting process, and then functions as a receptor for the molting hormone [5].

Since the 20E-dependent growth control is unique to arthropods including insects, this regulatory network is a good candidate for disruption by insecticides that are selectively toxic to insects and therefore environment-friendly. There are various ecdysone agonists, and some of them have insecticidal activity by interfering with the function of 20E [6–9]. Among ecdysone agonists, the class of diacylhydrazine (DAH)-type compounds is extensively investigated for its effect on insect molting. Interestingly, DAHs have selective toxicity among insect orders, even though natural steroid hormones such as 20E and ponasterone A (PoA) have similar potency among all insects [8]. The majority of DAH compounds are selectively toxic to Lepidoptera, and the discovery of new compounds that are toxic to other pests than Lepidoptera is desired to achieve more comprehensive selective crop protection strategies. The high toxicity of some DAH compounds to Lepidoptera is thought to be due to the high binding affinity to the Lepidoptera ecdysone receptor [8]. However, halofenozide which has no high binding affinity to the EcR/USP, is also marketed as insecticide to control coleopterans. Thus, it looks that binding affinity is not always correlated with larval toxicity, especially for coleopteran insects.

20E and its analog PoA bind to the binding pocket of the ligand binding domain of EcR [10–12]. It is believed that the induction of the metamorphosis cascade is caused by the activation of early genes [13]. The transcription factors constitute a conserved regulatory cascade that is carried out to accomplish the physiological

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process of molting or metamorphosis [14,15]. 20E directly decides the timing of development via this gene transcription network, and a number of previous studies have identified the various transcriptional factors involved in this network [15–18].

Assays for discovery of new ecdysone agonists could be based on displacement of binding of radioactive PoA to protein extracts of cells [19]. In such case, however, binding activity would give no information with respect to the ability of the compounds to activate or inhibit the EcR/USP complex. For instance, it will not be known whether compounds with strong binding affinity represent agonist or antagonist activity. Therefore, it is important to link the binding activity of compounds with gene activation to understand their mode of action.

For such a purpose, the molting hormone agonistic activity of numerous chemical compounds is studied by the quantitative structure–activity relationship (QSAR) technique using activities measured by a reporter gene assay (RGA) [19–22] as well as by the induction of chitin synthesis in the cultured integument [23]. The binding activity is also analyzed for the limited number of DAHs with various substituents at the *para*-position of the benzene ring [24]. RGA is convenient because the experiment can be done without using radioactive materials, but its use has been mainly limited to lepidopteran and dipteran cell lines so far. Only one report of an RGA exists that is based on cells derived from a coleopteran species [25], despite the abundance of important pests of agricultural products within this order. The establishment of RGAs using coleopteran cell lines would be helpful for the development of new insecticides with selective toxicity against such pests. Among them, the Colorado potato beetle *Leptinotarsa decemlineata* is a major pest of potato plants and other Solanaceae. Thus, various studies that employed SAR have been carried out to analyze activities of DAHs against *L. decemlineata* and to design more potent DAH compounds, but these studies have only provided limited insights because they employed either binding assays or larval toxicity assays [26–29]. More specifically, we showed that the strength of the binding affinity of DAHs to the *in vitro* translated EcR/USP of *L. decemlineata* was not correlated to the larvicidal activity against *L. decemlineata* [30]. While the cause of the inconsistency between the receptor binding and toxicity of DAHs in *L. decemlineata* is unclear, its elucidation is considered important in view of the development of novel 20E agonistic/antagonistic insecticides which show selective toxicity to Coleoptera.

Investigation of the transcription-regulating activity of DAHs in *L. decemlineata* could be insightful with respect to the mechanism of toxicity of these compounds. Although it needs to be kept in mind that the RGA system corresponds only to the very first step in the ecdysone-responsive cascade (activation of EcR/USP) and does not provide any information regarding activation of endogenous ecdysone-responsive early and early-late genes, the cell-based assay can provide valuable insights as it can be considered as an useful intermediate level between ligand-receptor binding and toxicity assay. In this study, we established an RGA system to quantitatively measure the transcriptional activation activity of ecdysone agonists using BCIRL-Lepd-SL1 cells of *L. decemlineata*, and compared this activity with their receptor-binding affinity and larvicidal activity.

2. Materials and methods

2.1. Materials

BCIRL-Lepd-SL1, a cell line derived from female pupae of *L. decemlineata* and a kind gift by Dr. Cindy Goodman (USDA-ARS, Columbia, MO), was maintained as reported previously [31]. The cell culture medium was prepared by adding 10% of inactivated FBS (Fetal Bovine Serum; CAMBREX Co., North Brunswick, NJ) to EX-CELL 401 (SAFC Bioscience, Lenexa, KS). Cells were maintained routinely in 25 cm^2-tissue culture flask (AGC TECHNO GLASS Co. Ltd., Chiba, Japan) at 25 °C. During RGA manipulations, the medium also contained 50,000 units of penicillin G potassium salt (Meiji Seika Kaisha Ltd., Tokyo, Japan) and 50 mg of sulfonyl streptomycin (Meiji Seika Kaisha) per 1 L of medium.

A reporter plasmid for ecdysone-dependent production of the luciferase, pBmbA/hsp27/firefly luciferase (hsp27/Fluc), which was previously referred to as ERE-b.act.luc [32], was used. This plasmid contains seven repeats of hsp27 EcRE sequence of *Drosophila melanogaster* [33] upstream of the basal promoter region from the actin A3 gene of *Bombyx mori* and a firefly luciferase coding ORF downstream. A plasmid for an internal control, pIZT/V5-His/*Renilla* luciferase (pIZT/Rluc), was a gift of Dr. Kamimura (National Institute of Agrobiological Science, Tsukuba, Japan). It was constructed by introducing *Renilla reinformis* luciferase gene into MCS of pIZT/V5-His plasmid vector (Invitrogen Co., Carlsbad, CA). *Renilla* luciferase gene is under the control of OpIE2 promoter from OpMNPV, a baculovirus of the lepidopteran *Orgyia pseudotsugata*, for constitutive expression. GFP (green fluorescent protein) coding sequence in fusion with the ampicillin coding sequence is also constitutively transcribed by OpIE1 promoter in this plasmid. These two plasmids were amplified in *Escherichia coli*, and purified by QIAGEN Plasmid Midi Kit (QIAGEN GmbH, Hilden, Germany).

Reagents for transfection by lipofection methods were Tfx-20™ (Promega Co., Madison, WI), TransFast™ (Promega), Lipofectamine™ 2000 and Plus Reagent (Invitrogen), GeneJuice (Merck KGaA, Darmstadt, Germany), TransIT®-Insecta (Mirus Bio Co., Madison, WI), DOTAP (Roche Co., Basel, Switzerland), DOSPER Liposomal Transfection Reagent (Roche), FuGENE 6 (Roche) and FuGENE HD (Roche) that are listed in Table 1. These reagents were purchased or kindly gifted from manufacturers. For detection of the luciferase activity, Dual-Luciferase Reporter Assay System (Promega) was used with GloMax™ 96 Microplate Luminometer (Promega Co., Madison, WI). All ecdysone agonists used in this study were from the stock of our laboratory at the University of Kyoto. Ecdysone and 20E are purchased from Sigma (St.-Louis, MO), and PoA from Invitrogen.

2.2. Transfection and induction of luciferase activity

The transfection ability of various transfection reagents for BCIRL-Lepd-SL1 was examined with hsp27/Fluc and 10 nM of PoA. Ethanol was used as the negative control of induction of the transcription. Experiments were conducted following the recommendations in the manufacturer's instruction for all transfection reagents. FuGENE HD Transfection Reagent (FuGENE HD) was selected for experiments for the rest of the study and the assay condition was optimized as shown below.

Table 1
Effect of transfection reagents on reporter expression in absence and presence of PoA. Indicated are RLUs after transfection under standard conditions by each transfection agent.

Transfection reagents	Control	Induction
Tfx-20	95	278
TransFast	283	1936
DOTAP	40	48
DOSFER	26	26
FuGene6	73	179
FuGENE HD	158006	538686
TransIT	108	302
Lipofectamine 2000	47	136
Lipofectamine 2000 + Plus Regent	113	194
GeneJuice	159	246