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In silico chemical library screening and experimental validation of novel compounds with potential varroacide activities

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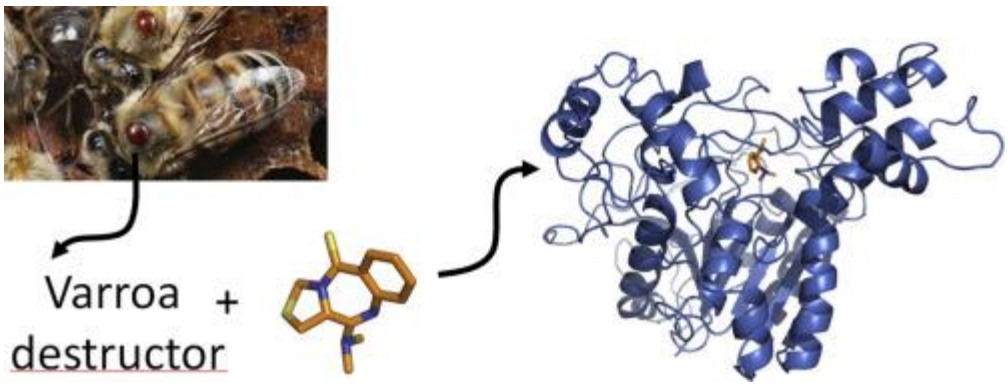
Abstract :

The mite *Varroa destructor* is an ectoparasite and has been identified as a major cause of worldwide honey bee colony losses. The use of yearly treatments for the control of varroosis is the most common answer to prevent collapses of honey bee colonies due to the mite. However, the number of effective acaricides is small and the mite tends to become resistant to these few active molecules. In this study, we have been looking for a new original varroacide treatment inhibiting selectively *Varroa destructor* AChE (*vd*AChE) with respect to *Apis mellifera* AChE (*am*AChE). To do this an original drug design methodology was used applying virtual screening of the CERMN chemolibrary, starting from a *vd*AChE homology sequence model. By combining the *in silico* screening with *in vitro* experiments, two promising compounds were found. *In vitro* tests of AChE inhibition for both species have confirmed good selectivity toward the mite *vd*AChE. Moreover, an *in vivo* protocol was performed and highlighted a varroacide activity without acute consequences on honey bee survival. The two compounds discovered have the potential to become new drug leads for the development of new treatments against the mite varroa. The method described here clearly shows the potential of a drug-design approach to develop new solutions to safeguard honey bee health.

Graphical abstract



Highlights

► This is the first study using a drug design approach to search for new compounds against the mite Varroa destructor. ► A homology model of vdAChE was used to perform virtual docking screening. ► Inhibitory activities on honey bee and on varroa mite AChE enzymes were investigated. ► Varroacidal hits were confirmed through *in vivo* experiments.

Keywords : Acetylcholinesterase, acaricide, in silico screening, docking, honey bees, varroa

1. Introduction

Varroa destructor (Acari: Varroidae) is a hemotophagous honey bee mite. It likely shifted from its original host *Apis cerana* to the Western honey bee *Apis mellifera* in the first half of the twentieth century [1]. Since then, varroa has spread worldwide, Australia remaining the only continent free from this mite.

Varroa is known to be one of the main culprits for the disastrous colony losses that have been reported in Europe and North America [2],[3]. The varroa mite has damaging physical and physiological effects and may weaken bees' immunity, allowing pathogens, especially viruses, to multiply unchecked [3]. In temperate climates, most of the colonies of Western honey bees will be damaged or even collapse within a few years if no control or inappropriate control methods are used [4]. Nowadays, beekeepers utilize a wide range of different chemical substances, application techniques and methods to keep mite populations under control [5]. Chemical control with synthetic acaricides remains a crucial component of an integrated pest management (IPM) of the mite varroa, but only a small number of compounds are suitable for mite control. Synthetic acaricides such as fluvalinate, flumetrine, coumaphos, cymiazole and amitraz have been successfully used to control varroa. Nevertheless, it has been observed that organisms can develop resistance via behavioural changes (e.g. avoiding the pesticide), reduced penetration, expression of certain detoxification enzymes, or target site desensitivitation by modifications of the active site [6]. The ability to develop resistance to a wide range of pesticides is a widespread phenomenon among mites [7] and it is almost inevitable that varroa becomes resistant against commonly used synthetic acaricides. It may in fact already be the case as an efficacy decrease to several active ingredients used for its control has been reported [8]. A way to limit this tolerance is to diversify the active molecules. Moreover, despite recent results [9],[10], no new active compounds have been registered for more than 25 years [11]. Therefore, there is an urgent need to find new acaricide compounds.

According to the IRAC (Insecticide Resistance Action Committee) mode of action classification scheme (www.irac-online.org [12]), currently available insecticides and acaricides can affect physiological functions such as nerve and muscle, growth and respiration. Most current chemical compounds act on nerve and muscle targets such as sodium channel, nicotinic acetylcholine receptors, octopamine receptors or acetylcholinesterase. The latter is the target we focused upon in this study.

Acetylcholinesterase (AChE, EC 3.1.1.7) plays a key role in cholinergic synapses in the insect central nervous system. AChE rapidly hydrolyses the neurotransmitter acetylcholine in the cholinergic system and terminates nerve impulses. Blockage of AChE leads to increased acetylcholine levels, causing a continuous stimulation resulting in muscular dysfunction, paralysis, and death. Due to its crucial role, AChE has been exploited as the target of organophosphate and carbamate pesticides to control many arthropod pests [13],[14],[12]. The overall structure of AChE is ellipsoidal and it belongs to the class of α/β proteins. It consists of a 12-stranded central mixed β-sheet surrounded by 14 α-helices. The 3D structure of AChE has revealed that, like other serine hydrolases, it contains a catalytic triad His, Ser, Glu [15]. This triad is located at the bottom of a 20 Å deep cavity, named the "aromatic gorge" as about 40% of its lining is composed of aromatic rings.

In this study, we have been looking for a new original varroacide treatment inhibiting selectively the *Varroa destructor* AChE (*vd*AChE) with respect to *Apis mellifera* AChE (*am*AChE). To do this, a structure-based virtual screening for drug discovery approach, currently used in human health, was applied (see supporting information Figure 1). A virtual screening of the CERMN chemolibrary, starting from *vd*AChE homology sequence model, was carried out. This screening was based on the active site topology, using the docking approach. The varroacide ability of selected compounds from virtual screening was validated by *in vitro* and *in vivo* biological tests.

2. Material and methods

2.1. Library

The chemical library (CERMN database, http://www.cermn.unicaen.fr/) contained at the date of screening 9788 compounds resulting from different research programs carried out by the CERMN laboratory in the field of medicinal chemistry. Standard tools of the ChemAxon Package were used (http://www.chemaxon.com/) to generate a 3D structure for each putative ligand as well as their ionization state at pH=7.4.

2.2. Docking Screening

In this study, the 3D model of *vd*AChE was generated using the homology sequence approach as by Dulin *et al.* [9]. To build this model, *vd*AChE sequence from the published genome sequences of *Varroa destructor* was used (accession number BRL_Vdes_1.0 (genome size 294.13 Mb)) [16]. The 3D model was built using the crystal structure (PBD: 1DX4) of *Drosophila melanogaster* AChE complex with tacrine [17]. The first sequence alignment (28% of sequence identity) was produced by the @TOME server [18] and then it was manually optimized (Figure 1). The homology model was generated with the Modeller software [19]. The folding quality of the *vd*AChE model was estimated using Verify3D [20] and Eval23D [21]. Qualitative Model Energy Analysis (QMEAN) score was also determined to estimate the quality of the homology model [22]. QMEAN scoring function is based on the linear combination of six structural descriptors and reflects the predicted global model reliability ranging from 0 to 1.

The model was next optimized by energy minimization and a quick molecular dynamic simulation using CHARMM software version c40b2 [23] using all-atoms CHARMM 36 force field [24]. The system was surrounded by a rectangular box of TIP3P water molecules [25] and neutralized with 0.15 M KCl using CHARMmGUI solvator [26]. Periodic boundary conditions were applied to the system using the IMAGE algorithm. Van der Waals interactions were truncated using a switching function between 10 and 12 Å with a cut-off distance of 16 Å and long-range electrostatic interactions were calculated with the particle-mesh Ewald (PME) method [27]. The vacuum dielectric was constant. After an energy minimization, dynamics simulation was performed using the Leapfrog Verlet algorithm with a 1 fs step. Systems were gradually heated until 300 K, by 10 K jumps during 20 ps and then the dynamic was temperature-equilibrated during 20 ps via heating reassignment under constant volume conditions. Finally, the system ran freely for 6 ns under NVT ensemble using Hover thermostat. An average structure was generated from the trajectory and resubmitted to a quick energy minimization to optimize its geometry. This minimized average structure was used in the docking screening.

The Gold program v5.3 [28, 29] was employed to generate a docked conformation for each library compound and calculate the fitness score to estimate its binding efficiency. This program applies a genetic algorithm to explore conformational spaces and ligand binding modes. Generally, the higher the fitness value calculated by the fitness function, the higher the predicted affinity should be. Four different fitness functions are available in the program v5.3 and after the docking tests on *vd*AChE, GoldScore fitness function was selected for our screening study and the docking was carried out using the default parameters. All compounds were docked within a sphere of 6 Å radius around the previously positioned ligand, pirimicarb. During the docking procedure side chains of three amino acids in the binding site were kept

flexible: Trp_{115}, Gln_{191}, Lys_{277}. For each ligand the best scoring position was selected for analysis.

2.3. Ligand position optimization by Molecular dynamics simulations.

To optimise the ligand position in the *vd*AChE binding site a molecular dynamic simulation was carried out using NAMD 2.12 [30] with the all-atom CHARMM 36 forcefield for proteins [24] and CGENFF for the synthetic ligands [31, 32]. To simulate aqueous solvent environment, the ligand/*vd*AChE complex was surrounded by a rectangular box of TIP3P water molecules [25] and 0.15 M of KCl as in model optimisation [26]. The chosen box size ensured, for each complex, that the simulated complex was at a minimum distance of 10 Å from the edge. Periodic boundary conditions were applied to the systems using the IMAGE algorithm. Van der Waals interactions were truncated using a force switching function between 10 and 12 Å and the PME approximation [27] was used to calculate long-range electrostatic interactions. The SHAKE algorithm was applied to restrain all bonds involving hydrogen atoms [33] and the vacuum dielectric constant was used during all calculations. The systems first underwent an energy minimization in 10000 steps. Then the minimized systems were heated to 303.15 K and the dynamics were temperature-equilibrated during 50 ps via heating reassignment under constant volume conditions. Finally, the systems ran freely for 20 ns under NPT conditions. Langevin dynamics with a damping coefficient of 1 ps^{-1} was used to maintain the system temperature and Nosé-Hover Langevin piston method to control the pressure at 1 atm. The trajectories generated were taken for subsequent analysis using CHARMM program version c40b2 [23].

2.4. In vitro tests of varroa and bee AChE biological activity

Inhibitory capacity of selected compounds on *vd*AChE and *am*AChE enzymatic activity was evaluated using an adapted spectrometric method of Ellman [34] in a 96 well plate. Acetylthiocholine iodide and 5,5-dithiobis- (2-nitrobenzoic) acid (DTNB) were purchased from Sigma Aldrich. Heads of bees or whole varroas were homogenized in Tris buffer (10 mM, pH=7.0) containing 1 M of NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA) and 1% (w/v) Triton X-100.

In our procedure, in each well, 100 µL of 0.3 mM DTNB dissolved in phosphate buffer pH 7.4 were added followed by 50 µL of test compound solution (or 50 µL of phosphate buffer for control) and 50 µL of enzyme solution. Initial absorbance was measured at 412 nm and used to correct the final absorbance. After 5 min of preincubation, the reaction was initiated by the injection of 50 µL of 10 mM acetylthiocholine iodide solution. The hydrolysis of acetylthiocholine was monitored by the formation of a yellow 5-thio-2-nitrobenzoate anion as the result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of acetylthiocholine, at a wavelength of 412 nm every minute for 30 min using a 96-well microplate plate reader (TECAN Infinite M200, Lyon, France). Test compounds were dissolved in analytical grade DMSO. Absorbance at 30 minutes (or at 10 minutes for honey bee samples) was used to evaluate the inhibition activity.

First screening of AChE activity was carried out at a 10^{-4} M concentration of selected compounds, with carbamate formetanate hydrochloride (10^{-4} M) used as positive control [9]. For compounds with significant inhibition at 10^{-4} M (> 50%), IC_{50} (concentration required to inhibit 50% of AChE activity) absorbance values were determined for a range of 10^{-3}-10^{-10} M concentrations. IC_{50} values were calculated by fitting the response points to nonlinear fit-log inhibitor concentration curve using GraphPad Prism 7.0 Software (GraphPad Software, San Diego, CA, USA).

2.5. Laboratory bioassays

In vivo tests were carried out in France (46°26'13.3"N 1°19'37.1"E). Introduction of honey bees and varroa mites in cages allowed us to evaluate the acute toxicity of the compounds simultaneously for both species. Phoretic varroas were collected the day of the experiment, using a modified icing sugar-shake method [35]. About 200 to 300 honey bees were collected using a varroa-counter jar (Swienty A/S). Approximately 30 g of powdered sugar (CristalCo, Daddy, France) was added and the jar gently rolled, to distribute the sugar, and then inverted and shaken for 1 to 3 minutes. This sugar shake method allows to separate varroas from honey bees without harming bees or mites. In parallel, workers bees of unknown age were collected using a ventilated transparent container, in colonies free from clinical symptoms of diseases and without any varroacide treatment for six months.

Immediately upon arrival from the field, within one hour after collection, varroas were gently brushed away from the sugar and kept on moist paper at room temperature until use. Inactive or moribund mites were discarded.

Prior to treatment, bees were anesthetized with carbon dioxide. A 3 L per hour carbon dioxide flow for 20 seconds, followed by 2 minutes in a hermetic container, allowed sufficient anaesthesia of honey bees. After anesthetization, each honey bee was topically exposed to the studied molecules by depositing 1 µL drop of selected compound dissolved in acetone. To solubilize both sr2091 **(**(3aR)-4-(dimethylamino)-3,3a-dihydro-1H-thiazolo[4,3-c][1,4]benzodiazepine-10-thione, 98% purity) and sr2093 (4-[2-(dimethylamino)ethylamino]-3,3a-dihydro-1H-thiazolo[4,3-c][1,4]benzodiazepine-10-thione, 80% purity), the addition of HCl was necessary (1 mole per mole of active substance). Solutions were vortexed vigorously before use. Before honey bees became active again, 10 varroa were deposited on their cephalothorax, then bees and varroa were immediately transferred into cages. The insects were

given approximately 15min to recover from the carbon dioxide treatment, and any dead bees were removed from the experiment. Solvent controls received 1 µl of acetone only, negative controls received no treatment and as positive control we applied a widely used varroacide molecule amitraz, at a concentration of 0.1 µg per honey bee. Amitraz targets octopamine receptors, not AChE, but it is effective on varroa mites without causing acute damage to honey bees. Any experiment with negative control mortality higher than 30% was discarded from the analysis.

Single-use cages consisted of 1 oz cups made of food grade and clear plastic. Approximately 60 holes were made into the side and lid of the plastic cups. Bees were provided ad libitum a 50% (weight/volume) sucrose solution made by dissolving refined sugar in water. The syrup was distributed through a pierced 1.5 mL Eppendorf tube, with one tube fixed in each cage lid.

Cages were maintained in darkness at room temperature, and mortality of honeybees and varroas was assessed 24h after treatments. At the end of these experiments, all cages were placed at -20 °C to kill honeybees, to assess the exact number of varroas in each cage and take in account any natural varroa infestation in the calculation of the mite's mortality rate.

A generalized linear model [36] with binomial distribution was used to compare the mortality of each group with the negative control group mortality. Post hoc comparison was conducted using the function *glht*, from the package "multcomp" to test for differences between all pairs of treatments. Analyses were performed using the statistical software R version 3.3.2 (R Core Team 2016).

3. Results

3.1. CERMN chemolibrary screening

In order to find new and original selective inhibitors of *vd*AChE, virtual screening of the CERMN chemolibrary was carried out. Structure-based screening using a docking approach was applied to the *vd*AChE homology model built using the *vd*AChE sequence from the varroa

genome [16] on a *dm*AChE template. The insertions/deletions (1 to 16 amino acids, Figure 1) were identified in the sequence alignment. These were however located in neighboring surface loops and have no effects on the secondary structure elements. The *vd*AChE homology model generated presented a root-mean-square (RMS) difference of 1.012Å (Cα atoms) from the *dm*AChE X-ray structure, taking into account all intracellular and extracellular loops. A QMEAN score of 0.62 (>0.50) for our model suggested that the homology model built was reliable.

Before docking, the *vd*AChE homology model was submitted to energy minimization and quick molecular dynamics simulation at 300 K (6 ns) under NVT conditions. An average structure was generated from the trajectory and resubmitted to a quick energy minimization to optimize side chain geometry.

Docking screening was carried out using the GOLD (Genetic Optimisation for Ligand Docking, [29]) program on this optimized average structure. GOLD is an automated ligand docking program that uses a genetic algorithm. GOLD's evolutionary algorithm modifies the position, orientation and conformation of a ligand to fit into one or more low energy states of the protein active site. It maps ligand geometry parameters onto populations of chromosomes and then runs evolutionary rounds of mutation, crossover, scoring and selection to optimise protein-ligand interactions. For solution selection, we applied the GoldScore score function and we kept the three binding site residues flexible, Trp_{115}, Gln_{191}, Lys_{277}. The value of the GoldScore gives a guide as to how good the pose is: the higher the score, the better the docking result is likely to be. The 3D structure for each compound from the CERMN chemical library (9788 compounds) was generated at its ionization state at pH=7.4 and docked into the *vd*AChE binding site. For each compound the best pose, with the highest score, was retained. The 1000 compounds representing the highest GoldScore values (varying from 99.77 to 66.69) from the total of 9788 in the CERMN chemolibrary were selected for subsequent analysis. We have

observed that the elongated shape of the AChE binding site causes the GOLD software to overestimate the Goldscore value for compounds with high molecular weights. The molecular weights among the 1000 selected compounds varied from 175.23 to 830.84 $g \cdot mol^{-1}$. In order to focus on moderate molecular weight compounds, which are easier to pharmacomodulate, a threshold of 350 $g \cdot mol^{-1}$ was applied on the selected 1000 compounds. We chose this value with reference to the molecular weight of pirimicarb (238.29 $g \cdot mol^{-1}$), a selective carbamate insecticide which has shown good affinity and selectivity for *vd*AChE in our previous studies [9, 37]. Among the 1000 compounds, 215 had a smaller molecular weight than 350 $g \cdot mol^{-1}$. To take into account the coherence between GoldScore and molecular weight, the GoldScore value was divided by the molecular weight [38, 39] and the 53 compounds with the highest GoldScore/Molecular Weight ratio were selected for biological evaluation. From the 53 selected compounds, 46 were evaluated at $1 \cdot 10^{-4}$ M ligand concentration by *in vitro* biological tests with the aim to detect *vd*AChE inhibition potency. Among the tested compounds, four had an inhibition activity higher than 20% and one close to 60% (see Table 1). As the goal of this screening was to find compounds able to inhibit *vd*AChE and not *am*AChE, not to compromise the health of bees, inhibition activity of *am*AChE was also evaluated. Among the three compounds with lesser inhibition potency (about 20%), sr2114 and sr2112 inhibited *am*AChE in the same way, while sr2090 inhibited *am*AChE very weakly. Compound sr2091 [40], which had the highest *vd*AChE inhibitor potency (59%) among the screened compounds, had at the same time a weak *am*AChE inhibition activity as desired (Table 1). Therefore, this compound presents the desired properties for a potentially new accaricide.

As the four compounds emerged from our screening have a tricyclic structure including a benzodiazepine ring, we assessed a larger part of the chemical space by *in vitro* tests on the whole chemical family related to these compounds [40], to ensure better understanding of the structure activity relationship (SAR). The *vd*AChE inhibition potency of 25 additional

compounds of this chemical family was measured (Table 2). Three additional compounds with *vd*AChE inhibition potency greater than 20% were discovered, of which one had inhibition activity of 53% (sr2093). All additional compounds tested presented small *am*AChE inhibition. Therefore, among the tested compounds, in addition to sr2091, sr2093 has also come out as a potential accaricide (Table 2). For both compounds, the IC_{50} for *vd*AChE inhibition was determined (see Table 3). The IC_{50} values obtained for sr2091 and sr2093 were respectively 16 ± 12 µM and 11 ± 6 µM, and they are of the same order as the IC_{50} of pirimicarb (IC_{50}=6.6 ±0.8 µM), another potential varroacide compound targeting *vd*AChE [9].

3.2. Laboratory bioassays

To confirm the *in silico* and *in vitro* hypotheses, the toxicity of sr2091 and sr2093 was assessed using cage tests. This device allows the evaluation of acute toxicity of compounds simultaneously for both species. Observed mean mortalities (±se) for both species are shown in Table 4. There was no honey bee mortality detected for any tested conditions. The varroa mean mortality in the negative control group was 8.6%. A solvent-control test was also carried out and no statistical difference with the negative control mortality was observed. The compounds sr2091 and sr2093 were effective at 87% ($p<0.001$) and 82% ($p<0.001$) respectively at an application of 10 µg per bee. However, there was no significant mortality of varroa mites when the deposit was reduced to 1 µg per bee ($p=0.88$ and $p=0.50$ for sr2091 and sr2093, respectively). In conclusion, a significant mortality of the mites was observed when 10 µg of both sr compounds was applied, and no effect was observed on honey bee survival.

3.3. sr2091/vdAChE and sr2093/vdAChE complexes optimized by Molecular Dynamic Simulation

To analyze in detail the fixation mode of sr2091 and sr2093 in the binding site of *vd*AChE, the initial result from screening was optimized by a molecular dynamic simulation. The interaction energy sr2091/*vd*AChE and sr2093/*vd*AChE along the trajectory was calculated and for each complex studied the one with the strongest interaction energy was saved for subsequent analysis (Figure 2). To investigate the sr2091 interaction mode in the *vd*AChE binding cavity, the interaction energies atom per atom with whole *vd*AChE were calculated (Figure 3A, 3B). Analyses highlighted as crucial atoms for compound binding: the sulphur and one nitrogen atom of the thiazolodiazepine ring (S4 and N21) and the sulphur of the thiolactame group (S10). Sulphur atoms interact principally through van der Walls interactions, while the interaction of N21 with *vd*AChE binding site is of an electrostatic type.

In parallel, interaction energies per *vd*AChE residue with whole sr2091 were calculated and they highlighted as key residues for sr2091 fixation: Lys277, Ala112, Gly111, Trp115, Arg62 and His432 (see Figure 2C). Visualization of the sr2091/*vd*AChE complex showed that Lys277 and His432 side chains are close to nitrogen atom N21 of the thiazolodiazepine ring, N21 distance from Lys227 Nζ is about 3.7 Å and from His432 Nδ1 about 3.7 Å (Figure 2). The Lys277 Nζ is also spatially close to sulphur atom S10 of the thiolactame group, $d_{S10\text{-}N\zeta}$=3.5 Å (Figure 2). The thiazolo diazepine ring sulphur atom S4 is situated in the proximity of Arg62 side chain, the distance between S4 and Arg Nε atom is about 3.4 Å. The hydrophobic residues Gly111, Ala112 and Trp115 were also suggested in our analysis as important for sr2091 fixation and their interactions are principally of the van der Walls type.

4. Discussion

In this study, we carried out a virtual screening of the CERMN chemolibrary, containing 9788 compounds, on the 3D homology sequence model of *vd*AChE with the aim of selecting a new

potential effective varroacide. A post processing treatment was applied on the thousands of selected potential *vd*AChE inhibitors by docking screening. The ACh binding cavity in *vd*AChE is elongated, with a second cavity entrance, and therefore the docking screening selected mainly rather bulky compounds (MW ranging from 175.23 to 830.84 $g \cdot mol^{-1}$). In order to focus our screening on moderate molecular weight compounds we have carried out post-processing based on the molecular weight of the compound as discussed previously by Pan *et al.* [38] and by Jhoti and Leach[39] and we have limited our selection to 53 compounds (MW ~ 175.23 - 300.9 $g \cdot mol^{-1}$).

Among the 46 compounds evaluated *in vitro* from the 53 virtually selected, four have shown an inhibition activity toward *vd*AChE higher than 20% at 10^{-4} M ligand concentration. As these four molecules were structurally related, all members of this compound family have also been tested *in vitro*. From the *in vitro* screening, sr2091 and sr2093 came out as potential varroacides, with IC_{50} for *vd*AChE at the ten micromolar range and they have presented only weak *am*AChE inhibition. As the purpose of new varroacide discovery is to find compounds inhibiting selectively *vd*AChE without altering honey bee health, the compounds highlighted during this screening were interesting and their acute toxicity for both species was studied.

During the laboratory acute toxicity biassays, a significant mortality of the mites, but not of honey bees, was observed when 10 µg of each sr compound was applied. Unlike amitraz, they were not effective when applied at 1 µg per bee. Differences in efficacy have already been observed by Papachristoforou *et al.* for different treatments against varroa [41]. A topical application of 1.81 µg/mite of amitraz completely inhibited a varroa mite's gravitational reflex, while the volatile accaricides formic acid, thymol crystals, and Apiguard® eliminated this reflex for doses of 13.83, 250 and 1000 mg/mite, respectively. Therefore, in our experiment, the gap between effective doses should not be considered as an eliminatory criterion. It was for instance proposed that acaricide treatments, that cause more than 70% mite mortality, with less

than 30% bee kill, should be considered as mite selective and acceptable for mite management [42]. Virtual screening of the CERMN chemolibrary has identified a new compound family inhibiting *vd*AChE at micromole concentrations and inhibiting *am*AChE only weakly. This new compound family is structurally different compared to other accaricides available on the market which could delay the appearance of resistances. Furthermore the sr family members are reversible AChE inhibitors which should guarantee fewer side effects.

Even if the sr family varroacide activity is rather moderate, the sr compounds discovered can serve as a lead for future optimization programs to generate new original varroacide compounds without effects on the honey bee. The *in vitro* screening on the whole chemical family allowed the determination of chemical groups responsible for *vd*AChE inhibition activity (Table 1 and Table 2). Firstly, both the sulphur atoms of the thiazolodiazepine ring and the thiolactame function are necessary for *vd*AChE inhibition. We observed that the replacement of the thiolactame function by a lactame function (entry 4 of Table 1 compared with entry 20 of Table 2) led to a loss of *vd*AChE inhibition and that the replacement of the sulphur atom of the thiazolodiazepine ring by a carbon atom decreases drastically the inhibition activity on *vd*AChE, from 60% to 20% (Table 1, entries 3 and 4) and from 63% to 0% (Table 2 entry 13 and 21). Our *in vitro* structure-activity analysis on the sr family did not allow us to evaluate the importance of the two nitrogen atoms in the tricycle, since in all tested compounds these two nitrogen atoms were present. Nevertheless, we could evaluate the influence of the nature of the substituent on the nitrogen atom in the aliphatic chain anchored on the tricyclic core on *vd*AChE inhibition: the replacement of the aliphatic chain by cyclic or aromatic substituents led to inactive, or less active, compounds (Table 1 entry 4 compared with Table 2 entries 10 and 22). The presence of a basic centre in the aliphatic chain led generally to a better inhibition potency, and the best results were obtained with compounds possessing an aliphatic chain with a basic centre (Table 1, entry 4 and Table 2, entry 21). In summary, the presence of

sulphur atom on the thiazolodiazepine ring and on the thiolactame function is necessary for *vd*AChE inhibition and the presence of nitrogen in the aliphatic chain is desirable.

To obtain a better understanding of the fixation mode of sr2091 and sr2093, molecular dynamics simulations were carried out on the complexes with *vd*AChE built by docking. The calculated interaction energy per atom for sr2091 highlighted the crucial role of three atoms in sr binding to *vd*AChE, two sulphur atoms and nitrogen N21 in the thiazolodiazepine ring. Molecular dynamics results have shown that sr compounds reach the bottom of the *vd*AChE binding groove and interact directly through nitrogen N21 of the thiazolodiazepine ring with His432 a residue of the catalytic triad. The sr compounds also established electrostatic interactions with two polar residues, Lys277 (the highest contribution) and Arg62. The Lys227 nitrogen is spatially close to nitrogen N21 of the thiazolodiazepine ring and sulphur S10 of thiolactame, while Arg62 interacts with sulphur S4 of the thiazolodiazepine ring (Figure 2). This result is in agreement with our observations on structure activity relation from *in vitro* tests on the sr family. We concluded from *in vitro* test results that the presence of both sulphur atoms is necessary for good *vd*AChE inhibition activity. Nevertheless, *in vitro* tests did not allow us to evaluated experimentally the importance of both nitrogen atoms in the thiazolo diazepine ring, as in all tested compounds these two nitrogen atoms were present. From the modelling results, nitrogen in position N21 plays a more important role for sr2091 binding than nitrogen in position N8, as it mainly participates in the electrostatic contacts with Lys277. Interestingly, sequence alignments of *vd*AChE with *am*AChE (Supporting Information, Figure S2) showed that these residues are not conserved in *am*AChE. They are substituted by two hydrophobic residues in *am*AChE, a Leu and a Trp, respectively, which could explain the poor *am*AChE inhibition activity of sr compounds compared to *vd*AChE.

5. Conclusion

With the aim to improve the diversity of varroa treatment, we have performed a computer aided drug-design approach, currently used for human health. This is the first time that this approach is developed for honeybee health.

The virtual screening of a chemolibrary, using a protein−ligand docking protocol, carried out on the *vd*AChE homology model, has identified a new, chemically original, compound family inhibiting *vd*AChE, with the best inhibition activity at a micromole concentration. Importantly, the discovered family inhibits *am*AChE only weakly. The structure-activity and molecular modelling analysis showed that the presence of both sulphur atoms and of two nitrogen atoms is crucial for sr compounds fixation. The modelling results suggested that sr compounds reach the bottom of the binding groove and interact directly with the histidine of the catalytic triad. From modelling results, two polar residues, Lys277 and Arg62, appeared overriding for sr compound binding to *vd*AChE. These two residues are replaced by hydrophobic ones in *am*AChE, which could explain the selectivity of sr compounds toward the mite. Biologically, these two compounds have the desired properties for a potentially new accaricide and they can serve as a lead for future optimization programs to generate new varroacid compounds without effects on the honey bee. Our results represent the first step towards the development of a new veterinary product. More field tests are still required to asses, inter alia, any sublethal and long-term side effects on adult bees and honey bee brood, and confirm the varroacide efficiency. However, the method described here clearly shows the potential of a drug-design approach to develop new solutions to safeguard honey bee health.

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Compliance with Ethical Standards

C. Riva has been co-funded by Normandy County Council and Véto-pharma.

The authors declare that they have no conflict of interest.

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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Figure captions

Figure 1. Sequences alignment between *dm*AChE and *vd*AChE used for the model building. β-strands are represented by orange arrows and α-helix by green ones. Amino acids of the catalytic triad are marked by an asterisk.

Figure 2. Sr2091 (A) and sr2093 (B) position in *vd*AChE binding sites after the optimization by molecular dynamic simulation. The compounds and the selected side chains of the binding site residues are in stick and the protein in ribbon representation. This figure was made with PYMOL (DeLano Scientific, 2002, San Carlo, USA).

Figure 3. (A) sr2091 compound formula (B) Calculated interaction energy per sr2091 atom with *vd*AChE (C) Calculated interaction energy per residue of *vd*AChE with sr2091.

Figure 1

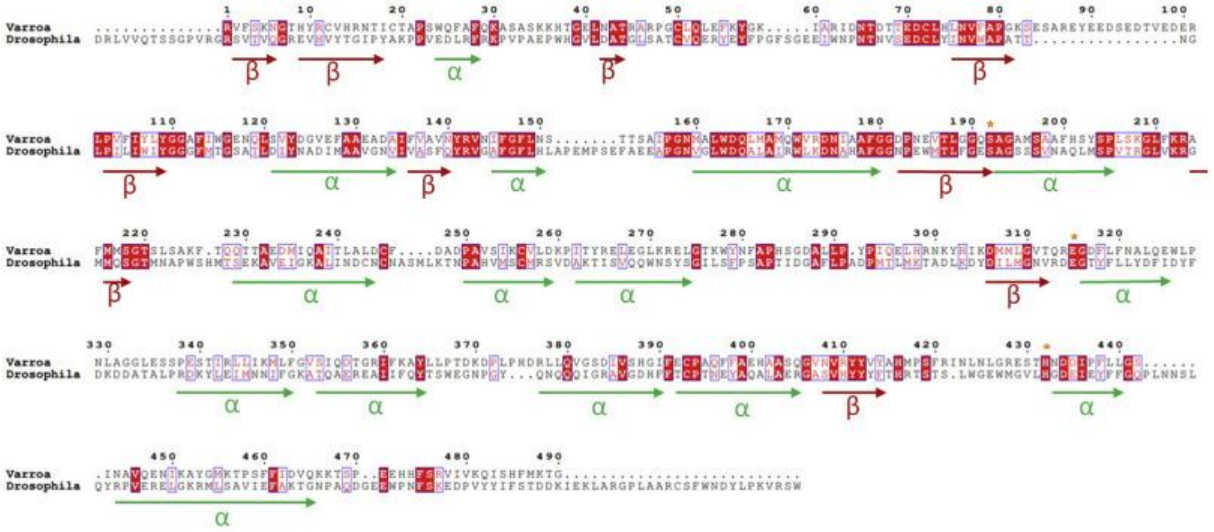


Figure 2

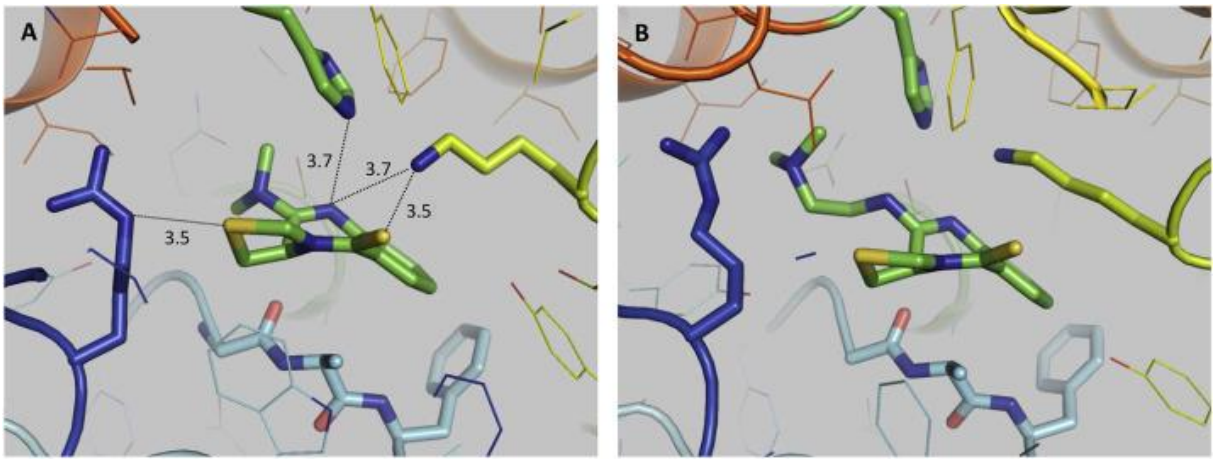
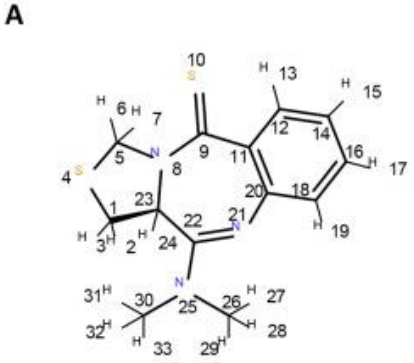


Figure 3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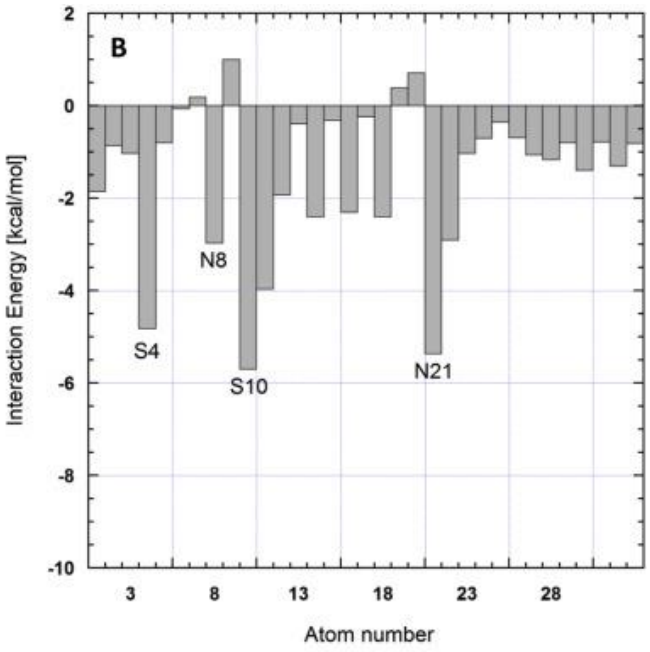

B
Interaction Energy [kcal/mol]
2
0
-2
-4
-6
-8
-10
3
8
13
18
23
28
Atom number
S4
N8
S10
N21

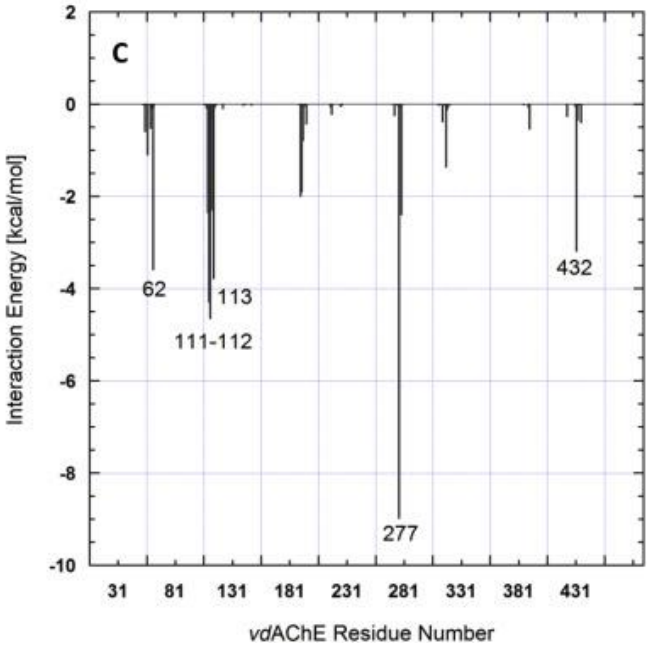

C
Interaction Energy [kcal/mol]
2
0
-2
-4
-6
-8
-10
31
81
131
181
231
281
331
381
431
vdAChE Residue Number
62
113
111-112
277
432

Table 1. Best Compounds from the Docking Virtual Screenings

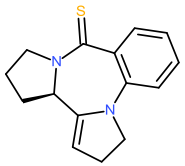
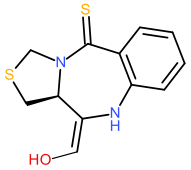
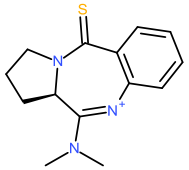
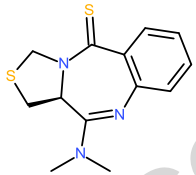
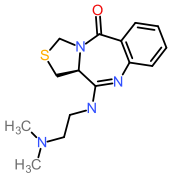
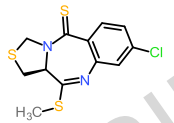
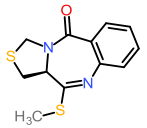
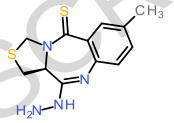
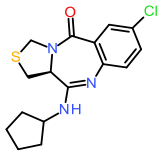
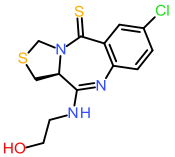
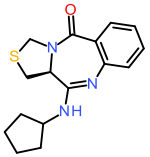
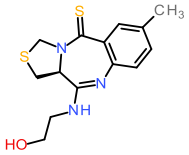
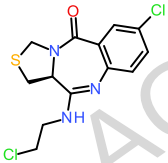
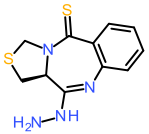
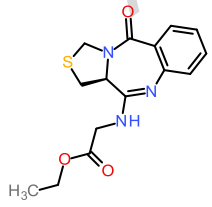
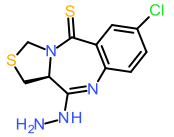
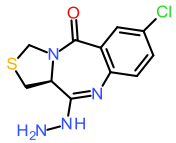
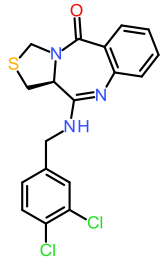
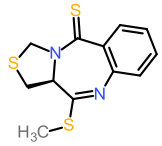
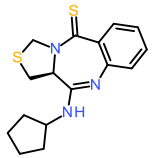
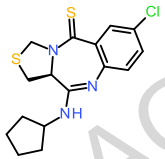
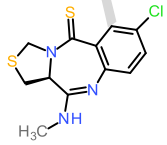
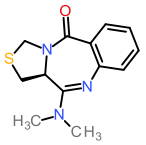
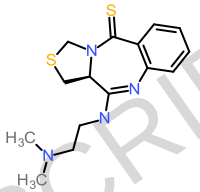
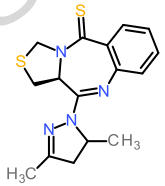
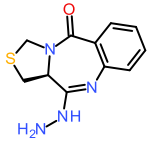
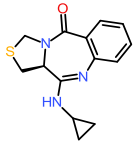
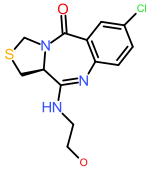
	Compound Name	Compound diagramme	Molecular Weight [g/mol]	Gold Score Fit	Gold Score/MW	*vd*AChE inhibition	*am*AChE inhibition
1	sr2114		257.35	71.67	0.2785	29%	18%
2	sr2090		265.35	73.37	0.2765	21%	5%
3	sr2112		260.38	71.92	0.2762	22%	25%
4	sr2091		277.41	73.44	0.2647	59%	21%

Table 2. Results of *in vitro* screening on sr2091 chemical family

	Compound Name	Compound diagramme	*vd*AChE inhibition	*am*AChE inhibition		Compound Name	Compound diagramme	*vd*AChE inhibition	*am*AChE inhibition
1	mr19199		5%	0%	14	sr2021		5%	0%
2	sr1975		5%	0%	15	sr2083		3%	0%
3	sr1982		4%	0%	16	sr2085		13%	0%
4	sr1984		3%	0%	17	sr2086		19%	1%
5	sr1986		4%	0%	18	sr2088		5%	0%
6	sr1989		1%	0%	19	sr2089		4%	0%

7	sr1990		3%	4%
8	sr1993		12%	0%
9	sr2020		20%	0%
10	sr2099		6%	0%
11	sr2100		4%	0%
12	sr2102		5%	0%

20	sr2092		3%	0%
21	sr2093		63%	15%
22	sr2097		5%	0%
23	mr17862		2%	0%
24	mr17861		3%	0%
25	mr19924		2%	0%

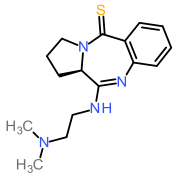
13	sr2109		0%	5%

Table 3. Inhibition percentage (± standard error) and IC_{50} (± standard error) values for two best compounds from the screenings (n=3)

		***am*AChE**	***vd*AChE**
sr2091	Inhibition (10^{-4} M)	21% (± 0.06)	59% (± 0.07)
	IC_{50} (µM)	ND	16 (± 12)
sr2093	Inhibition (10^{-4} M)	15% (± 0.02)	63% (± 0.06)
	IC_{50} (µM)	ND	11 (± 6)
Formetanate (control)	Inhibition (10^{-4} M)	97% (± 0.005)	84% (± 0.04)

Table 4. Results of *in vivo* tests. Data correspond to six replicates, each carried out with 10 honey bees and 10 varroas.

Compound		**Mortality[a]**	
		Honey bee	**Varroa**
sr2091	10 µg	0%	86.7% (± 3.3)*
	1 µg	0%	14.2% (± 1.1)
sr2093	10 µg	0%	81.7% (± 2.1)*
	1 µg	0%	19.1% (± 1.0)
Acetone control		0%	9.6% (± 0.9)
Amitraze control	1 µg	0%	100%*

[a] percent mean mortality (± standard error) * indicate significant difference determined by post-hoc test (* p-value < 0.01)

Highlights

- This is the first study using a drug design approach to search for new compounds against the mite Varroa destructor.
- A homology model of vdAChE was used to perform virtual docking screening.
- Inhibitory activities on honey bee and on varroa mite AChE enzymes were investigated.
- Varroacidal hits were confirmed through in vivo experiments.