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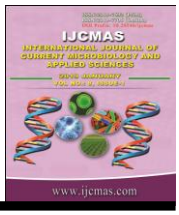

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# *In silico* Approaches for the Ecdysone Receptor of Hemiptera: The First Step for Rational Pesticide Discovery

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**A B S T R A C T**

Ecdysteroids are hormones with an important role in the molting, reproduction and immunological defense of arthropods. Ecdysone Receptor (EcR) is a protein that belongs to the superfamily of nuclear receptors, widely studied for pesticide discovery. Currently, several non-steroidal molecules, belonging to the chemical group of diacilhydrazines (DAH), are commercially available for pest control. Such molecules are specific for lepidopteran or coleopteran pests. Hemipterans are important pests in most crops and many cases of pesticide resistance are reported. There is no pesticides targeting hemipteran EcR and such strategy would be interesting in the point of view of controlling sucker insects. In this context, this work aimed to explore hemipterans EcR for *in silico* study for rational pesticide design. Amino acid residues of binding site are mostly conserved among different insect orders, which explains the unspecificity of ecdysteroids, such as 20-hydroxyecdysone or Ponasterone-A. Hemipteran EcR presents several cavities around the binding pocket. Those cavities can be explored as a target for allosteric modulators/inhibitors. Further, conserved amino acids in hemipteran EcR binding pocket are interesting targets for pharmacophore-based pesticide discovery. Analysis of specific characteristics of hemipteran EcR is the first step for novel and selective pesticide discovery.

## Introduction

Hemipterans are one of the main groups of insect pests in agriculture due to the mode of attack and virus transmission to the plants. In this sense, synthetic pesticides are massively sprayed to control this group of insects. The inadequate use of such pesticides has caused several reports of resistance in hemipterans, such as aphids (Silva *et al*., 2012), pentatomids (Sosa-Gómez and Silva, 2010) and aleyrodids (Basit *et al*., 2013). The white-fly *Bemisia tabaci* (Hemiptera: Aleyrodidae) are the hemipteran specie with most cases of resistance, totaling more than 600 records (APDRD, 2018).

Another cause for pesticide resistance in hemipterans is the lack of modes of actions of commercially available pesticides for use in rotation, once especially systemic neurotoxic pesticides are used for controlling harmful

populations. Furthermore, cyantraniliprole, the most recent and selective mode of action discovered for control of sucker insects, decreased efficacy to known populations due the constantly and inadequate use (Grávalos *et al*., 2015).Therefore, new approaches are required to decrease cross resistance of the limited number of pesticides available for hemipterans.

Ecdysone receptors (EcRs) have been the subject of many studies involving the rational design of insecticides. Ecdysteroids bind to EcRs triggering several physiological processes in arthropods, such as embryogenesis, molting, and metamorphosis. The knowledge about the function and structure of ecdysteroids has allowed the development of non-steroidal compounds, mainly diacylhydrazines (DAHs) (Wing *et al*., 1988). Such compounds have been widely used as insecticides for pest control, mostly targeting Lepidopterans, acting as a hormonal disruptor.

In EcR, the Ligand Binding Domain (LBD) is a conserved region responsible for receiving hormonal signalization (Verhaegen *et al*., 2011). The structural analysis of the ecdysteroid binding site in LBD shows a linkage through the aliphatic chain of ecdysteroids in a large lobe located at the upper end of the site (Zotti *et al*., 2012; Carmichael *et al*., 2005; Verhaegen *et al*., 2011). However, lepidopteran EcR LBD is structurally different to some orders due to the presence of a second cavity in the superior region of the binding site. Such second cavity is not present in Hemiptera (Camirchael *et al*., 2005) or Phthiraptera (Ren *et al*., 2014).On the other hand, *Tribolium castaneum* (Coleoptera: Tenebrionidae) has this second lobe (Iwema *et al*., 2007), similarly to Lepidoptera, which allows the activity of non-steroidal agonists like halofenozide (Smagghe and Swevers, 2013). This second lobe forms a sort of additional binding site, where the B-ring of DAHs binds (Soin *et al*., 2010).

Recently, Hu *et al*., (2018) discovered candidate molecules with antagonistic activity based on Diptera EcR structure, highlighting that, different orders from Lepidoptera and Coleoptera can be used for EcR-based pesticide discovery. The three-dimensional structure of the EcR of *B. tabaci* in complex with Ponasterone-A (PonA) was published by Carmichael *et al*., (2005), and such crystal structure may be explored as a target for selective pesticides.

Three-dimensional model of *B. tabaci* EcR LBD is presented and analyzed in this study, aiming to describe it as a model for rational pesticide discovery. Further, different cavities in the receptor were exposed as potential allosteric sites.

## Materials and Methods

For three dimensional and alignment analyses, primary and tertiary structures of EcR were obtained from Protein Data Base (PDB) according to the following PDB codes: *B. tabaci* (1Z5X), *Heliothis virescens* (Lepidoptera: Noctuidae) (1R1K, 3IXP), *T. castaneum* (2NXX), *Bovicola ovis* (Phthiraptera: Trichodectidae) (4OZT) (Billas *et al*., 2003; Browning *et al*., 2007; Carmichael *et al*., 2005; Iwema *et al*., 2009; Ren *et al*., 2014). Progressive amino acid multiple sequence alignment were created with CLUSTA-X and edited with BIOEDIT v.7.2.6 software (Thompson *et al*., 1994; Hall, 1999). Highlighted amino acids represent hydrogen bonds (H-bonds), which are directly related to the activation of the receptor, accordingly to the binding mode obtained from PDB structures. The three-dimensional virtual analyses were performed with the Microsoft Windows 7® operating system. Analyses of EcRs, such as binding pocket,

hydrogen bond pattern, amino acid composition and H-bond based pharmacophore were performed by Discovery Studio 4.5® (Accelrys, San Diego, CA). Autodock VINA software (Trott and Olson, 2010) was used for molecular docking of tebufenozide towards lepidopteran EcR (non-steroidal EcR agonist). Subsequent to molecular docking, binding sites of PonA and tebufenozide were overlapped by using Matchmaker tool of Chimera® software (Pettersen *et al*., 2004).

## Results and Discussion

The EcR of *B. tabaci* shares a high degree of identity to *B. ovis*, (80%), and such similarity is unsurprising, once both insects share the same type of metamorphosis and are phylogenetically very close to another (Misof *et al*., 2014). On the other hand, *B. tabaci* EcR shares the lowest identity with *H. viresscens* (58%), a holometabolous insect belonging to the distinct clade of mecopterida (Misof *et al*., 2014). The amino acids involved to form H-bonds to Ponasterone-A (PonA) are mostly conserved, but in this case, the only exception is the residue Val110 of *T. castaneum* (Fig. 1).

It is important to highlight the particularities in lepidopteran EcR before start to explore hemipteran features. Besides the binding pocket, the EcR of *H. virescens* presents a small indentation, forming a second three-dimensional cavity (Fig. 2) formed by a lepidopteran-specific torsion of amino acids Leu134, Met95, Asn218 and Val130 (Fig. 1). This second cavity fits specific non-steroidal molecules used as pesticides, like DAHs pesticides, such as tebufenozide and methoxyfenozide for example. An overlapping among the steroidal and non-steroidal binding site exists (Fig. 2), where the non-steroidal compound BY108346 binds to the amino acids Thr57 and Tyr122 of *H. virescens* EcR (Fig. 1) forming H-bonds and conferring a high specific agonistic activity. The knowledge concerning site-specific binding is important to understand that features of each EcR, like the tertiary structure, are crucial for pesticide exploration and discoveries.

The crystal structure of the revealed EcRs LBD by X-ray of four species from different orders is available at Protein Data Base (PDB). Despite, researchers use molecular modeling for elucidation of secondary and tertiary structures of different arthropod groups. The basis of computational protein modeling is that different proteins with similar amino acid sequences would adopt similar tertiary structures (Blundell *et al*., 1987; Sali; and Overington, 1994). The canonical structure of the EcR LBD of different species has the same three-dimensional structure, composed mostly by twelve α-helix and two or three β-sheets (Fig. 3).

Beyond the binding pocket, there are seven surrounding cavities (or sites) in EcR LBD of *B. tabaci* (Fig. 4A). Theoretically, the seven extra cavities are capable to receive a specific allosteric ligand, once there are specific sets of functional groups like, sites volumes, amino acids arrangement and hydrogen donors and acceptors.

The second possibility relies on the main binding pocket, composed by several specific and conserved amino acids, where a molecular docking can be applied for a site-specific pesticide design (Fig. 4B). The last approach, and the most used in studies of pesticide discovery, is the pharmacophore-based molecules. In this approach, the physical-chemical features of ligand-receptor interaction, i.e., H-bonds and distances, are used for docking molecules from databases. Our analysis showed that PonA forms four H-bonds to the donor residues Tyr118, Thr53, Thr56 and Glu21, and two to the acceptor residues Arg93 and Ala108 (Fig. 4C).

The difference between the structures of the EcR analyzed are notable, specially in the primary structure, once the secondary and tertiary three-dimensional structures are similar, composed by twelve α-helix and two or three β-sheet surrounding a hydrophobic binding site (Fig. 2).

In figure 4A, eight sites are shown, including the ecdysteroid binding domain and the extra pockets surrounding. Allosteric sites are unexplored for pesticide design at EcR approaches, but it is a reality for several pharmaceuticals (Hardy and Wells, 2004) and other pesticides modes of action (Salgado and Saar, 2004; Kato *et al*., 2009; Tao *et al*., 2013).

Given the central role of EcR in the metamorphosis of insects, the exploitation of peculiarities between species may enable the development of specific pesticides for pest management. The canonical structure of the EcR LBD of *B. tabaci*, revealed by Carmichael *et al*., (2005), is structurally similar to another also revealed by X-ray (Fig. 3). However, it is important to note that the amino acid sequence of *B. tabaci* EcR shows low similarity to *H. virescens* EcR (58%) if compared to another species. Nevertheless, residues involved to ligand binding pocket are conserved, which remarkably points that, theoretically, ligand-based pharmacophore can generate unselective molecules.

The crystal bound conformation of PonA (Fig. 4C) is useful as alignment template for virtual screening of EcR site-specific molecules (Harada *et al*., 2013), as well as BYI06830, a non-steroidal EcR activator (Hu *et al*., 2018). The aliphatic chain of PonA binds to Tyr118, suggesting a critical ligation for EcR activation (Hu *et al*., 2017). Based on ligand-receptor interactions, molecular docking can be applied as a useful and costless tool for pesticide discovery.

For using molecular docking, it is suggested to use more than one program because different poses can be identified (Houston and Walkinshaw, 2013). Thus, it is possible to explore the space of binding pocket at different niches by uncountable classes of small molecules (Holmwood and Schindler, 2009).

Different cavities can be targeted by different ligands according to the functional groups affinities (Billas *et al*., 2003; Holmwood and Schindler, 2009). Further, identification of potential allosteric sites in proteins can generate opportunities for pesticide discovery. When *B. tabaci* EcR LBD was analyzed, we found seven extra pockets surrounding the ecdysone binding pocket.

For example, the screening of a massive database of small molecules through to a human liver protein resulted to a discovery of functional allosteric sites (Oikonomakos *et al*., 2000; Rath *et al*., 2000). The inhibition mechanisms of allosteric sites are similar to activators, based specially on H-bonds formation and hydrophobic interactions (Hardy and Wells, 2004).

Allosteric sites are successfully used for pest control in different modes of action. For example, the spinozines obtained from secondary metabolism of *Saccharopolyspora spinose*, act as allosteric modulator of nicotinic receptor of the post-synaptic nerve in insects (Salgado and Saar, 2004). Further, diamides, the newest mode of action to be introduced for pest control, act as modulator of ryanodine channels with high selectivity for natural enemies (Ramos *et al*., 2018; Pazini *et al*., 2016).

**Fig.1** Sequence alignment of EcR LBD from *B. ovis (Trichodectidae: Phthiraptera); T.castaneum (Coleoptera: Tenebrionidae); H. virescens (Lepidoptera: Noctuidae) and B. tabaci (Hemiptera: Aleyrodidae).*Protein Data Base (PDB) codes are: 4OZT, 2NXX, 3IXP and 1Z5X, respectively. Conserved amino acids residues involved in H-bonds in the ecdysone-binding site for PonA are indicated by a black square. Residues involved in hydrogen bonds for the non-steroidal agonist BY108346 in Lepidoptera are indicated by a red circle. Residues involved in extra pocket formation in Lepidoptera are indicated by a green square

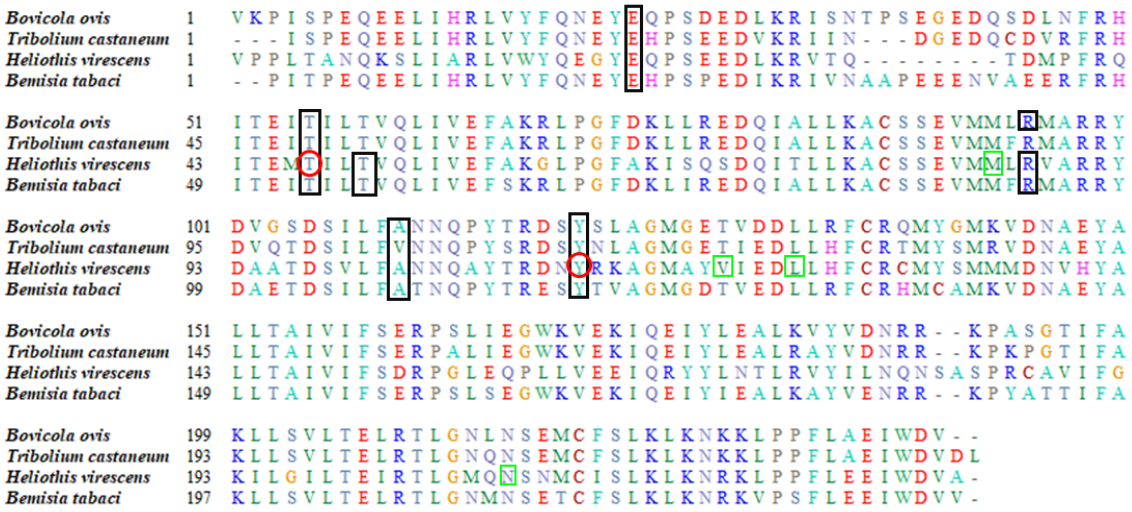



**Fig.2** Overlap of tebufenozide and PonA in lepidopteran EcR-binding sites. A: a macro view of the EcR with two overlapped molecules complexed. B: a detailed view of the overlapping sites. The area circled in red corresponds to the exact location where the binding sites of the tebufenozide A-ring and the ecdysteroid aliphatic chain overlap occurs

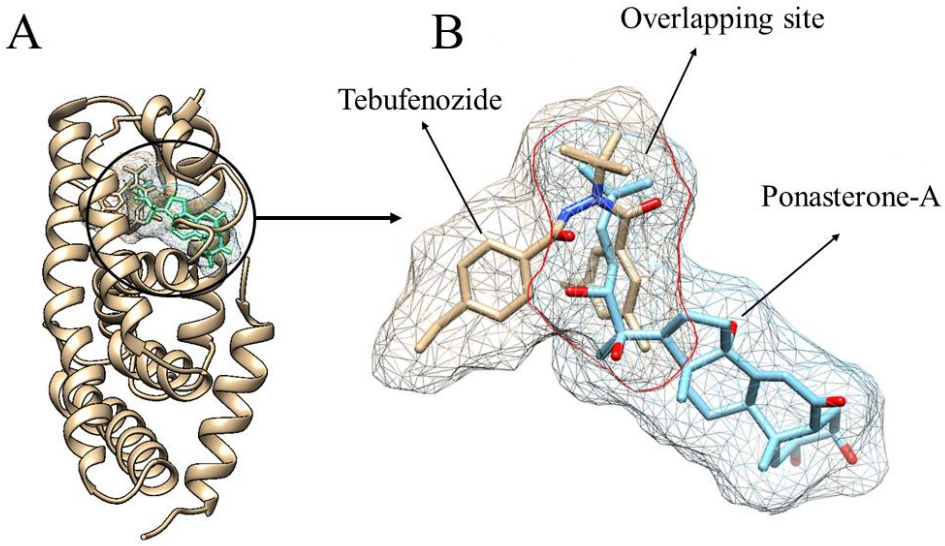


**Fig.3** Comparison of EcR LBD X-ray structures available at PBD. Ribbon diagrams of the EcR LBD canonical structure of *B. tabaci* (1Z5X), *B. ovis* (4OZT), *T. castaneum* (2NXX) and *H. virescens* (3IXP). N and C correspond to the N- and C-terminus of the polypeptide chains, respectively

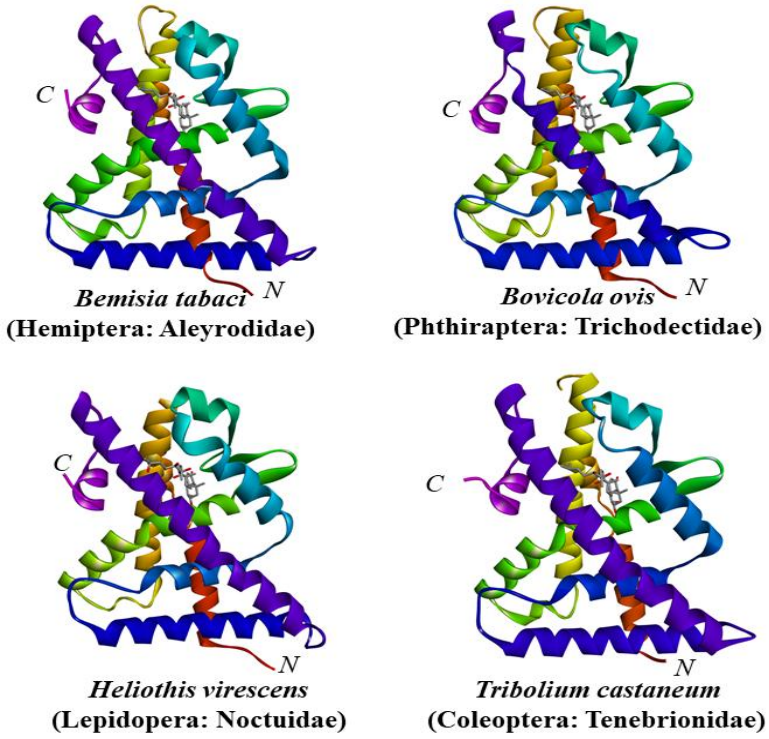
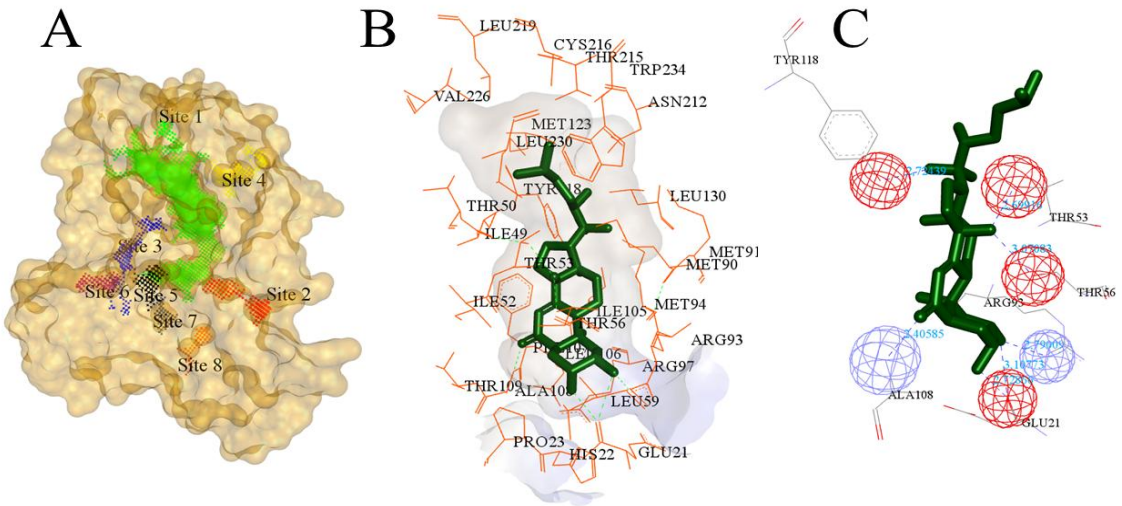



**Fig.4** Three-dimensional features of hemipteran (*B. tabaci*) EcR. A- Site formed for tridimensional conformation of the amino acid residues; B- Site 1, or the ligand binding pocket and amino acid composition and distribution; C- H-bond based pharmacophore model showing functional groups and distances of the hemipteran EcR interacting to PonA



Studies have shown that mutations in amino acids G4946 and I4790M in *Plutella xylostela* (Lepidoptera: Plutellidae) and in homologues G4903E and I4746M in *Tuta absoluta* (Lepidoptera: Gelechiidae) are related to the development of diamine resistance (Roditakis *et al*., 2017; Troczka *et al*., 2012). In addition, a region close to the N-terminal (aa183-290 of *Bombix mori*) and two located within the C-terminus of the Drosophila transmembrane region (aa4610-4655) also showed sensitivity to such insecticides (Kato *et al*., 2009; Tao *et al*., 2013), indicating several allosteric binding sites in ryanodine receptor.

Cell-based assays coupled to virtual screening are useful tools for pesticide discovery. Cell lines secrete endogenously all of the components necessary for activation and transactivation of the EcR (Zotti *et al*., 2013). Thus, cell cultures transfected with a reporter plasmid (Swevers *et al*., 2004) are widely used in high-throughput screening systems (HTSS), since it allows the screening of a massive amount of molecules *in situ* (Zotti *et al*., 2013; Hu *et al*., 2018, Harada *et al*., 2011; Soin *et al*., 2010; Smagghe and Swevers, 2013).

Continuous cell lines derived from hemipterans like aleyrodis, aphids and leafhoppers (Adam and Sander, 1976; Mitsuhashi, 1989; Hunter and Polston, 2001; Kimura, 1984) have already been stablished, and EcR-based screening systems as described by Zotti *et al*., (2013) and Soin *et al*., (2010) may be applied for rational pesticide discovery for sucker pests. Further, virus containing recombinant EcR can be applied to deliver the gene into cell lines for subsequent gene expression and hormonal activity detection (Tohidi-Esfahani *et al*., 2011).

It is hoped that hemipteran EcR, in the future, will be useful as a target for novel pesticides, designed based on specific features of the receptor. Potential allosteric sites or ligand binding pocket, which has already been explored for Lepidoptera and Coleoptera, may provide an important subject for rational pesticide discovery for sucker pests.

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