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Towards Coleoptera-specific high-throughput screening systems for compounds with ecdysone activity: development of EcR reporter assays using weevil (*Anthonomus grandis*)-derived cell lines and *in silico* analysis of ligand binding to *A. grandis* EcR ligand-binding pocket

Thomas Soin [a], Masatoshi Iga [a], Luc Swevers [b], Pierre Rougé [c], Colin R. Janssen [d], Guy Smagghe [a,*]

[a] *Laboratory of Agrozoology, Department of Crop Protection, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, B-9000 Ghent, Belgium*
[b] *Insect Molecular Genetics and Biotechnology, Institute of Biology, National Centre for Scientific Research "Demokritos", Aghia Paraskevi Attikis, Athens, Greece*
[c] *Surfaces Cellulaires et Signalisation chez les Végétaux, UMR Université Paul Sabatier CNRS 5546, Castanet Tolosan, France*
[d] *Laboratory of Environmental Toxicology and Aquatic Ecology, Department of Applied Ecology and Environmental Biology, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium*

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ABSTRACT

Molting in insects is regulated by ecdysteroids and juvenile hormones. Several synthetic non-steroidal ecdysone agonists are on the market as insecticides. These ecdysone agonists are dibenzoylhydrazine (DBH) analogue compounds that manifest their toxicity via interaction with the ecdysone receptor (EcR). Of the four commercial available ecdysone agonists, three (tebufenozide, methoxyfenozide and chromafenozide) are highly lepidopteran specific, one (halofenozide) is used to control coleopteran and lepidopteran insects in turf and ornamentals. However, compared to the very high binding affinity of these DBH analogues to lepidopteran EcRs, halofenozide has a low binding affinity for coleopteran EcRs. For the discovery of ecdysone agonists that target non-lepidopteran insect groups, efficient screening systems that are based on the activation of the EcR are needed. We report here the development and evaluation of two coleopteran-specific reporter-based screening systems to discover and evaluate ecdysone agonists. The screening systems are based on the cell lines BRL-AG-3A and BRL-AG-3C that are derived from the weevil *Anthonomus grandis*, which can be efficiently transduced with an EcR reporter cassette for evaluation of induction of reporter activity by ecdysone agonists. We also cloned the almost full length coding sequence of *EcR* expressed in the cell line BRL-AG-3C and used it to make an initial *in silico* 3D-model of its ligand-binding pocket docked with ponasterone A and tebufenozide.

1. Introduction

Molting in insects is regulated by ecdysteroids; in most insects the active ecdysteroid is 20-hydroxyecdysone (20E). Several synthetic non-steroidal ecdysone agonists are on the market as insecticides (Dhadialla et al., 1998; Yanagi et al., 2006). These dibenzoylhydrazine (DBH) compounds have been shown to manifest their toxicity via interaction with the ecdysone receptor (EcR) in susceptible insects. Like 20E, they transactivate a succession of molt initiating transcription factors that, in turn, induce the expression of a group of molt-related genes. As a result of the expression of these up-regulated genes, the larva undergoes premature apolysis and head capsule slippage and takes on the appearance of the pharate larva. However, unlike 20E, which is cleared at this juncture, allowing the down-regulated genes to be expressed, these synthetic agonists are not cleared easily. Therefore, all the down-regulated events that occur as the titer of 20E decreases are repressed by the presence of the ecdysone agonist. The result is that the insect remains trapped in the molting process and dies slowly from starvation and desiccation (Dhadialla et al., 1998).

Four DBH ecdysone agonists are currently available on the market. Tebufenozide (RH-5992), methoxyfenozide (RH-2485) and chromafenozide (ANS-118) are highly lepidopteran specific (Dhadialla et al., 1998; Yanagi et al., 2006). Halofenozide (RH-0345) is used to control coleopteran (scarabid larvae) and lepidopteran insects in turf and ornamentals (Dhadialla et al., 1998). The success of these compounds in insect control programs validates EcR as a valuable target for the development of environmentally friendly biorational insecticides (Nakagawa, 2005). However, as mentioned above, current available ecdysone agonists target mainly lepidopteran insects together with a limited number of coleopteran insects

* Corresponding author. Tel.: +32 9 2646150; fax: +32 9 2646239.
E-mail address: guy.smagghe@ugent.be (G. Smagghe).

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(Dhadialla et al., 1998). For the discovery of ecdysone agonists that target other insect groups, efficient screening systems that are based on the activation of EcR are needed.

Important to note is that DBH non-steroidal ecdysone agonists have an unusual high affinity for the EcR of lepidopteran insects. Binding studies have indicated that the dissociation equilibrium constant (K_d) of binding of DBH analogues can differ by 1–2 orders of magnitude between coleopteran and lepidopteran insects (Ogura et al., 2005). While this illustrates the specificity of DBH analogues for lepidopteran insects, the mechanism by which the higher binding affinity is achieved is unknown. Deeper insight into the mechanism of activation of coleopteran and lepidopteran EcRs would provide the basis for the rational design of new molting hormone agonists with increased specificity and efficiency for coleopteran insects. Important tools to achieve this goal include (1) the availability of coleopteran-specific cell-based ecdysone reporter assays to allow fast screening for activity of compounds and (2) appropriate models of the EcR ligand-binding domain (LBD) to allow *in silico* docking studies of candidate ligands.

In this article, we report the molecular cloning and analysis of the *EcR* of an embryonic cell line (BRL-AG-3C) derived from the cotton boll weevil *Anthonomus grandis* (Stiles et al., 1992b) and the use of this cell line in two assays with an ecdysone responsive reporter construct to screen for ecdysone agonistic activity. We compared these two reporter assays: one where we transfected the cells with a plasmid with the reporter construct, and the other where we infected the cells with a recombinant baculovirus that has incorporated the same reporter construct in its genome. Similar assays are already reported with the use of lepidopteran (Swevers et al., 2004) or dipteran cells (Soin et al., 2008), but to our knowledge not with coleopteran cells. As is the case with binding assays, activities of DBH analogue compounds show much lower activity in coleopteran than in lepidopteran reporter assays (Swevers et al., 2004; Wheelock et al., 2006). Finally, we present a model of the LBD of AgEcR bound to ecdysteroid and DBH analogue. Specifically, it was of interest to compare the binding mode of a DBH analogue to the AgEcR ligand-binding pocket (LBP) with the interactions observed in the lepidopteran EcR-LBP to indicate differences that could explain the differences in activity of DBH analogues recorded in coleopteran and lepidopteran binding and cell-based reporter assays (Ogura et al., 2005; see further below).

The weevil *A. grandis* (Curculionidae) is a very important pest insect in cotton, although almost eradicated in the USA by an eradication program from which the trials started in 1978 (Smith and Swink, 2003). However, this species represents the order of Coleoptera comprising agricultural pests such as the Colorado potato beetle *Leptinotarsa decemlineata* and the corn rootworm *Diabrotica virgifera*, storage pests such as *Sitophilus oryzae* and *Tribolium castaneum*, and many tree and forest pests.

2. Material and methods

2.1. Chemicals

A technical grade of the ecdysone agonists RH-5849 (~100%), halofenozide (>90% pure), tebufenozide (>95% pure) and methoxyfenozide (>95% pure) were a kind gift of Rohm and Haas Co. (Spring House, PA). 20E (≥95% pure) was purchased from Sigma–Aldrich and ponasterone A (PonA) from Invitrogen. Serial dilutions of these test compounds were prepared in ethanol.

2.2. Cell lines

The embryonic cell lines derived from the cotton boll weevil *A. grandis*, BRL-AG-3A and BRL-AG-3C (Stiles et al., 1992b), and a Colorado potato beetle cell line established from pupal tissue, BCIRL-Lepd-SL1 (Long et al., 2002), were cultured in EX-CELL® 420 (Sigma–Aldrich) supplemented with 5% FBS (Invitrogen). The cell lines were obtained from the Biological Control of Insects Research Laboratory, USDA-ARS, Columbia, MO.

To confirm the species origin of the cell line BRL-AG-3C, we cloned a fragment of the mitochondrial 16S rRNA gene from this coleopteran cell line using the following universal primers: 5′-CGACTGTTTATCAAAAACAT-3′ or 5′-CGACTGTTTAACAAAAACAT-3′ as forward primer and 5′-GGTCTGAACTCAGATCATGT-3′ as reverse primer (Douris et al., 1998). DNA from the cell line was isolated with the Tissue DNA Kit (Omega Bio-Tek). PCR amplification conditions were 20 s at 94 °C, 1 min at 46 °C, 40s at 72 °C for 30 cycles. PCR products were cloned into the pGEM®-T vector (Promega) and sequenced by Agowa. The partial sequence (GenBank accession no. FJ423738) that was obtained showed very high identity to the partial 16S rRNA sequences of *Anthonomus rubi* (GenBank accession no. AJ495539; 91% identity), *Anthonomus pomorum* (GenBank accession no. AJ495540; 89% identity) and *Furcipes rectirostris* (GenBank accession no. AJ495541; 88% identity). These results confirm the *A. grandis* origin of the cell line.

2.3. Testing ecdysone agonists on transfected or infected coleopteran cell lines

Stiles et al. (1992a) reported that Lipofectin™ was very efficient to transfect the BRL-AG-3C cell line. So we tried to transfect the *A. grandis* cell lines BRL-AG-3A and BRL-AG-3C and the *L. decemlineata* cell line BCIRL-Lepd-SL1 with Lipofectin™ (Invitrogen). A well of a 6-well plate was filled with 1×10^6 cells. After the cells were attached the cells were washed twice with serum-free EX-CELL® 420 (Sigma–Aldrich). For one well or 1×10^6 cells, 15 μl Lipofectin™ was incubated together with 85 μl of the same medium for 45 min at room temperature and then 15 min together with 1.5 μg of the reporter construct ERE-b.act.luc (Soin et al., 2008) before adding to the cells. The cells were incubated for 5 h with the transfection medium. Transfected cells at a density of around 50,000 cells in 100 μl were incubated for 24 h with 20E or an ecdysone agonist. The Steady-Glo® Luciferase Assay System kit (Promega) was used for measuring the luciferase expression and the luminescence was measured with an Infinite M200 luminometer (Tecan). For every concentration four replicates were used and each experiment was repeated three times.

We also tried to infect the same coleopteran cell lines with a genetically modified baculovirus, BmNPV/A.GFP/ERE-b.act.luc (Swevers et al., 2008), and to use these infected cells to test the ecdysone responsiveness of (potential) ecdysone agonists. Cells were seeded in a 96-well plate at a density of 500,000 cells/ml. After 1 h incubation to let the cells adhere to the substrate, 50 μl medium of each well was aspirated and replaced by 'virus medium'. This 'virus medium' is the supernatant of a Bm5 cell culture after infection with the modified baculovirus (Swevers et al., 2008). Then 1 μl of a concentration range of ecdysone agonist or 20E was added to the cell suspension in the wells. After 24 h of incubation the luciferase expression was measured with the Steady-Glo Luciferase Assay System kit (Promega). For every concentration four replicates were used and each experiment was replicated three times.

Median effective concentrations, EC_{50}s, were calculated with GraphPad Prism Version 4.00 (GraphPad software) using sigmoid dose–response (variable slope). One should be cautious when comparing the activities of ecdysone agonists with only using these EC_{50}-values. An EC_{50}-value is calculated by calculation of the point of inflection of the sigmoid dose–response curve and it does not say anything about the plateau of the curve (i.e. the maximal induction). It is possible when comparing two compounds that