Properties of ecdysteroid receptors from diverse insect species in a heterologous cell culture system – a basis for screening novel insecticidal candidates

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Abstract:

Insect development is driven by the action of ecdysteroids on morphogenetic processes. The classic ecdysteroid receptor is a protein heterodimer composed of two nuclear receptors, the ecdysone receptor (EcR) and Ultraspiracle (USP), the insect ortholog of retinoid X receptor. The functional properties of EcR and USP vary among insect species, and provide a basis for identifying novel and species-specific insecticidal candidates that disrupt this receptor's normal activity. A heterologous mammalian cell culture assay was used to assess the transcriptional activity of the heterodimeric ecdysteroid receptor from species representing two major insect orders: the fruit fly, *Drosophila melanogaster* (Diptera), and the Colorado potato beetle, *Leptinotarsa decemlineata* (Coleoptera). Several nonsteroidal agonists evoked a strong response with the *L. decemlineata* heterodimer that was consistent with biochemical and *in vivo* evidence, whereas the *D. melanogaster* receptor's response was comparatively modest. Conversely, the phytoecdysteroid muristerone A was more potent with the *D. melanogaster* heterodimer. The additional presence of juvenile hormone III potentiated the inductive activity of muristerone A in the receptors from both species, but juvenile hormone III was unable to potentiate the inductive activity of the diacylhydrazine methoxyfenozide (RH2485) in the receptor of either species. The effects of USP on ecdysteroid-regulated transcriptional activity also varied between the two species. When it was tested with *D. melanogaster* EcR isoforms, basal activity was lower and ligand-dependent activity was higher with *L. decemlineata* USP than with *D. melanogaster* USP. Generally, the species-based differences validate the use of the cell culture assay screen for novel agonists and potentiators as species-targeted insecticidal candidates.

Keywords: cell culture; Drosophila; insecticide; juvenile hormone; nonsteroidal agonist

Abbreviations:

20E, 20-hydroxyecdysone; bHLH-PAS, basic helix–loop–helix Per-Arnt-Sim; CHO, Chinese hamster ovary; DBD, DNA-binding domain; DmEcR, *Drosophila melanogaster* EcR; DmUSP, *Drosophila melanogaster* USP; EcR, ecdysone receptor; EcRE, ecdsyone response element; EMSA, electrophoretic mobility shift assay; JH, juvenile hormone; LBD, ligand-binding domain; LdEcR, *Leptinotarsa decemlineata* EcR; LdUSP, *Leptinotarsa decemlineata* USP; MakA, makisterone A; MET, Methoprene-tolerant; MurA, muristerone A; PonA, ponasterone A; RXR, retinoid X receptor; USP, ultraspiracle

Article:

Insect development is largely driven by the action of ecdysteroids and its modulation by juvenoids. For all insects and many other arthropods, ecdysteroid action is mediated by the heterodimerization of two nuclear receptors, the ecdysone receptor (EcR) and its partner, ultraspiracle (USP), the insect ortholog of the vertebrate

retinoid X receptor (RXR). Many essential characteristics of ecdysteroid action are well described in *Drosophila melanogaster* [1,2], and have since been confirmed and further investigated in other insect species [3,4]. Generally, one or more isoforms of EcR and USP in a given species trigger an orchestrated and multitiered hierarchy of transcriptional changes in target cells that ultimately mediate the morphogenetic changes associated with molting, metamorphosis, and reproductive physiology [5].

Although the basic molting mechanism is highly conserved, it is apparent that the characteristics of the EcR–USP heterodimer vary among species. This is readily seen in the species-specific effects of the diacylhydrazines, nonsteroidal agonists that show order-specific differences in receptor affinity and *in vivo* toxicity [6]. Biochemical and cell culture studies of EcR and USP have also revealed species-specific functional characteristics that presumably underlie differences in ecdysteroid-driven developmental events [7–11]. Steroids and nonsteroidal agonists bind exclusively to the EcR ligand-binding domain (LBD), although the presence of USP increases ligand-binding affinity [12–15].

The diversity of ligand-responsive characteristics seen among ecdysteroid receptors from various insect species suggests a basis for identifying and screening for compounds that perturb normal receptor function [12,13,15,16]. Ecdysteroid receptor-mediated transcriptional activity has been measured in mammalian cells, which have no endogenous response to insect ecdysteroids, by transfecting them with the genes encoding EcR and USP, along with an ecdysteroid-inducible reporter [17–19]. An analysis of species-specific versions of EcR and USP and site-directed mutations in this heterologous cell system has generally established that the effects of ecdysteroids and other diacylhydrazine-based agonists can be measured by reporter gene activity [8,19,20]. Furthermore, the *Drosophila* EcR–USP heterodimer is potentiated by the presence of juvenile hormone (JH) in mammalian cells; that is, JH dramatically reduces the ecdysteroid concentration necessary to attain maximal induction from an ecdysteroid-inducible reporter gene [9,21]. The mechanism for potentiation has not been elucidated, although it reveals a modulatory action that may be useful for identifying novel insecticides acting as disruptors of normal ecdysteroid action. This possibility increases the importance of evaluating the heterologous cell culture assay as a valid tool for the assessment of ecdysteroid receptor capabilities from specific species.

Hundreds of phytocompounds that act as nonsteroidal and steroidal agonists of the insect ecdysteroid receptor have been identified [22,23], and a large number of JH analogs and mimics have also been isolated from plants [24]. If the cell culture assay has utility as a method for detecting novel inducers and/or JH potentiators of EcR–USP, then receptors from an insect species such as the Colorado potato beetle, *Leptinotarsa decemlineata*, are expected to evoke a profile of response that varies considerably from those previously reported for *D. melanogaster*. Furthermore, these characteristics are expected to be consistent with *in vivo* measurements of ecdysteroid activity in *L. decemlineata* [16,20,25–27]. *L. decemlineata* belongs to a relatively primitive insect order, the Coleoptera. Owing to its worldwide importance as a pest insect and its well-established ability to develop resistance to insecticides, the species has been well studied for its susceptibility to a variety of agonists [28,29].

The *L. decemlineata* ecdysteroid receptor shows the general structural features shared by all EcR and USP sequences characterized among insects and other arthropods [5,30,31]. Two EcR isoforms (A and B) have been identified so far in the *L. decemlineata* genome. *L. decemlineata* USP (LdUSP) carries an LBD that is remarkably similar to the vertebrate RXR, and lacks many of the features found in *D. melanogaster* USP (DmUSP), such as glycine-rich regions and a B-loop between helices 2 and 3 [30–32]. This divergence between the Coleopteran USP LBD (often referred to as RXR in this order) with those of the Lepidoptera and Diptera has been noted, suggesting a concomitant functional divergence [32]. Whereas the cell culture assay has been employed to survey the responses of ecdysteroid receptors from several species, this work focuses on a direct and thorough comparison of several attributes associated with well-described ecdysteroid receptors from two insect species for which relevant biochemical and *in vivo* information exists. The comparative profiles demonstrate an approach for developing a screening system to identify and characterize candidate insecticidal

compounds showing both inductive and potentiative activity.

Results

The DNA-binding domains (DBDs) of *Leptinotarsa* and *Drosophila* EcR and USP are identical at every amino acid position that is conserved among all EcR and USP DBD sequences, respectively, and share an overall identity of over 90% in both cases [31]. Therefore, it was expected that the canonical *hsp27* ecdsyone response element (EcRE) would allow direct comparisons of agonist inducibility when tested with EcR–USP from each of the two species. Sequence conservation is not as extensively shared in the LBD, where the identity between *D. melanogaster* EcR (DmEcR) and *L. decemlineata* EcR (LdEcR) is about 67% [21] (Fig. S1). USP LBD conservation is < 39% between the two species [21] (Fig. S2).

The N-terminal (A/B) domains of EcR are also divergent in the two insect species [31] (Fig. 1), although all of the isoforms from both species share almost complete identity over a stretch of 35–37 amino acids that lie just to the N-terminal side of the DBD (Fig. 1C). The EcRA isoforms from the two species share a few similar motifs in the middle region of the A/B domain (Fig. 1A), whereas LdEcRB shares some identity with DmEcRB1 only in the most N-terminal region (Fig. 1B).

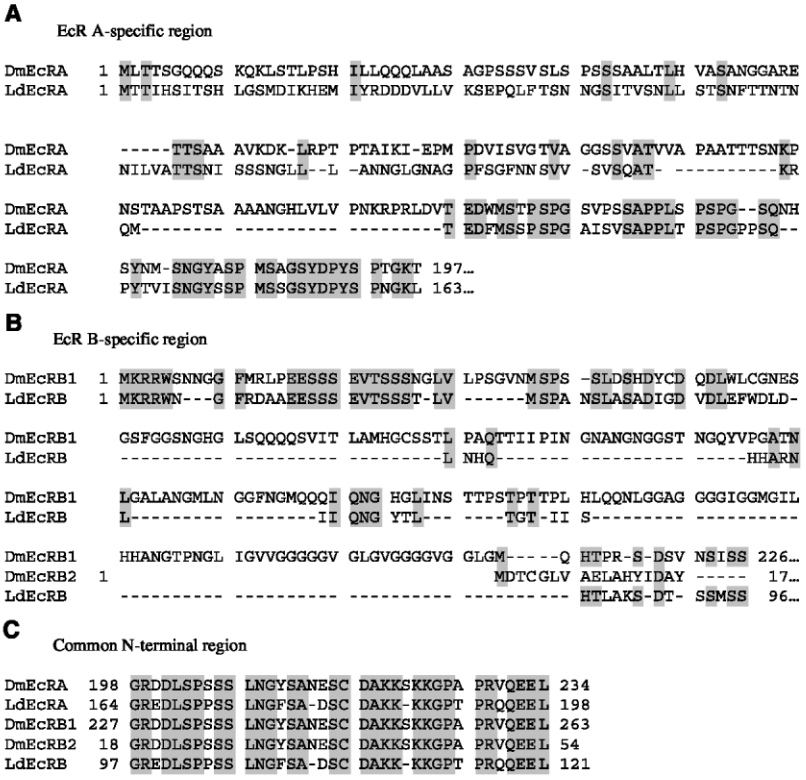

Fig. 1. CLUSTALW amino acid alignment of N-terminal (A/B) domains of LdEcR and DmEcR. (A) Alignment of EcRA from the two species. (B) Alignment of DmEcRB1, DmEcRB2, and LdEcRB. (C) Alignment of most carboxy-terminal side of the A/B region shared among all isoforms of both species.

Effects of selected agonists on EcR–USP transcriptional activity in the two species

In an initial series of experiments, the basal and ligand-induced properties of the three *D. melanogaster* isoforms (DmEcRA, DmEcRB1, and DmEcRB2) with the VP16-DmUSP heterodimer used in earlier studies were compared with those of the *L. decemlineata* isoforms (EcRA and EcRB) paired with the equivalent VP16-LdUSP construct [18]. Activity was determined by measuring reporter gene (luciferase) activity mediated by the *hsp27* EcRE after normalization for cell mass using β-galactosidase activity registered via a constitutive promoter.

In order to compare the efficacy of agonists, maximally inducing doses of several ecdysteroids and the most inductive nonsteroidal agonist, methoxyfenozide (RH2485), based on preliminary experiments, were tested.

The pattern of response was similar for each of the three *D. melanogaster* isoforms (Fig. 2A). In all cases, muristerone A (MurA) (2.5 μm) evoked the strongest fold induction, and the greatest absolute level of transcriptional activity. RH2485 also evoked a response from all three DmEcR isoforms, with lesser responses from the natural molting hormone, 20-hydroxyecdysone (20E), and makisterone A (MakA), the latter being the most abundant ecdysteroid in late third instar whole body titers of *D. melanogaster* [33]. The relatively modest response to natural ecdysteroids such as 20E has been noted in previous cell culture studies. Also, differences in the quantitative levels of transcription were previously reported, with DmEcRB1 showing the highest levels of basal and induced activity, and EcRA displaying the lowest levels of activity [9].

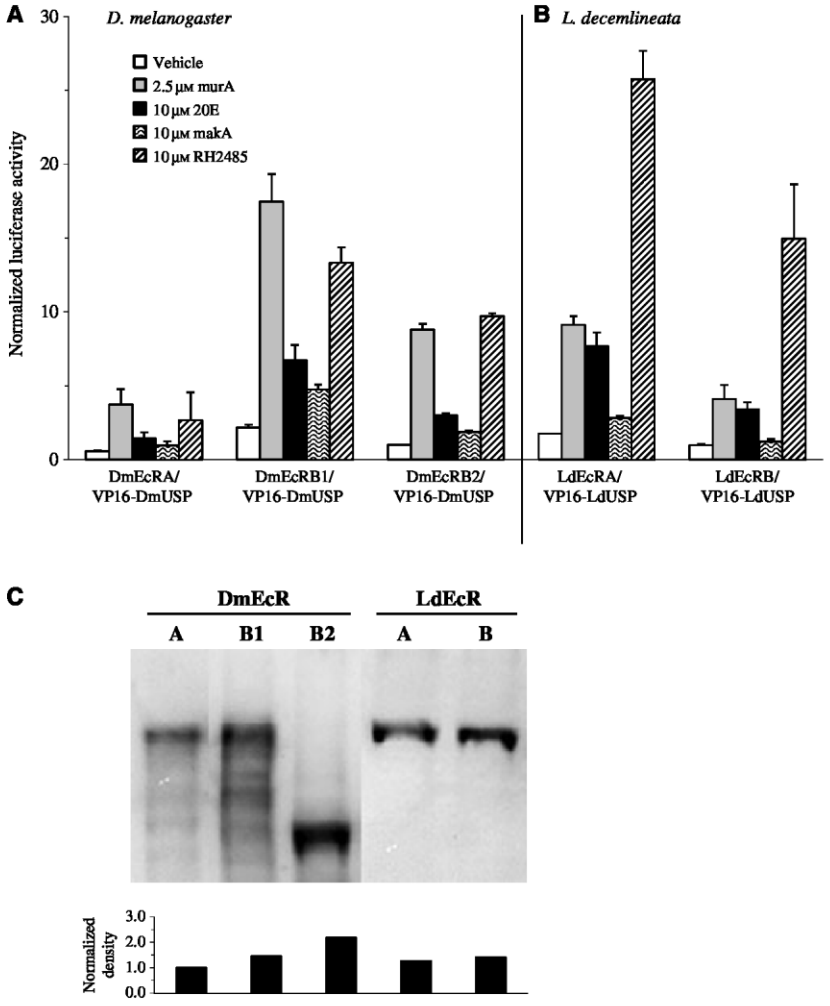

Fig. 2. Effects of maximal dosages of selected agonists (20E, MurA, MakA, and RH2485) upon normalized ecdysteroid receptor-mediated transcriptional activity with DmEcR–DmUSP or LdEcR–LdUSP expressed in CHO cells. All transcriptional activity values are normalized on the basis on cell mass as measured by β-galactosidase reporter gene activity. Levels of all activities were then adjusted relative to DmEcRB2–DmUSP in the absence of hormone (assigned a value of 1.0), to allow for direct comparison of quantitative transcriptional activity. All data points are based on $n = 3$; error bars indicate one standard deviation. (C) Western immunoblot of CHO cell extracts expressing the EcR vectors used in this study as detected with 9B9 (LdEcR) and DDA2.7 (DmEcR) monoclonal antibodies, as described in the text. Extracts from cells grown in culture medium with no added agonist were equalized for gel loading on the basis of β-galactosidase reporter gene activity. Densitometry readings for individual signals are adjusted relative to DmEcRB2 (equals 1.0).

The response profile observed for each of the two LdEcR–LdUSP heterodimers varied considerably from those seen with the DmEcR–DmUSP heterodimers (Fig. 2B). RH2485 evoked a much higher fold induction (up to 25-fold) from the *L. decemlineata* heterodimers. By contrast, the response of LdEcR–LdUSP to MurA and 20E was relatively modest as compared with that of DmEcR–DmUSP. Minimal induction was seen with MakA with receptors from either species.

Differences in normalized induction in this experiment and others are not attributable to differences in cell growth caused by the effects of the individual ligands. The β-galactosidase reporter gene measurements used to normalize transcriptional activity (by providing an estimate of cell mass) varied by < 20% for all the ligand regimens applied. Also, the absolute β-galactosidase values varied by < 20% between experiments; that is, cell growth rates were relatively constant (data not shown).

Immunoblots were also performed with cell extracts expressing the EcR isoforms employed in this study, to determine whether transcriptional activity levels are related to expression levels. Although the signal evoked

from individual isoforms varied to some degree, as noted in previous work [9], the strength of signal did not correlate with differences in transcriptional activity (Fig. 2C). In summary, each of the isoforms within a species generated a similar responsiveness to maximal dosages of individual agonists. Whereas the EcR N-terminal domain influences the quantitative level of transcription for a given isoform, it had no effect on relative ligand responsiveness. Importantly, the relative induction by individual agonists was species-specific for all of the tested ligands, and the responsiveness to RH2485 was much higher in *Leptinotarsa* than in *Drosophila*, whereas DmEcR–DmUSP was more responsive to MurA than to any other agonist.

Effects of selected ecdysteroids and nonsteroidal ecdysteroid agonists on transcriptional activity in the two species

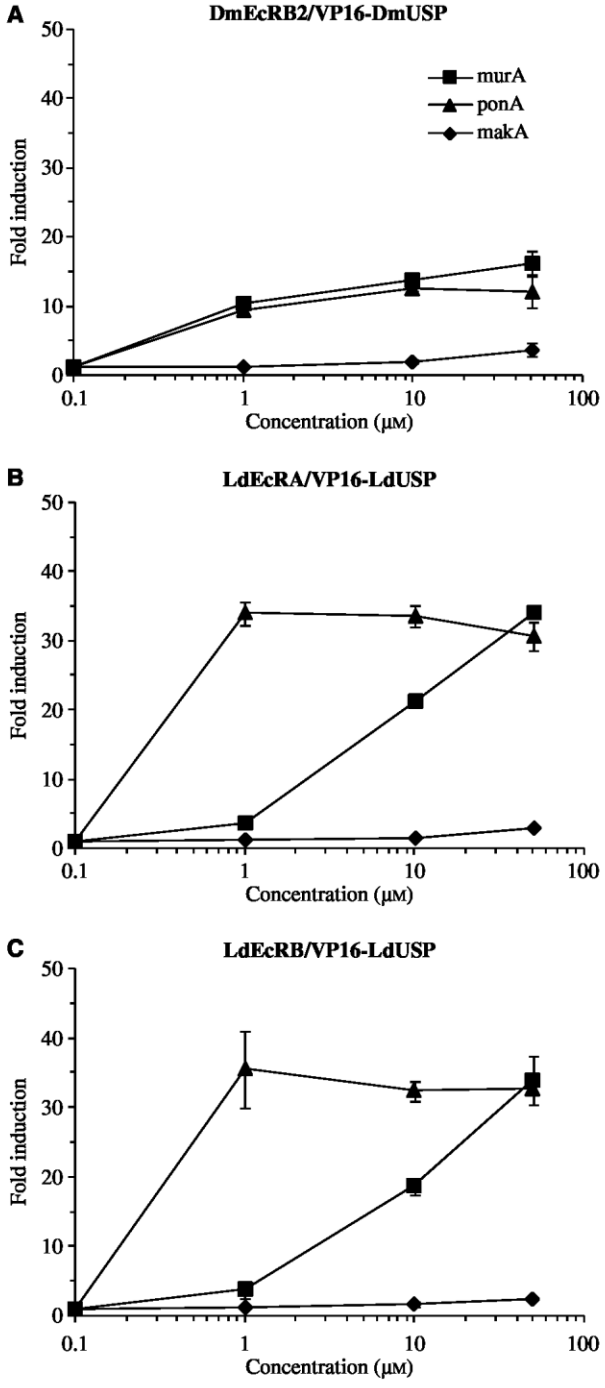

Fig. 3. Fold induction caused by the natural ecdysteroids 20E, MurA, PonA and MakA of ecdysteroid receptor-mediated transcriptional activity in CHO cells over a dosage range. (A) DmEcRB2. (B) EcRA. (C) LdEcRB. All luciferase activity levels were normalized on the basis of β-galactosidase activity as a measure of cell mass. For each agonist, fold inductions are shown relative to the normalized luciferase activity observed in the absence of the test agonist (assigned a value of 1). All data points are based on $n = 3$ that were tested at the same time; error bars indicate one standard deviation.

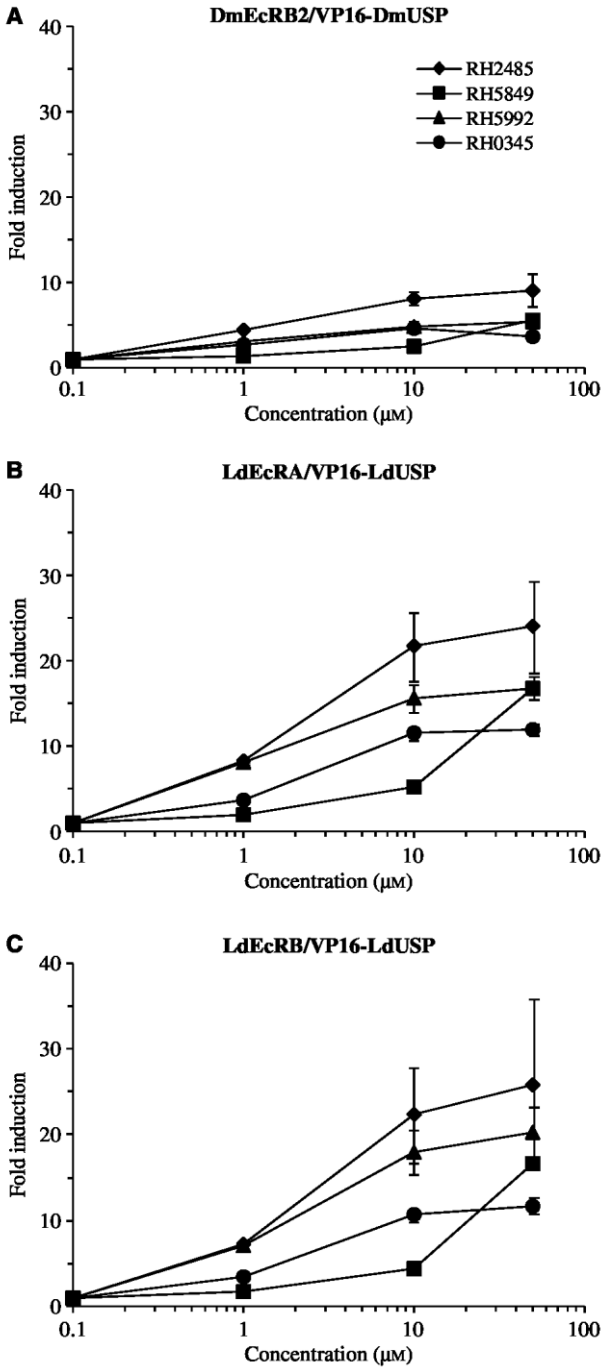

Fig. 4. Fold induction caused by the nonsteroidal agonists RH0345, RH2485 and RH5849 of ecdysteroid receptor-mediated transcriptional activity in CHO cells over a dosage range. (A) DmEcRB2. (B) LdEcRA. (C) LdEcRB. All luciferase activity levels were normalized on the basis of β-galactosidase activity as a measure of cell mass. For each agonist, fold inductions are shown relative to the normalized luciferase activity observed in the absence of the test agonist (assigned a value of 1). All data points are based on $n = 3$ that were tested at the same time; error bars indicate one standard deviation.

The potency of natural and nonsteroidal agonists was further evaluated by comparing the dose response of DmEcRB2–DmUSP with those of the two LdEcR–LdUSP complexes. Three natural ecdysteroids, MurA, ponasterone A (PonA), and MakA, were tested in receptors from both species (Fig. 3A–C). MurA was significantly more potent with receptors of *D. melanogaster* than with those of *L. decemlineata*. Whereas DmEcR–DmUSP showed a maximal response in the range of 1–10 μm MurA, LdEcR–LcUSP required about

50 μm MurA to show a maximal response. Nevertheless, the maximal induction evoked by MurA at 50 μm was over 30-fold with *L. decemlineata*. Receptors from both species were maximally induced by 1 μm PonA, and neither species responded strongly to MakA, even at 50 μm.

Four nonsteroidal ecdysteroid agonists, halofenozide (RH0345), methoxyfenozide (RH2485), RH5849, and tebufenozide (RH5992), were also tested over a range of dosages with receptors from both species (Fig. 4A–C). The maximal fold induction evoked by nonsteroidal compounds was considerably higher among the LdEcR dimers than it was for the compared DmEcRB2–DmUSP heterodimer. Except for RH5849, each of the RH compounds evoked a maximal induction at 10 μm with the LdEcR–LdUSP dimers that was > 10-fold. The order of fold induction obtained for the pooled results (i.e. LdEcRA and LdEcRB) was RH2485 = RH5992 > RH0345 > RH5849; one-way ANOVA, $P \leq 0.01$). By contrast, the *Drosophila* receptor showed a more modest induction with all of the nonsteroidal ecdysteroid agonists, never exceeding 10-fold (Fig. 4A).

An electrophoretic mobility shift assay (EMSA) was also performed using cell culture extracts expressing DmEcRB1–DmUSP and DmEcRB2–DmUSP or the LdEcR–LdUSP combinations to verify their interaction with the *hsp27* EcRE. The observed shifts associated with the *hsp27* EcRE revealed that DmEcRB1–VP16-DmUSP showed an increased shift intensity in the presence of agonist, and that that of DmEcRB2–VP16-DmUSP was modestly increased by the presence of agonist (Fig. 5) [9]. Under identical experimental conditions, the two LdEcR–LdUSP complexes showed little change in shift intensity when an agonist was present. The variability among the individual EcR–USP pairings could be attributed to the selected conditions, which had been optimized for testing DmEcR–DmUSP.

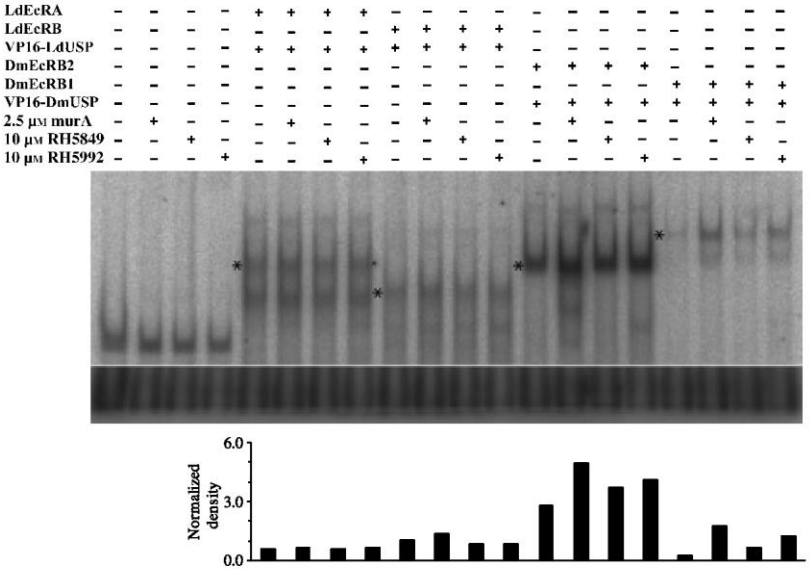

Fig. 5. EMSA using CHO cell extracts following transfection and incubation in the absence and presence of MurA, RH5849, and RH5992, using the *hsp27* EcRE as a labeled probe. Asterisk designates shift band. All extracts were equilibrated by β-galactosidase activity prior to loading. Densitometry readings corresponding to designated shift bands are indicated below the image and adjusted relative to the signal generated by LdEcRB (equals 1.0).

Effect of JH on EcR–USP transcriptional activity in the two species

When Chinese hamster ovary (CHO) cells expressing DmEcR–DmUSP are challenged with JHIII alone, no effect on transcriptional activity is observed [9]. However, the simultaneous presence of JHIII in a cell culture medium that already contains ecdysteroids reduces the concentration of ecdysteroids necessary for maximal transcriptional activity by about 10-fold. In other words, JHIII potentiates the responsiveness of EcR–USP to ecdysteroids [9,14,21]. Using the same paradigm employed for measuring potentiation in the *Drosophila* system, a submaximal dosage of MurA together with JHIII was simultaneously tested with cells expressing LdEcR–LdUSP. Under these conditions, partial and significant potentiation by JHIII was observed in the *L. decemlineata* receptor (Fig. 6A; $P \geq 0.01$, *t*-test).

The potentiation testing paradigm was then modified by testing the nonsteroidal agonist RH2485 instead of MurA. No potentiation by JHIII was seen in either *D. melanogaster* or *L. decemlineata*, using RH2485 as an

agonist (Fig. 6B). This result indicates that potentiation by JHIII is not a general cellular effect, but depends upon the specific agonist–EcR interaction.

Effects of *L. decemlineata* and *D. melanogaster* USP constructs on ecdysteroid-inducible transcriptional activity

As noted, when VP16-DmUSP/DDBD is tested with the three D. *melanogaster* EcR isoforms, EcRA and EcRB2 heterodimers form a relatively inactive dimer [9] (Fig. 7A). However, DmUSP/ΔDBD retains nearly normal activity when paired with EcR-B1, indicating that the nature of the EcR–USP interaction is isoformspecific [9,34] (Fig. 7A). The analogous VP16-LdUSP/ΔDBD was tested with LdEcRA and LdEcRB. In both cases, the expression of VP16-LdUSP/ΔDBD, as verified by immunoblots (data not shown), resulted in a heterodimer with severely reduced transcriptional activity (Fig. 7B).

In order to compare the capabilities of DmUSP and LdUSP further, cross-species heterodimers were tested for transcriptional activity (Fig. 7C). At least four functional differences were observed: (a) the DmEcRB1 and DmEcRB2 isoforms display a higher level of ligand-dependent (induced) transcriptional activity with VP16-LdUSP than with the equivalent VP16-DmUSP; (b) the same EcRB1 and EcRB2 isoforms display a lower level of ligand-independent (basal) transcriptional activity with VP16-LdUSP than with VP16-DmUSP; (c) VP16-LdUSP/ΔDBD forms a relatively inactive dimer with DmEcRB1, unlike VP16-DmUSP/ΔDBD; and (d) VP16-DmUSP consistently evokes a lower quantitative level of transcriptional activity, with both its own EcR isoforms, and with the two *L. decemlineata* EcR isoforms.

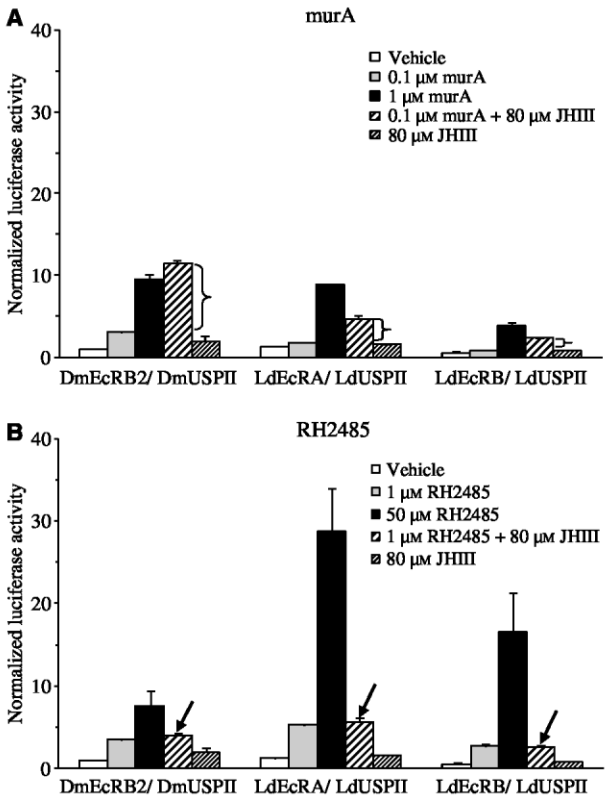

Fig. 6. Effects of JHIII on transcriptional activity induced by (A) MurA and (B) RH2485 of DmEcRB2–VP16-DmUSP and analogous LdEcR–VP16-LdUSP complexes. Parentheses in (A) indicate a potentiation effect, and arrows in (B) indicate an absence of potentiation when RH2485 is the agonist. All transcriptional activity levels are adjusted to DmEcRB2–VP16-DmUSP in the absence of ligand (assigned a value of 1.0). No effect upon transcriptional activity was observed when JHIII was tested with RH2485.

Discussion

A controlled assessment and comparison of the *Leptinotarsa* and *Drosophila* EcR–USP heterodimers in this study reveals a variety of distinctions between them in terms of quantitative level of transcriptional activity, ligand responsiveness, and capability for potentiation by JHIII. These findings are generally consistent with expectations from other *in vivo* and biochemical work with the two species' receptors, and indicate that the CHO cell culture assay system can be validly employed to characterize individual insect EcR–USP heterodimers for their responsiveness to agonists and potentiators.

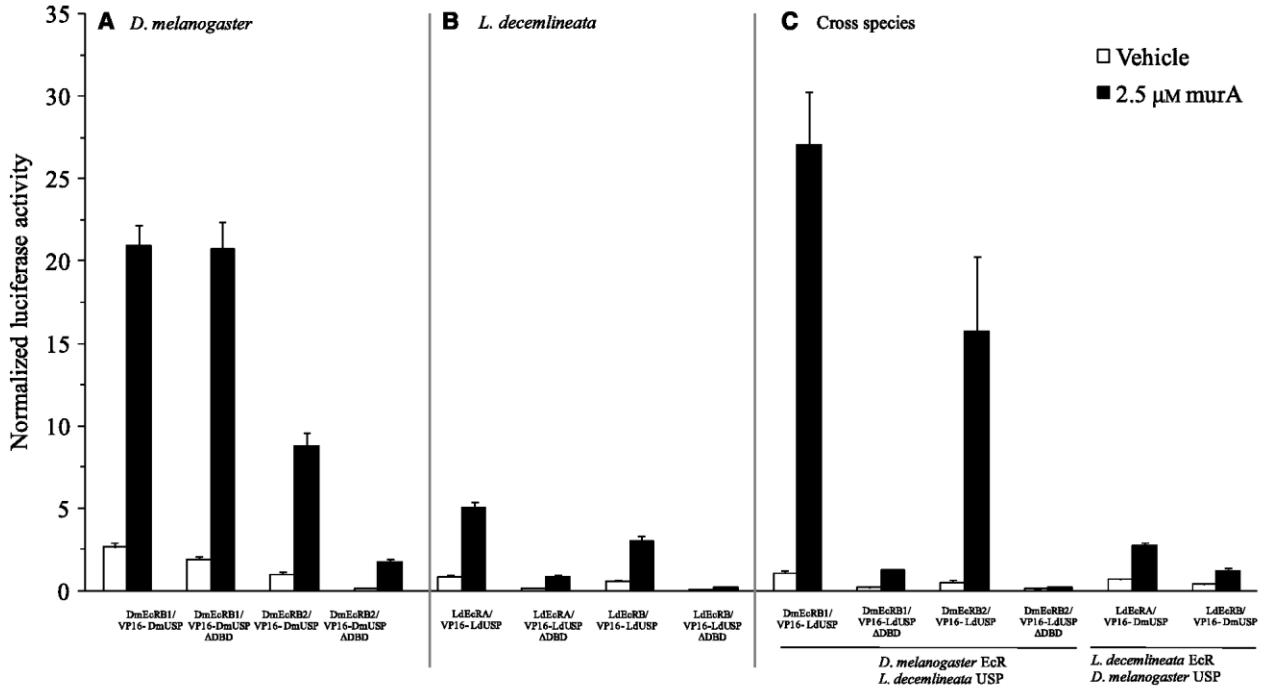

Fig. 7. Effects of VP16-USP and VP16-USP/ΔDBD on MurA-inducible transcriptional activity at 2.5 μM. (A) DmEcRB1 and DmEcRB2 with VP16-DmUSP and VP16-DmUSP/ΔDBD. (B) LdEcRA and LdEcRB with VP16-LdUSP and VP16-LdUSP/ΔDBD. (C) Cross-species EcR–USP heterodimers, as designated. All levels are adjusted to the activity observed in EcRB2–VP16-DmUSP in the absence of agonist (equals 1.0). All data points are based on $n = 3$ and replicates were run simultaneously. Error bars indicate one standard deviation.

Utility of the cell culture as a screening assay for novel agonists

The differences in characteristics of the ecdysteroid receptors from the two species studied here, and the general consistency with previously published results [25–27], suggest a basis for screening plant extracts and candidate insecticides affecting EcR–USP-mediated induction or potentiation in either or both species.

The fold induction evoked by the tested RH compounds on transcriptional activity of LdEcR approximately corresponded with their ligand affinity [12,19]. Nevertheless, although RH0345 is not the most efficacious of the RH compounds in the cell culture assay, it is actually the most toxic of these compounds in *L. decemlineata*, owing to its relative persistence in target tissues [35]. This observation highlights the reality that a robust fold induction in the assay is not necessarily the best indication of toxicity. The study alternatively suggests that ligand potency may be the best primary criterion for isolating insecticidal candidates within a given species, even if fold induction is modest. The potency of RH0345 with the LdEcR isoforms was similar to those of RH2485 and RH5992, and all three of these RH compounds showed greater potency and efficacy than RH5849, which is weakly toxic in *L. decemlineata*. Finally, all of the RH compounds yielded a higher fold induction with the *L. decemlineata* receptor than with the receptor of *D. melanogaster*, which is relatively unresponsive to the effects of RH compounds [36], thus suggesting that fold induction can serve as a basis for predicting differences in the toxicity of a compound between species. The weak inductive effects of the natural ecdysteroids (MurA, PonA, MakA, and 20E) further show a lack of correspondence between fold induction and ligand affinity, as the affinities of the natural ecdysteroids for EcR are higher than the affinities of the diacylhydrazines [12].

The differences in fold induction observed between the natural steroids and the nonsteroidal agonists is predictable, as these agonist classes involve different amino acid interactions in the ligand-binding pocket. Nevertheless, both DmEcR and LdEcR carry the same residue at each of the putative binding sites ascribed to the RH compounds [8], consistent with the suggestion that other features of the ligand-binding pocket account for species differences in responsiveness to RH compounds [13].

EcR and USP

Transcriptional activity levels varied widely among the three Drosophila isoforms and two Leptinotarsa isoforms. Such quantitative differences may prove important for in vivo functions. In Manduca, the presence of a B-isoform increases transcriptional activity normally mediated by the A-isoform alone, heightening the possible relevance of these differences for in vivo regulation [37].

There is growing evidence that changes in net activity induced by ecdysteroids and nonsteroidal agonists in the cell culture system involve not only allosteric changes in the receptor itself, but also factors such as the effect of DNA and ligand on receptor stability and the regulation of nuclear receptor transport in the cell [38–41]. Therefore, differences between basal and induced transcriptional activity must be viewed as a net effect resulting not only from changes in the level of receptor molecule activity, but also from changes in stability and intracellular localization. Possible differences in these parameters among EcR–USP dimers from different species have not been explored extensively, although the relationship between protein stability and ligand interactions has been noted for *Drosophila* E75 and its interaction with heme [42]. Degradation of DmEcR is seen at specific developmental periods [43].

The studies also demonstrated that DmUSP and LdUSP are not interchangeable in terms of transcriptional activity, although USP does not affect ligand affinity when tested in cross-species dimers [12]. Species-specific differences in USP structure have already been implicated in the regulation of developmental events associated with larval growth and subsequent metamorphosis [44]. The effects observed in cross-species EcR–USP dimers further suggest that USP plays a role in determining the quantitative level of transcriptional activity.

Implications for a mechanism of potentiation

As noted earlier, the effects of potentiation suggest a low-affinity interaction between EcR–USP and JHIII. A similar effect for DmEcR–DmUSP has been observed for methyl farnesoate and other substrates within the mevalonate pathway [14]. The mechanism for this effect upon EcR–USP activity remains unknown, although the ability of JHIII to potentiate ecdysteroid inducibility has also been observed with polychlorinated biphenyls, whose activity is associated with members of the basic helix–loop–helix Per-Arnt-Sim (bHLH-PAS) transcription factor family [45]. Members of this family, in turn, include the *Drosophila* methoprene-tolerant (MET) gene product [46], and MET is known to bind to JHIII [47]. Mutations of the MET gene in *Drosophila* block the normally lethal effects of methoprene application [46]. Mammalian bHLH-PAS transcription factors bind to nuclear receptors, leaving the possibility for a MET–EcR–USP interaction. A physical interaction between MET and both EcR and USP has been reported [48], although its relevance for the functional effects of JHIII remains to be explored. The homolog of MET in *Tribolium castaneum* mediates JH action, further raising the possibility of a similar role in modulating ecdysteroid receptor action [49]. Nonsteroidal ecdysteroid agonists are known to confer a markedly different shape upon the ligand-binding pocket of EcR than natural ecdysteroids [8] that could prevent interactions with regulatory cofactors such as MET via the LBD. It is important to recognize that USP itself binds to JH and methyl farnesoate under certain experimental conditions [50]. Alternatively, the effect of RH2485 on EcR is to alter the shape of its ligand-binding pocket, thus blocking potentiation mediated by USP binding to JHIII. Finally, although MET explains some JH-mediated activities in *T. castaneum*, it does not account for all of them [49], leaving open the possibility that JH acts via multiple modes of action. The inability to see potentiation with nonsteroidal compounds at least demonstrates that the effects of JHIII cannot be attributed to a generalized cellular action upon the transcriptional complex that includes EcR and USP. Rather, the occurrence of potentiation depends upon the specific agonist.

Summary

The comparative study of the *Leptinotarsa* and *Drosophila* EcR–USP complexes further establishes the utility of the heterologous CHO cell culture system for assessing the effects of agonists/antagonists and other modulators on EcR–USP-mediated transcriptional activity. The insect ecdysteroid receptor is a commercially proven target for insecticidal action, and the assay provides a conceptual basis for high-throughput screening and identifying compounds that perturb receptor function, not only in terms of classic ecdysteroid agonist functions, but also for those compounds that are capable of mimicking or evoking the potentiation effect induced by JHIII in this assay.

Experimental procedures

Cell culture, EMSA, and western immunoblotting

All aspects of cell culture methodology, ligand application, transfection, reporter gene measurement, western immunoblotting and EMSAs have been previously reported [9,21]. Briefly, CHO cells were grown to confluence and transfected (250 ng each) with: (a) a plasmid vector containing the luciferase gene controlled by the canonical *hsp27* EcRE and a weak constitutive promoter [51]; (b) a vector containing the β-galactosidase gene controlled by a constitutively active promoter; (c) one of the EcR-encoding vectors described below; and (d) one of the USP-encoding vectors described below. After transfection for 6 h, cells were incubated with or without agonists and/or JHIII for 24 h, cells were harvested, and extracts were processed for the studies. The reagents tested included: MurA (Alexis Biochemical, San Diego, CA, USA), PonA, MakA (AG Scientific, San Diego, CA, USA), and JHIII (Sigma Chemical, St Louis, MO, USA). The diacylhydrazine-based agonists that were tested included RH0345, RH2485, RH5849, and RH5992, all > 95% pure, and kindly provided by Rohm and Haas Co. (Spring House, PA, USA). Western immunoblots of LdEcR and DmEcR were performed with the 9B9 and DDA 2.7 monoclonal antibodies, respectively, obtained from the Developmental Studies Hybridoma Bank at the University of Iowa.

Band densities were measured, using BioRad (Hercules, CA, USA) quantity one software from the EMSA and western immunoblot images. The pixel intensity of the band signal was determined for the defined band area and adjusted relative to one of the signals, as designated, to calculate the relative band density.

Vector description and construction

All DmEcR and DmUSP expression vectors and the luciferase (and β-galactosidase) reporter gene vectors have been described previously [9,21]. The expression vectors encoding the natural isoforms of DmEcR are denoted DmEcRA, DmEcRB1, and DmEcRB2.

The following protocols were used to construct the LdEcR cell culture vectors encoding its two natural isoforms (LdEcRA and LdEcRB). The LdEcRA ORF was isolated by PCR from pBluescriptKS + LdEcRA [31], using the forward primer 5′-TTTT GGATCC ACC ATG ACC ACC ATA CAC TCG ATC-3′ and the reverse primer 5′-TTTT TCTAGA CTA TGT CTT CAT GTC GAC GTC-3′. The underlined portions of the primers represent the inserted *Bam*HI and *Xba*I restriction sites, respectively. The vector pcDNA3.1+ and the LdEcRA amplicon were digested with the restriction endonucleases *Bam*HI and *Xba*I. The digestion products were purified from an agarose gel excision, and then ligated to create the vector pcDNA3.1 + LdEcRA. The LdEcRB fragment was removed from pBluescriptKS + LdEcRB [31], and the vector pcDNA3.1- (Invitrogen, Carlsbad, CA, USA) was linearized by restriction digestion with *Xba*I and *Bam*HI. Both restriction products were purified by excision from an agarose gel and then ligated to produce the vector pcDNA3.1-LdEcRB.

The vectors encoding DmUSP have also been described previously [9]. For these vectors, the N-terminal (A/B) domain of DmUSP was replaced with the VP16 activation domain, as the DmUSP A/B domain displays minimal transcriptional activity in CHO cells [18]. Two constructs were produced; VP16-DmUSP includes the USP DBD, whereas VP16-DmUSP/ΔDBD has had the DBD deleted.

The analogous VP16-LdUSP and VP16-LdUSP/ΔDBD vectors were constructed for this study as follows. The LdUSP and LdUSP/ΔDBD fragments were isolated by PCR from pBluescriptKS + LdUSP [31], using the

forward primer 5′-TTTT GAATTC TGC TCG ATTTGC GGG GAC AAG-3′for LdUSP (which is the 5′-end of the DBD-encoding DNA sequence) or 5′-TTTT GAATTC AAG CGG GAG GCG GTT CAA GAA-3′ (which lies just to the 3′-side of the DBD-encoding sequence). Each primer was paired with the reverse primer 5′-TTTT AAGCTT CTA AGT ATC CGA CTG GTT TTC-3′, which is the complement of the 3′-end of the LdUSP LBD. The respective *Eco*RI and *Hin*dIII restriction sites inserted by the PCR primers are underlined. The resulting LdUSP amplicon includes the entire DBD, whereas LdUSP/ΔDBD includes the entire ORF beginning at the first amino acid following the LdUSP DBD. Both amplicons and the pVP16 vector were digested with *Eco*RI and *Hin*dIII restriction endonucleases. Ligation of the products into the linearized pVP16 vector (Clontech, Mountain View, CA, USA) resulted in the pVP16-LdUSP and pVP16-LdUSP/ΔDBD constructs. All constructs were subsequently verified by DNA sequencing.

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References:

1. *Thummel CS (2002) Ecdysone-regulated puff genes 2000. Insect Biochem Mol Biol 32, 113–120.*
2. *Lezzi M, Bergman T, Mouillet J-F & Henrich VC (1998) The ecdysone receptor puzzle. Arch Insect Biochem Physiol 41, 99–106.*
3. *Riddiford LM, Cherbas P & Truman JW (2000) Ecdysone receptors and their biological actions. Vitam Horm 60, 1–73.*
4. *Billas I & Moras D (2005) Ligand-binding pocket of the ecdysone receptor. Vitam Horm 73, 101–29.*
5. *Henrich V (2005) The ecdysteroid receptor. In: Comprehensive Insect Physiology, Biochemistry, and Molecular Biology Series. Vol. 3. pp. 243–286.Elsevier Press, Oxford, UK.*
6. *Palli SR, Hormann RE, Schlattner U & Lezzi M (2005) Ecdysteroid receptors and their applications in agriculture and medicine. Vitam Horm 73, 60–100.*
7. *Mouillet J-F, Henrich VC, Lezzi M & Vogtli M (2001) Differential control of gene activity by isoforms A, B1, and B2 of the Drosophila ecdysone receptor. Eur J Biochem 268, 1811–1819.*
8. *Billas IM, Iwema T, Garnier JM, Mitschler A, Rochel N & Moras D (2003) Structural adaptability in the ligand-binding pocket of the ecdysone hormone receptor. Nature 426, 91–6.*
9. *Beatty J, Fauth T, Callender JL, Spindler-Barth M & Henrich VC (2006) Analysis of transcriptional activity mediated by the Drosophila melanogaster ecdysone receptor isoforms in a heterologous cell culture system. Insect Mol Biol 15, 785–795.*
10. *Graham LD, Pilling PA, Eaton RE, Gorman JJ, Braybrook C, Hannan GN, Pawlak-Skrzecz A, Noyce L, Lovrecz GO, Lu L et al. (2007) Purification and characterization of recombinant ligand-binding domains from the ecdysone receptors of four pest insects. Protein Expr Purif 53, 309–24.*
11. *Graham D, Johnson WM, Pawlak-Skrzeca A, Eaton RE, Bliese M, Howell L, Hannan GN & Hill RJ (2007) Ligand binding by recombinant domains from insect ecdysone receptors. Insect Biochem Mol Biol 37, 611–626.*
12. *Minakuchi C, Ogura T, Miyagawa H & Nakagawa Y (2007) Effects of the structures of ecdysone receptor (EcR) and ultraspiracle (USP) on the ligand-binding activity of the EcR/USP heterodimer. J Pestic Sci 32, 379–384.*
13. *Carmichael JA, Lawrence MC, Graham LD, Pilling PA, Epa VC, Noyce L, Lovrecz G, Windler DA, Pawlak-Skrezecz A, Eaton RE et al. (2005) The X-ray structure of a hemipteran ecdysone receptor*

ligand-binding domain: comparison with a lepidopteran ecdysone receptor ligand-binding domain and implications for insecticide design. J Biol Chem 280, 22258–22269.

14. *Henrich VC, Beatty JM, Ruff H, Callender J, Grebe M & Spindler-Barth M (2009) The multidimensional partnership of EcR and USP. In: Ecdysones, Structures and Functions. pp. 361–375. Springer-Verlag, (in press).*
15. *Grebe M, Przibilla S, Henrich VC & Spindler-Barth M (2003) Characterization of the ligand-binding domain of the ecdysteroid receptor from Drosophila melanogaster. Biol Chem 384, 93–104.*
16. *Nakagawa Y (2005) Nonsteroidal ecdysone agonists. Vitam Horm 73, 131–173.*
17. *Christopherson KS, Mark MR, Bajaj V & Godowski PJ (1992) Ecdysteroid-dependent regulation of genes in mammalian cells by a Drosophila ecdysone receptor and chimeric transactivators. Proc Natl Acad Sci USA 1992, 89: 6314–18.*
18. *Yao TP, Forman BM, Jiang ZY, Cherbas L, Chen JD, McKeown M, Cherbas P & Evans RM (1993) Functional ecdysone receptor is the product of EcR and ultraspiracle genes. Nature 366, 476–479.*
19. *Hormann RE, Smagghe G & Nakagawa Y (2008) Multidimensional quantitative structure–activity relationships of diacylhydrazine toxicity to Lepidopteran and Coleopteran insect pests. QSAR Comb Sci 27, 1098–1112.*
20. *Wheelock CE, Nakagawa Y, Harada T, Oikawa N, Akamatsu M, Smagghe G, Stefanou D, Iatrou K & Swevers L (2006) High throughput screening of ecdysone agonists using a reporter gene assay followed by 3-D QSAR analysis of the molting hormonal activity. Bioorg Med Chem 14, 1143–1159.*
21. *Henrich VC, Burns E, Yelverton DP, Christensen E & Weinberger C (2003) Juvenile hormone potentiates ecdysone receptor-dependent transcription in a mammalian cell culture system. Insect Biochem Mol Biol 33, 1239–1247.*
22. *Dinan L, Savchenko T & Whiting P (2001) On the distribution of phytoecdysteroids in plants. Cell Mol Life Sci 58, 1121–1132.*
23. *Elbrecht A, Chen Y, Jurgens T, Hensens OD, Zink DL, Beck HT, Balick MJ & Borris R (1996) 8-O-Acetylharpagide is a nonsteroidal ecdysteroid agonist. Insect Biochem Mol Biol 26, 519–523.*
24. *Staal GB (1986) Anti-juvenile hormone agents. Annu Rev Entomol 31, 391–429.*
25. *Smagghe G & Degheele D (1994) Action of a novel nonsteroidal ecdysteroid mimic, tebufenozide (RH-5992), on insects of different orders. Pestic Sci 42, 85–92.*
26. *Smagghe G & Degheele D (1994b) The significance of pharmacokinetics and metabolism to the biological activity of RH-5992 (tebufenozide) in Spodoptera exempta, Spodoptera exigua, and Leptinotarsa decemlineata. Pestic Biochem Physiol 49, 224–234.*
27. *Nakagawa Y, Smagghe G, Tirry L, Kugimiya S, Ueno T & Fujita T (1999) Quantitative structure–activity studies of insect growth regulators: XVI. Substituent effects of dibenzoylhydrazines on the insecticidal activity to Colorado potato beetle Leptinotarsa decemlineata. Pestic Sci 55, 909–918.*
28. *Alyokhin A, Dively G, Patterson M, Castaldo C, Rogers D, Mahoney M & Wollam J (2007) Resistance and cross-resistance to imidacloprid and thiamethoxam in the Colorado potato beetle Leptinotarsa decemlineata. Pest Manag Sci 63, 32–41.*
29. *Khelifi M, Lague C & de Ladurantaye Y (2007) Physical control of colorado potato beetle: a review. Appl Eng Agric 23, 557–569.*
30. *Laudet V & Bonneton F (2005) Evolution of nuclear hormone receptors in insects. In: Comprehensive Insect Physiology, Biochemistry, and Molecular Biology Series. Vol. 3. pp. 287–318. Elsevier Press, Oxford, UK.*
31. *Ogura T, Minakuchi C, Nakagawa Y, Smagghe G & Miyagawa H (2005) Molecular cloning, expression analysis and functional confirmation of ecdysone receptor and ultraspiracle from the Colorado potato beetle Leptinotarsa decemlineata. FEBS J 272, 4114–28.*
32. *Iwema T, Billas IML, Beck Y, Bonneton F, Nierengarten H, Chaumot A, Richards G, Laudet V & Moras D (2007) Structural and functional characterization of a novel type of ligand-independent RXR-USP receptor. EMBO J 20, 3770–3782.*
33. *Pak MD & Gilbert LI (1984) A developmental analysis of ecdysteroids during the metamorphosis of Drosophila melanogaster. J Liq Chromatogr 20, 2591–2597.*

34. *Ghbeish N, Tsai CC, Schubiger M, Zhou JY, Evans RM & McKeown M (2001) The dual role of ultraspiracle, the Drosophila retinoid X receptor, in the ecdysone response. Proc Natl Acad Sci USA 98, 3867–3872.*
35. *Nakagawa Y, Smagghe G, van Paemel M, Tirry L & Fujita T (2001) Quantitative structure–activity studies of insect growth regulators: XVIII. Effects of substituent on the aromatic moiety of dibenzoylhydrazines on larvicidal activity against the Colorado potato beetle Leptinotarsa decemlineata. Pest Manag Sci 57, 858–865.*
36. *Nakagawa Y, Minakuchi C, Takahashi K & Ueno T (2002) Inhibition of [3H] ponasterone A binding by ecdysone agonists in the intact Kc cell line. Insect Biochem Mol Biol 32, 175–180.*
37. *Hiruma K & Riddiford LM (2004) Differential control of MHR3 promoter activity by isoforms of the ecdysone receptor and inhibitory effects of E75A and MHR3. Dev Biol 272, 510–21.*
38. *Azotei A & Spindler-Barth M (2009) DNA affects ligand binding of the ecdysone receptor of Drosophila melanogaster. Mol Cell Endocrinol 303, 91–99.*
39. *Ruff R, Tremmel C & Spindler-Barth M (2009) Transcriptional activity of ecdysone receptor isoforms is regulated by modulation of receptor stability and interaction with AB and C domains of the heterodimerization partner Ultraspiracle, Arch Insect Biochem Physiol (in press).*
40. *Braun S, Azotei A & Spindler-Barth M (2009) DNA binding properties of Drosophila ecdysone receptor isoforms and its modification by the heterodimerization partner ultraspiracle. Arch Insect Biochem Physiol (in press).*
41. *Nieva C, Gwozdz T, Dutko-Gwozdz J, Wiedenmann J, Spindler-Barth M, Wieczorek E, Dobrucki J, Dus D, Henrich V, Ozyhar A et al. (2005) Ultraspiracle promotes the nuclear localization of ecdysteroid receptor in mammalian cells. Biol Chem 386, 463–470.*
42. *Reinking J, Lam MMS, Pardee K, Sampson HM, Liu S, Yang P, Williams S, White W, Lajoie G, Edwards A et al. (2005) The Drosophila nuclear receptor E75 contains heme and is gas responsive. Cell 122, 195–207.*
43. *Koelle MR, Talbot WS, Segraves WA, Bender MT, Cherbas P & Hogness DS (1991) The Drosophila EcR gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. Cell 4, 59–77.*
44. *Henrich VC, Vogtli ME, Antoniewski C, Spindler-Barth M, Przibilla S, Noureddine M & Lezzi M (2000) Developmental effects of chimeric ultraspiracle gene derived from Drosophila and Chironomus. Genesis 28, 125–133.*
45. *Oberdorster E, Cottam DM, Wilmot FA, Milner MJ & McLachlan JA (1999) Interaction of PAHs and PCBs with ecdysone-dependent gene expression and cell proliferation. Toxicol Appl Pharmacol 160, 101–108.*
46. *Ashok M, Turner C & Wilson TG (1998) Insect juvenile hormone resistance gene homology with the bHLH-PAS family of transcriptional regulators. Proc Natl Acad Sci USA 95, 2761–2766.*
47. *Miura K, Oda M, Makita S & Chinzei Y (2005) Characterization of the Drosophila methoprene- tolerant gene product. Juvenile hormone binding and ligand-dependent gene regulation. FEBS J 272, 1169–1178.*
48. *Li Y, Zhang Z, Robinson GE & Palli SR (2007) Identification and characterization of a juvenile hormone response element and its binding proteins. J Biol Chem 282, 37605–37617.*
49. *Konopova B & Jindra M (2007) Juvenile hormone resistance gene methoprene-tolerant controls entry into metamorphosis in the beetle Tribolium castaneum. Proc Natl Acad Sci USA 104, 10488–10493.*
50. *Jones G, Jones D, Teal P, Sapa A & Wozniak M (2006) The retinoid-X receptor ortholog, ultraspiracle, binds with nanomolar affinity to an endogenous morphogenetic ligand. FEBS J 273, 4983–4989.*
51. *Riddihough G & Pelham HRB (1987) An ecdysone response element in the Drosophila hsp27 promoter. EMBO J 6, 3729–3734.*