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Insecticide compounds selection based on activation of ecdysteroid receptors in Diptera (S2) and Lepidoptera (Sf9) cell lines

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Abstract

Nuclear receptors are important targets in the research for the discovery of brand-new insecticides, and the ecdysteroid receptor is one of the most important targets nowadays. In this study, nine organocalcogenic compounds analogs of dibenzoylhydrazine were tested in a screening system, that presented at least 80% of compatibility in at least one of the three micromolar concentrations tested. We optimized in embryonic cell lines of the order Lepidoptera (SF9) and Diptera (S2), and these cell lines were transfected with a specific reporter plasmid. The nine compounds tested did not present agonist activity in both cell lines tested. In S2 cells the DAF-2 molecule inhibited the ECR-USP luminescence in 29.91%, and the DAF-4 molecule significantly inhibited the nuclear receptor luminescence in 51 to 55% compared to 20-Hydroxyecdysone (control). In SF9 cells, DAF-2 inhibited ECR-USP luminescence in 19.62%, and DAF-4 inhibited the receptor luminescence by 35.14% compared to 20-Hydroxyecdysone (control). In silico analysis of the Drosophila melanogaster receptor with the compound, DAF-2 showed inhibition of amino acids THR467 and MET-508 and with compound DAF-4 inhibition of ARG-511. For the of *Heliothis virescens* receptor for dendritic compounds, DAF-2 promoted inhibition of amino acids THR-343 and ARG-387 and DAF-4 inhibited ASN-504 and ARG-387.

Keywords: Dibenzoyl hydrazine, Reporter plasmid, Cell biotechnology, 20-Hydroxyecdysone, Ponasterone-A, Bioprospection of new compounds.

Introduction

The process of growth and reproduction of insects could be described by the combination of several hormones that belong to several distinct chemical classes. The major hormones that trigger chemical processes are steroids, terpenoids, and peptides (Nijhout and Emlen, 1998). The endocrine hormone 20-Hydroxyecdysone (20He) and the juvenile hormone regulate the molting and metamorphosis of arthropods. The hormone receptor is formed by the ecdysteroid receptor (EcR) and the ultraspiracle, which together form a nuclear receptor heterodimer, ecdysone receptor (EcR, NR1H1), and ultraspiracle (USP, NR2B4) (Koelle et al. 1991, Yao et al. 1993).

Nuclear receptors function as ligand-activated transcription factors that regulate the expression of target genes responsive to the processes of development and reproduction in insects (Mangelsdorf et al. 1995). The ecdysteroids and juvenile hormone (JH) exert regulatory functions in initiating and triggering the molting and metamorphosis processes. Juvenile hormone (JH) shows the unique structure and the diversity of its effects on insect development and reproduction (Wyatt et. al 1996). The insect nuclear hormone receptor is not present in mammals (Yao et al. 1992) and therefore represents a logical target for the development of insecticides.

This observation led to the development of several compounds as dibenzoylhydrazines that have been developed and marketed over the years, e.g., tebufenozide (RH-5992), halofenozide (RH-0345), and methoxyfenozide (RH-2485). Its activity is based on the binding of the EcR receptor and induces the process of molting on developing insects, being lethal in larval phases and interfering with reproduction in adults (Dhadialla et al. 1998, Smagghe et al. 2012). Consequently, these insecticides are usually included in Integrated Pest Management (IPM) programs and used in different pests and crops. A virtual screening system is a tool for searching new ecdysteroids receptor agonist compounds using computational parameters that compare the differences of a molecule in inhibiting the amino acids of the nuclear receptor through in silico analysis (Harada et al. 2011).

The exploration of intrinsic differences of each receptor targets the specificity of the planned molecule (Smagghe et

al. 2012). This procedure directed exclusively at a single specific target, as a nuclear receptor, has improved high-throughput screening procedures (Swevers and Smagghe, 2016). In this way, screening new compounds with cell lines is advantageous due to the endogenous production of every component needed for the organism to function (Zotti et al. 2013b). The screening of compounds in cell lines is a high-throughput tool for the search of new compounds obtained through organic synthesis and virtual screening, serving as a model for the rational development of pesticides for the agrochemical industries, becoming a necessary tool to search for selective molecules for insect pests control (Swevers et al. 2004, Soin et al. 2010, Smagghe et al. 2012).

Results and Discussion

Citotoxicity assay

Initially, 23 structural organocalcogenic chemical compounds were tested in S2 cells bioassays at 10^{-3} μM, 10^{-6} μM and 10^{-9} μM concentrations. Through viability results, we observed that nine compounds tested at the three concentrations presented partial toxicity (M2, M9, M14, M16, M17, DAF-2, DAF-4, SE-CHOL and SE-CHOL-AC). Non-steroidal compounds may have partial cytotoxicity at some micro molar concentrations or be extremely cytotoxic, interfering with viability (Soin et al. 2010). The micro molar concentrations that presented a viability of at least 80%, were added in our bank of new molecules and tested using the high throughput screening system, seeking agonist or antagonist activity on lepidopteran and dipteran nuclear receptors (Swevers et al. 2004).

Ecdysteroids agonist activity

The reporter bioassays to express the luciferase gene in S2 and SF9 cells were obtained from a dose-response curve with the natural hormone 20He and the Phytosteroid Pon A (Fig 2). The curves of these hormones were calculated with the aid of the GraphPad Prism software to evaluate the activity of the insect-specific natural hormone 20He and the phytosteroid PonA, both ecdysteroids had an excellent responsiveness in transfected Diptera and Lepidoptera cells with the reporter gene plasmid ERE-b.act.luc (Zotti et al. 2013a). The steroidal compounds 20He and PonA presented similar patterns for the two strains, and PonA proved to be an excellent agonist (Fig 2). The EC50 of 20He was 7.185 and 6.958, and PonA was 6.24 and 5.653 for Diptera and Lepidoptera cells, respectively (Table 2). Thus, it can be observed that both steroids, in both cell lines, presented a pattern of activity. The result obtained indicates that both 20He and PonA are non-specific for these two orders of insects.

Secondary compounds obtained from plants, produce properties similar to agonist or antagonist activity of the ecdysteroid receptor. Dinan et al. (1999) identified more than 4500 species of plants with potential to serve as a model for the development of new control agents. Cyasterone is a phytosteroid which had agonist activity in S2 and Bm5 (Lepidoptera) cell ecdysteroid receptors, however, its activity was lower than tebufenozide, which makes it unfeasible for the development of new insecticides in the agrochemicals industry (Zotti et al. 2013a). Plants produce steroidal compounds, some of which may have activity in insect ecdysteroid receptor and may compete for the binding site of the Diptera and Lepidoptera receptor ligand, being an evolutionary adaptation in insect orders (Dinan et al. 2001).

In relation to the agonist activity of the new organocalcogenic compounds, both S2 and SF9 cells showed no significant effect when compared to the concentration of 100μM of 20He and PonA (Fig 3). The compounds were tested at the maximum non-toxic micro molar concentration for cell cultures (Table 3), the concentration of the standard hormones used for these bioassays, since larger concentrations may cause cytotoxicity in cells (SOIN et al 2010). As the concentrations used showed not respond, we concluded that the nine new molecules (Fig 3) have no potential to become commercial agonists both for the lepidoptera and Diptera cell lines, being economically unfeasible to continue the research, as several optimizations in the chemical structures must be carried out to possibly obtain expressive results, making the molecules active.

The agonist activity for the natural hormone 20He and the phytosteroid PonA in both cell lines, showed no significant difference (Fig 3). Thus, for a non-steroidal molecule to have the potential to be commercially used, a response of at least 80% compared to the natural 20He hormone is expected.

Non-steroidal ecdysteroid receptor agonist molecules such as dibenzoylhydrazine (DHA) analogs represent an important group of insecticides in pest insect control, their highly selective mode of action and make it a useful tool for the control of arthropod pests. However, the commercially available DHA analogs are only highly active against Lepidoptera, except for halofenozide, which has activity against coleoptera and Lepidoptera. However, these non-steroidal commercial agonist insecticidal molecules also have a certain activity in Diptera (Smagghe et al. 2012). Diptera-specific reporter-based screening system was developed in order to discover and evaluate compounds that have agonist or antagonist activity in the ecdysteroid receptor (DINAN *et al* 2001).

Ecdysteroids antagonist activity

In relation to the antagonistic activity of the new organocalcogenic compounds, in both S2 and SF9 cells only two molecules had a significant effect when compared to the concentration of 100μM of 20He and PonA (Fig 4). The compounds were tested at the maximum non-toxic micro molar concentration for the cell cultures (Table 4). In S2 cell line, the results showed that DAF-2 molecule inhibited the nuclear receptor luminescence in 29.91% and DAF-4 molecule inhibited in 51.55%, comparing to the hormone 20He (Figure 6A). The results obtained in the SF9 cell line showed that DAF-2 inhibited the nuclear receptor luminescence in 19.62%, and DAF-4 inhibited the receptor luminescence in 35.14% (Figure 6B). The compounds DAF-2 and DAF-4 are described as synthetic modulators DAF-12, with potential use in controlling nematodes. The compound DAF-2 and DAF-4 were tested for their activity in HEK-293T cells co-transfected with a vector containing luciferase reporter gene under regulation of Gal4 promoter (Dansey et al. 2015).

Bioassays with insect cell lines targeting nuclear receptor antagonist activity are generally scarce (Dinan and Hormann 2005, Soot et al 2009, Zotti et al 2013a). With the advance of bioinformatics, a virtual screening based on form and activation power of the ligand to the EcR can be performed based on a database of more than 2 million compounds to identify novel non-steroidal compounds like the P-bonding phytosteroid A in response to EcR. The parameter set for an antagonist molecule has an acceptable inhibitory effect of at least 50% of the hormonal activity (Harada et al. 2011). However, due to the lack of studies focused on antagonistic

Table 1. Agonist activity of 20-Hydroxyecysonium (20He) and Ponasterone-A (PonA).

	S2			SF9		
	-logEC50	95%CI	R^2	-logEC50	95%CI	R^2
20He	7,185	-7.696 -6.675	0,8530	6,958	-7.331-6.584	0,8901
PonA	6,424	-6.593 -6.255	0,9746	5,653	-5.792 -5.514	0,9908

Data expressed as response averages at the 95% confidence interval (both in -logEC50). R^2 represents the accuracy of the data plotted on the sigmoid curve.

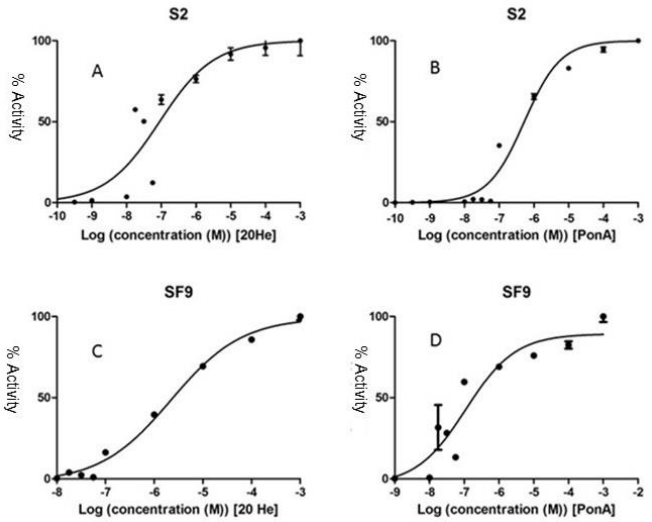

Figure 1. Dose-response sigmoid curves concerning the activity of two ecdysteroids in S2 and SF9 cells.

Table 2. Activities of compounds in bioassays in S2 and SF9 cell culture for ecdysteroid agonists.

Compound	Max. Conc.(M) [1]	Agonist Activity	Cytotoxic Conc.(M) [2]	Supplier
M2	10^{-9}	Inactive	10^{-3} and 10^{-6}	LabSOL
DAF-4	10^{-6}	Inactive	10^{-3}	Dr. Burton
DAF-2	10^{-6}	Inactive	10^{-3}	Dr. Burton
M9	10^{-9}	Inactive	10^{-3} and 10^{-6}	LabSOL
M14	10^{-9}	Inactive	10^{-3} and 10^{-6}	LabSOL
M16	10^{-9}	Inactive	10^{-3} and 10^{-6}	LabSOL
M17	10^{-9}	Inactive	10^{-3} and 10^{-6}	LabSOL
SE-CHOL-AC	10^{-9}	Inactive	10^{-3} and 10^{-6}	Dr. Isabella
SE-CHOL	10^{-9}	Inactive	10^{-3} and 10^{-6}	Dr. Isabella
DMSO	*	Inactive	----------	Synth
PonaA	10^{-3}	Active	----------	Sigma Aldrich
20 He	10^{-3}	Active	----------	Sigma Aldrich

[1] Maximum concentration. [2] Cytotoxic concentration * Solvent used to dilute compounds.

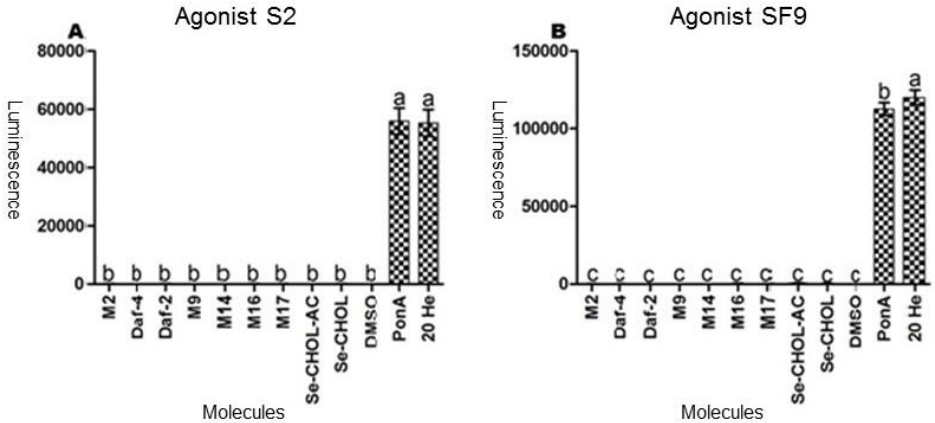

Figure 2. Observed agonist effect on the nine new molecules on S2 (A) and SF9 (B) cell lines. The agonist activity of the molecules was compared to the concentration of 100µM of the 20-Hydroxyecdysone (20He), Ponasterone A(PonA) and DMSO (Solvent) as a control. Values that are followed by letters were significantly different (Tukey test = 0.05). The significant difference in the measured luminescence measured in comparison to the 20He control was analyzed through One Way Anova. (F = 402.7, gl = 11, GL residue = 24, P <0.0001) and (Tukey test = P <0.05), SF9 agonist (F = 1913, gl = 11; GL residue = 24 ; P <0.0001) and (t = significant test P <0.05).

Table 3. Identification of the compounds used in this study according to their chemical formulas (C.F.), chemical name, molar mass (M.M.), initial purity (I.P.) and solvent used for dilutions.

Comp.*	C.F. [1]	Chemical Name	M.M. [2] ($g.mol^{-1}$)	I.P. (%)	Solvent
20H [4]	C27 H44 O7	2|A,3|A,14|A,20|A,22,25-Hexahydroxy-7-cholesten-6-one	480,648621	95	Ethanol[6]
PonA [5]	C27 H44 O6	Cholest-7-en-6-one,2,3,14,20,22-pentahydroxy-, (2b,3b,5b,22R)	464.643000	>65	Ethanol[6]
M2	C18 H27 S1	2-phenyl-3-(phenylthio)imidazo[1,2-a]pyridine	302,00	95	DMSO
M9	C20 H38 N2 S1 F1	2-(4-fluorophenyl)-3-(phenylthio)imidazo[1,2-a]pyridine	320,00	95	DMSO
M14	C21 H42 N3 Se1	1,3-diphenyl-4-(phenylselanyl)-1H-pyrazol-5-amine	391,00	95	DMSO
M16	C21 H38 N3 Se1	1-benzyl-5-phenyl-4-(phenylselanyl)-1H-1,2,3-triazole	391,00	95	DMSO
M17	C22 H44 N5 S1 N5	(Z)-1-((phenylselanyl)methyl)-15H-dibenzo[d,h][1,2,3]triazolo[1,5-a][1,3,6]triazonine	431,00	95	DMSO
DAF-2	C22 H38 O3	3β-(t-Butyldimethylsilyloxy)-5-cholenic acid methyl ester	417,00	95	DMSO
DAF-4	C21 H36 O2	3β-(t-Butyldimethylsilyloxy)-5,24-cholestadien-27-oic acid ethyl ester	360,00	95	DMSO
SE-CHOL	C26 H33 O2 Se1	(5R,6R,10R,13R)-10,13-dimethyl-17-(6-methylheptan-2-yl)-6-(phenylselanyl)hexadecahydro-5H-cyclopenta[a]phenanthrene-3,5-diol	559,00	95	DMSO
SE-CHOL-AC	C30H31O3 Se1	5R,6R,10R,13R)-5-hydroxy-10,13-dimethyl-17-(6-methylheptan-2-yl)-6-(phenylselanyl)hexadecahydro-1H-cyclopenta[a]phenanthren-3-yl acetate	601,00	95	DMSO

[1]Chemical formula, [2]Molar Mass, [3]Inicial Purity, [4]20-Hidroxyecdysone, [5]Ponasterone-A, [6]Absolute Ethanol, [7]Dimethyl sulfoxide.
* Compounds tested in S2 and SF9 cells screening system, cell viability greater than or equal to 80%.

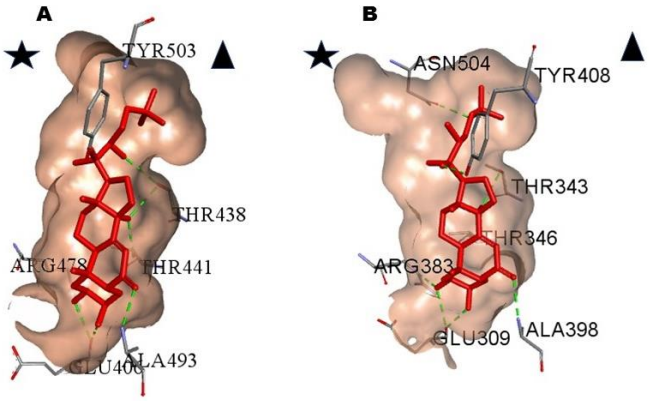

Figure 3. Binding site cavity inside ecdysteroid receptor (EcR). In the image, 20-Hydroxyecdysone anchored in the EcR of *Drosophila melanogaster* (A) and *Heliothis virescens* (B). The two lobes located in the upper portion of the cavity are indicated by a star (left side) and by a triangle (right side). In the Lepidoptera receptor (B) it is possible to observe the protruding left lobe, a site which binds to the dibenzoylhydrazines.

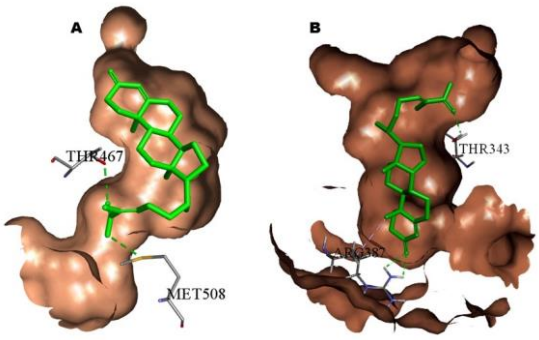

Figure 4. Graphical interface of the possible interaction between the DAF-2 molecule with the *Drosophila melanogaster* (A) and *Heliothis virescens* (B) ecdysteroid receptors. The illustration shows the inhibition of THR467 and MET508 in *D. melanogaster*, and THR343 and ARG387 in *H. virescens* EcR.

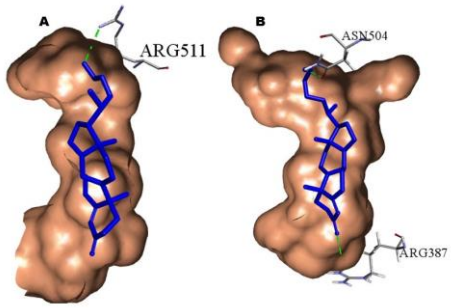

Figure 5. Graphical interface of the possible interaction between the DAF-4 molecule and the *Drosophila melanogaster* (A) and *Heliothis virescens* (B) ecdysteroid receptor. The illustration shows the inhibition of ARG-511 in *D. melanogaster*, and ASN-504 and ARG-387 in *H. virescens* EcR.

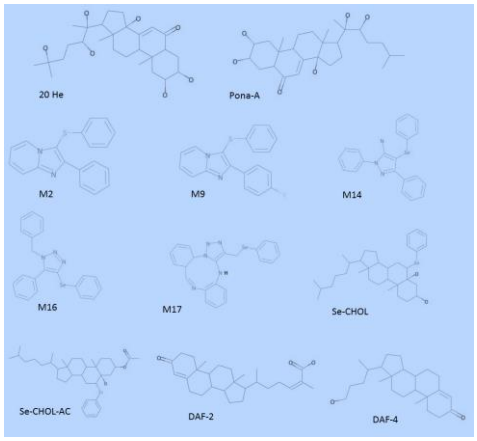

Figure 6. Steroidal and non-steroidal compounds used for screening in Diptera (S2) and Lepidoptera (SF9) cell lines.

bioassays, we considered active compounds that obtained at least 15% inhibition of the nuclear receptor in the two cell lines S2 and SF9. Dinan et al. (2001), in one of its main works in cell line BII, from *D. melanogaster*, identified 36 plant-extracted secondary compounds from the alkaloid, brassinosteroids, cardenolides, bufadienolides, cucurbitacins, picracins, flavonoids, lignans, limonoids, phenylalkanoids, saponins and whitasteroids chemical classes presenting ecdysteroid antagonistic activity.

There are steroidal compounds such as castasterone which has affinity for ecdysteroid receptors of S2 (Diptera) and BM5 (Lepidoptera) cells due to similarity on binding sites, in contrast to dibenzoylhydrazines (Zotti et al. 2013a). Hetru et al (1986) found that catasterone can cause antagonism to the natural hormone 20He in *Phormia terranovae* Robineau-Desvoidy, 1830 (Diptera: Calliphoridae). Compounds extracted from seeds of *Carex pendula* (Cyperaceae) inhibited the nuclear receptor presenting an antagonistic activity in dipteran cells (Meng et al. 2001). This demonstrates a broad repertoire of non-steroidal dibenzoylhydrazines-like compounds, with the ability to inhibit the activity of insect's nuclear receptors in various insect orders.

Three-dimensional modeling of Diptera and Lepidoptera EcR-LBD

The virtual analysis of EcR binding site shows a bond with an aliphatic chain of 20He hormone occurs in a large lobe located at the upper end of the site, observable in both the dipteran and lepidopteran receptors (Figure 5). However, when we compare the structure of dipteran (Figure 5A) and lepidopteran (Figure 5B) receptors, it is possible to observe that there is a second cavity in the superior region of the binding site of Lepidoptera receptor, absent in the Dipteran. This second cavity forms a kind of second binding site. Soin et al (2010) verified that it is in this second cavity that the benzene ring of dibenzoylhydrazines bind, allowing expressive activity of these molecules in Lepidoptera.

Three-dimensional modeling of DAF-2 and DAF-4 compounds

Based on the results obtained in the agonist and antagonist activity bioassays, virtual simulations of the interactions of the DAF-2 and DAF-4 compounds were carried out at the ecdysteroid receptors of diptera and lepidoptera, the only compounds which showed activity in the cellular bioassays. The *D. melanogaster* receptor has not yet been elucidated by the X-ray diffraction method, however, there are computational tools that can model the quaternary structure of proteins based on the alignment of the amino acid sequence of other species, serving as a model. For the modeling of the *D. melanogaster* ecdysteroid receptor in the present study, the Swiss-Model was used, which is an automated system for modeling structures (Biasini et al 2014). The simulations were performed at the nuclear receptor of *D. melanogaster* and *Heliothis virescens* for DAF-2 (Figure 6) and DAF-4 (Figure 7) compounds.

The graphical interface of the simulated interaction between the DAF-2 molecule and the *D. melanogaster* EcR shows two hydrogen bonds with THR-467 and MET-508 amino acids (Figure 6A). The DAF-2 molecule interacts with the *H. virescens* EcR (Figure 4), forming two hydrogen bonds and inhibiting the amino acids THR-343 and ARG-387.

Graphical interface of the possible interaction between the DAF-4 molecule and the *D. melanogaster* ecdysteroid receptor (Figure 7A). The results of DAF-4 simulated binding

to diptera EcR shows the inhibition of amino acid ARG-511, forming one hydrogen bond. The DAF-4 molecule in interaction with the *H. virescens* EcR (Figure 5) forms two hydrogen bonds and inhibits the amino acids ASN-504 and ARG-387.

With the use of computational tools allowing for massive *in silico* screening of chemical compounds, Harada et al. (2011) sought compounds with similar physicochemical characteristics as the phytosteroid PonA. In this study, the authors used a database of 2.1 million molecules and based on pre-established parameters the authors identified 24 compounds for organic synthesis. Of the 24 synthesized compounds only 3 had antagonist activity. Deng et al. (2016) used the virtual screening to modify radicals existing dibenzoylhydrazine molecules based on pre-established parameters. After the screening, synthesized 38 molecules, and only one molecule showed activity like tebufenozide in caterpillars of the order Lepidoptera. It is important to point out that *in silico* (Virtual screening), *in situ* (cell biotechnology) insecticidal activity experiments may diverge from *in vivo* (insects) assays, since whole insect organism is complex and there are enzymes with ability for detoxification of compounds (SWEVERS and SMAGGHE 2016).

Materials and Methods

Chemical compounds

Nine organocalcogenic dibenzoylhydrazines-like compounds (with ~ 95% purity) were tested in the screening system. Five molecules were synthesized and purified in the Laboratory of Clean Organic Synthesis (LabSOL) of the Federal University of Pelotas in Brazil. Dr. Isabella from the University of Bialystok in Poland provided the compounds SE-CHOL-AC and SE-CHOL synthesized with ~ 95% purity. Compounds DAF-2 and DAF-4 were provided by Dr. Gerardo Burton and Dr. Olga A. Castro, from the University of Buenos Aires, the molecules were synthesized with ~ 95% purity. The ecdysteroid hormones Ponasterone A (PonA) (> 65% purity) and 20-Hydroxyecdysone (~ 95% purity) were purchased from Sigma Aldrich (Bornem, Belgium) (Figure 1). The molecules were diluted in molar concentrations according to their molecular weight in absolute ethyl alcohol or Dimethyl sulfoxide (DMSO) (Table 1). The initial dilution was performed at a molar concentration of 10^3, and serial dilutions were performed to reduce the concentrations, forming a spectrum, mainly in the hormones, which required several concentrations for the preparation of dose-response curves.

Cell lines

Cell lines were provided by Dr. Guy Smagghe from the Crop Protection Department of Ghent University (Belgium). The cell cultures were established in the Department of Plant Protection at the University of Pelotas (Brazil), in the laboratory of Molecular Entomology (Lab Entomol-UFPel). SF9 cells from embryonic cells of *Spodoptera frugiperda* (JESmith, 1797) (Lepidoptera: Noctuidae) were maintained at 27 ° C with InsectXpress ™2 (LONZA®) cell culture medium in culture flasks with filter cap vent (KASVI®) of 25cm² and 75cm². S2 cells from embryo cells of *D. melonogaster* were incubated at 27ºC in InsectXpress ™ 2 (LONZA®) culture medium in 25cm² and 75cm² vent filter caps (KASVI®).

Cytotoxicity assays

To determine the cytotoxicity of the compounds, cytotoxicity assays were performed using the Neubauer chamber also known as a hemocytometer. Concentrations of S2 and SF9 cells to prepare cytotoxicity assays were 500,000 cells/ml and 300,000 cells/ml, respectively. Cells were conditioned in 24-well cell culture plates (KASVI®).

Viability was measured using the trypan blue dye solution 0.4% (Sigma Aldrich), a reagent which can penetrate the cytoplasmic membrane of dead cells, giving a dark blue coloration thereto. After cell count and cell deposition in the wells, 1 and 1.5 µl, for S2 and SF9, respectively, of the compounds diluted in absolute alcohol or DMSO were added to the respective wells and incubated for 24 hours at 27 °. In each cell line, the cytotoxic effect of each molecule was tested in standard treatments with concentrations of 1µM, 10µM and 100µM.

Each treatment was performed in triplicate, with each experiment reproduced at least 2 to 3 times. Statistical analysis was performed by t-test, with multiple comparison of the means of the compounds with the mean of the control, within each group (concentration). Data were analyzed with Prism v5 software (GraphPad Software Inc. La Jolla, Ca).

Cells transfection

The compounds were tested for their ability to activate the transcription of an ecdysteroid inducible luciferase reporter gene in S2 or SF9 cells, which are responsive to the ecdysteroid receptor. Prior to exposure, the cells were transfected with the reporter plasmid using Escort ™ IV reagent (Sigma Aldrich). The formulation of this reagent consisted of a unique polycationic lipid and an untransfected neutral lipid. This liposome forming compound is used for transfection of nucleic acids into a wide variety of eukaryotic cell types. Transfection was conducted following manufacturer instructions as described. Briefly, the transfection protocol is described: A 6-well flat bottom cell culture plate with a cell growth area of 9.60 cm^2 is used. One well was filled with 3,000,000 S2 cells or 2,000,000 SF9 cells. The cells were allowed to stand for 30 minutes to adhere to the bottom of the well. Escort ™ IV was incubated with the solution containing the reporter plasmid ERE-b.act.luc for 45 min in cell culture medium. The reporter construct ERE-b.act.luc is composed of seven copies of the ecdysone response element (ERE) derived from the *Drosophila* hsp27 promoter, a basal actin promoter derived from *Spodoptera* (b.act), followed by the reporter gene for firefly luciferase (luc) and a termination signal. The cells were incubated at 27°C for 16h, and the transfection medium was removed and replaced with normal cell culture medium.

Bioassays for ecdysteroid receptor agonists and antagonists and the ability to respond to ECR-USP

Transfected cells were treated with ecdysteroids, or organocalcogenic compounds, at molar concentrations according to the treatment. After removal of the transfection medium and replacement by normal means, 1µl of the molecule diluted in alcohol or DMSO for S2 and 1.5µl for SF9 was added to the well. The agonist / antagonist data were measured in numerical form of luminescence emitted by the luciferase enzyme after the addition of the Luciferin substrate. The greater the activation of the ecdysteroid receptor, the greater the luminescence. For the reporter assay, a 10 ml solution was prepared with 50,000 cells/ml Diptera (S2) cells, and 30,000 cells/ml Lepidoptera (SF9) cells

transfected with the reporter plasmid. After transfection, 100 µl of the solution was added to each well in a white 96-well microtiter plate (Thermo Scientific, Roskilde, DN, Denmark).

For dose response and agonist activity experiments, luminescence was measured 24 hours after addition of the treatments (compounds). For the antagonistic activity assay, first, 1µl and 1.5µl of a given molecule was added to S2 and SF9, respectively, in the concentration of 100µM and incubated for 24h at 27ºC. After the incubation, the hormone 20He and PonA (control) were added at a concentration of 100 µM and incubated again, this time for 24 hours at 27 ° C. After the incubation periods, 100 µl of the cell suspension was added per well in a 96-well plate. To measure luciferase expression, the luciferase substrate Steady-Glo luciferase assay system kit (Promega, Leiden, The Netherlands) was used. 100 µl of the luciferase substrate was added to the 96-well plate with the cells. Measurement was performed with the SpectraMax M5e luminometer, (Molecular Devices, USA). Each treatment was performed with three technical replicates, with each experiment being performed at least 2 times. EC50 values (Mean effective concentration to induce 50% luminescence) with 95% reliability were calculated with Prism v4 software (GraphPad Software Inc. La Jolla, Ca), the sigmoid curve data accuracy was assessed based on the R^2 values. The agonist effect data were calculated by the t-test at 5% of reliability, with a positive control of 20-Hydroxyecdysone at a concentration of 100µM. For the antagonistic effect, efficient compounds were those that reduced >15% luminescence when compared to the positive control with 20He or PonA.

Three-dimensional (3D) modeling of nuclear receptors of Diptera and Lepidoptera

To confirm the ability of the organocalcogenic compounds tested, to activate or block the ecdysonium receptor the three-dimensional structures of the compounds were used to construct a 3D-QSAR model. This model describes the activity of Daf-2 and Daf-4 compounds and the hormones with the ecdysteroid receptor. Compounds that did not show activity at the nuclear receptor of Diptera and Lepidoptera were excluded from the virtual analyzes. The way the Daf-2 and Daf-4 compounds and the 20-H hormone bind to the Lepidoptera ecdysteroid receptor was revealed by structural X-ray crystallography analysis (BILLAS *et al* 2003). These structures are found in the Protein Data Bank (Berman *et al* 2000) and used for the following analysis. The three-dimensional model of the *D. melanogaster* ecdysteroid receptor was generated with the Swiss-Model online tool (Biasini *et al* 2014), using the amino acid sequence available online (GeneBank # P34021). All virtual simulations were performed on the Microsoft Windows 7® operating system using Discovery Studio® and Chimera® software.

Conclusions

The cell screening system is effective for testing a high amount of steroidal and non-steroidal compounds. The hormones 20-Hydroxyecdysone and Ponasterone-A have similar activity patterns in the two cell lines used. The molecules DAF-2 and DAF-4 are antagonists to the hormone 20-Hydroxyecdysone in Diptera (S2) and Lepidoptera (SF9) cell lines. The observed interactions will serve as a basis for the rational design of new insecticidal molecules.

Author Contribution Statement

MRRM, EJL and MJZ contributed with the concepts and experiment designs. MRRM and EAS conducted the laboratory experiments. MRRM and MJZ conducted the three-dimensional modelling. MVL, JAJ and IRC conducted the data analysis. JAGS, DJH, VSB and DAM wrote sections of the manuscript.

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