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Ecological toxicity reduction of dinotefuran to honeybee: New perspective from an enantiomeric level

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ABSTRACT

In last decade, there has been a concerted effort to reduce the potential threats of honeybees' population due to exposure to neonicotinoid pesticides. A new perspective was put forward to reduce the potential ecological toxicity of neonicotinoid dinotefuran to honeybee in terms of an enantiomeric level in the study. Toxicity of dinotefuran was enantioselective, and *S*-dinotefuran was 41.1- to 128.4-fold more toxic than *R*-dinotefuran to honeybee *Apis mellifera* (*Apis mellifera* Linnaeus), whereas *R*-dinotefuran exhibited comparative insecticidal activities (1.7–2.4 times) to typical sucking pests *Aphis gossypii* and *Apolygus lucorum* compared to racemic mixtures. Our data suggested that use of *R*-dinotefuran could have a good efficacy in controlling target pests while minimizing hazard to honeybees. The mechanism for chiral specific toxicity to honeybee was further characterized by electrophysiological studies and molecular docking. *S*-dinotefuran appears to be more toxic by binding to α8 subunit of nAChR of *Apis mellifera*. The α8 also have a more stable, functional binding cavity to *S*-dinotefuran with a higher binding score of 7.15, primarily due to an extensive hydrogen bond network. Therefore, new chiral products with a high proportion of or an enantiomeric pure *R*-dinotefuran are recommended to achieve effective pests control reducing hazard to honeybee populations.

1. Introduction

Honeybees are crucial for global agroecosystems, which contribute over $200 billion in pollination services for approximately 2/3 of crop species and most wild flowering plants (Connolly, 2013; Palmer et al., 2013; Rundlöf et al., 2015). These contributions of honeybees in agricultural products have been increasing steadily, especially for those crops with highly economical values and nutritional contents (Lautenbach et al., 2012; Stanley et al., 2015). Nonetheless, numerous studies have indicated a negative correlation between honeybee health and neonicotinoid pesticides exposure, illustrating dramatic reductions in honeybee foraging (Feltham et al., 2014; Henry et al., 2012; Kessler et al., 2015), reproductive performance (Rundlöf et al., 2015; Whitehorn et al., 2012), and colony survival (Goulson, 2015; Stanley et al., 2015). The severe threats and mass death of honeybee may leave us to face a “pollination crisis”, ultimately resulting in serious consequences for biodiversity maintain, ecosystem function and food security (Goulson et al., 2015; Vanbergen and Initiative, 2013). Generally, much of the crisis in honeybee populations have been attributed to neonicotinoid pesticides, the most widely used chemical class of insecticides, which account for 80% of the global insecticidal seed treatment market share in > 120 registered countries (Jeschke et al., 2010). The main measures adopted to minimize the risk of neonicotinoids are the restriction in use of these pesticides in agroecosystems, especially in the European Union and in the United States (Gewin, 2008). Would a better solution be to reduce the toxicity of neonicotinoids to nontarget honeybees without sacrificing their use in controlling target pests in agricultural system?

In view of this concern, a new perspective was put forward to in term of chirality. Chiral characteristics had been increasingly found in neonicotinoid insecticides with the synthesis of more complex molecular structures in the last few years (e.g., dinotefuran, sulfoxaflor and cycloxaprid). For example of chiral neonicotinoid dinotefuran (Fig. 1), (EZ)-(*RS*)-1-methyl-2-nitro-3-(tetrahydro-3-furylmethyl) guanidine, is

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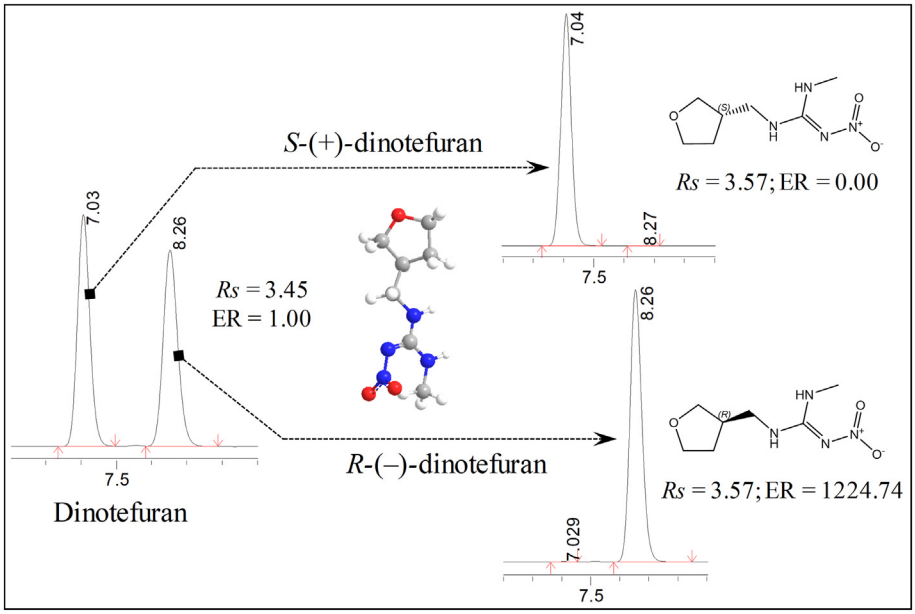

Fig. 1. Typical chromatograms, molecular structures and enantioseparation parameters of dinotefuran, *S*-(+)-dinotefuran and *R*-(−)-dinotefuran. (Arrows show the beginning and end of each enantiomer collected in the chiral preparation.)

widely used around the world towards both sucking insects and health pests (Z. Chen et al., 2015b; Kiriyama et al., 2003). It consists of a pair of enantiomers, *S*-dinotefuran and *R*-dinotefuran. Dinotefuran acts on the nicotinic acetylcholine (ACh) receptor (nAChR), which is the principal insecticide target and the ligand-gated ion channel, mediating fast cholinergic synaptic transmission in the nervous systems (Shao et al., 2013; Wakita, 2010). High toxicity of racemic dinotefuran to honeybees has also been confirmed according to the International Union of Pure and Applied Chemistry (IUPAC, 2019). However, little is known about the toxicities of dinotefuran enantiomers to honeybees. There are substantial knowledge gaps with regard to the molecular mechanism for the enantioselective toxicity of dinotefuran to honeybees.

To evaluate the potential toxicity reduction of neonicotinoid pesticides to honeybees, dinotefuran was chosen as a representative neonicotinoid to understand the mechanism of toxicity to *Apis mellifera* in terms of chiralspecificity. We proposed a transdisciplinary approach that effectively combines electrophysiological recording and molecular docking with traditional lethality evaluation and chromatographic method. The mechanism for the chiralspecific toxicity of dinotefuran to *Apis mellifera* was elucidated progressively from honeybee individual to target receptor nAChR subunit and finally to molecular binding interaction. Our research works were carried out as follows: 1) Chiral preparation and identification of *S*-dinotefuran and *R*-dinotefuran were achieved to obtain optical pure individual enantiomers of dinotefuran. 2) Enantioselective lethality of dinotefuran was evaluated for honeybee (*Apis mellifera*) and two typical sucking pests, cotton aphid (*Aphis gossypii*) and Mirid bug (*Apolygus lucorum*), to clarify the variability between enantiomers under two typical exposure modes. 3) Electrophysiological recording was applied to explore the toxicological properties of the *S*-dinotefuran and *R*-dinotefuran with each nAChR subunits of *Apis mellifera* by expressing the hybrid nAChR in *Xenopus* oocytes, to screen the chiralspecific target subunit(s) and to confirm the origin of enantioselective toxicity. 4) Homology modeling and molecular docking was used to investigate the binding ligand and affinity between the dinotefuran enantiomers and the target receptor subunit(s) of *Apis mellifera*. 5) Comprehensive toxicological assessments were finally employed to propose a better solution to reduce the potential threats and mass death of honeybees due to neonicotinoid pesticides exposure. New chiral pesticide products were also recommended to achieve effective insect control, while minimizing ecological toxicity of neonicotinoids to honeybee populations in agroecosystems.

2. Materials and methods

2.1. Chiral preparation and identification

Dinotefuran enantiomers were separated and prepared using a supercritical fluid chromatography (SFC) MultiGram II system from Berger Technologies (Newark, DE, USA) equipped with a Daicel chiral preparative column (Tokyo, Japan) and a Knauer K2501 UV detector (Berlin, Germany). The enantiomeric resolution, retention time and peak area of dinotefuran enantiomers were calculated using SFC Method Station (Berger Technologies). The optical rotation of each enantiomer was further identified by an Advanced Laser Polarimeter from PDR-Separations LLC (Palm Beach Gardens, FL, USA). The absolute configuration (*S* and *R*) of dinotefuran enantiomers was clarified by the corresponding relationship with the optical rotation (+ and −) indirectly based on a previous study by Kiriyama in the early synthesis stage of dinotefuran (Kiriyama et al., 2003).

2.2. Enantioselective lethality evaluation

The honey bees *Apis mellifera* were provided by the Institute of Apicultural Research, Chinese Academy of Agricultural Sciences. Young adult worker bees were collected from the same queenright colony, of similar age and feeding status, free of disease, and had no history of dinotefuran and other chemical substances before test. Two typical sucking pests, cotton aphid (*Aphis gossypii*) and Mirid bug (*Apolygus lucorum*), were collected from an established laboratory colony of the Institute of Plant Protection, Chinese Academy of Agricultural Sciences. The *Aphis gossypii* were reared on cotton plants *Gossypium hirsutum* Linnaeus at 25 ± 1 °C, 70% ± 5% relative humidity and 16/8 h of light and dark. The *Apolygus lucorum* were reared on green beans *Phaseolus vulgaris* Linnaeus with a 10% sucrose solution at 27 ± 0.5 °C, 70% ± 5% relative humidity and 14/10 (L/D) photoperiod. More than twenty-five generations of the two pests were used for the enantioselective bioassays. Two independent methods by physical contact and oral ingestion were respectively applied to examine the enantioselective lethality of dinotefuran enantiomers to *Apis mellifera*,

Aphis gossypii and *Apolygus lucorum*. The physical contact test was carried out to evaluate the contact poisoning effect from the insect's surface exposure, whereas the oral ingestion test was correspondingly used for the assessment of stomach poisoning characteristics by direct dispersion in food of the selected insects. Details about the two methods of topical and oral exposure routes were summarized in the Supporting information.

2.3. Cloning the full length cDNAs of nAChR subunits and phylogenetic analysis

Total RNA was isolated from the dissected brains of adult bees using TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Synthesis of first-strand cDNA was carried out according to the reverse transcriptase XL (AMV) (Fermentas, Glen Burnie, MD, USA) protocol with oligo dT18. The specific primers to amplify the *Apis mellifera* L. α subunits were designed according to the genome sequence data (Jones et al., 2006), while the primers for the *Rattus norvegicus* β2 subunit were obtained based on the nucleotide sequences reported by Boulter J (Boulter et al., 1987). PCR were carried out on 1 μL of cDNA template, 5 mM of each primer, 0.1 mM of each deoxynucleoside triphosphate (dNTP), and 1.0 U of prime STAR DNA polymerase (Takara, Dalian, Liaoning, China) in the supplied buffer, containing 1.0 mM $MgCl_2$, in 25 μL of total volume. PCR conditions were as follows: initial denaturation at 98 °C for 10 s, 35 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 2 min, and a final extension at 72 °C for 10 min. The amplified product was visualized by ethidium bromide staining and purified from 1.5% agarose gels using the Wizard PCR Preps DNA Purification System (TianGen, Beijing, China). *Escherichia coli* competent cells TOP10 was transformed with purified DNA which was ligated into the pEASY-Blunt vector (TianGen, Beijing, China). Several independent sub-clones were sequenced from both directions. Phylogenetic analysis was performed using the amino acid sequences of nAChR subunits in *Apis mellifera*, *Drosophila melanogaster* Meigen, *Bombyx mori* and *Anopheles gambiae* Giles, which were obtained from GenBank. A phylogenetic tree was constructed with the neighbor-joining method using MEGA 5.1 (The Biodesign Institute, Center for Evolutionary Functional Genomics, Tempe, AZ.) Branch support was assessed by bootstrap analysis based on 2000 replications. Gene names and their GenBank No. were shown in Table S1.

2.4. Expression and electrophysiological recording in Xenopus oocytes

The nAChR α subunit genes of the *Apis mellifera*, referred to as Amelα1–9, and the *Rattus norvegicus* β2 subunit, referred to as ratβ2 (L31622), were sub-cloned into the expression vector pT7TS. The sequences of all the primers were listed in Table S2. All plasmid constructs were verified by nucleotide sequencing. Plasmids were linearized with *Eco*RI. To generate cRNA, in vitro transcription was performed using the mMESSAGE mMACHINE T7 transcription kit (Thermo Fisher Scientific, Shanghai, China). Transcripts were recovered by precipitation with isopropanol, then dissolved in nuclease-free water at a final concentration of 4 μg/μL and stored at −80 °C prior to use. Ovarian lobes were isolated from female *Xenopus laevis* frogs. Stage V–VI oocytes for clumps were dissected in a sterile modified washing buffer (NaCl 96 mM, KCl 2 mM, $MgCl_2$ 5 mM, and 5 mM HEPES, pH 7.4 adjusted with NaOH). The dissected oocytes were treated with 2 mg/mL collagenase I (GIBCO, Carlsbad, CA, USA) in a sterile modified buffer for about 65 min at room temperature, rinsed thoroughly, and then stored in Ringer's buffer (NaCl 96 mM, KCl 2 mM, $MgCl_2$ 5 mM, $CaCl_2$ 0.8 mM and HEPES 5 mM, pH 7.4) supplemented with 5% dialysed horse serum, 50 μg/mL tetracycline, 100 μg/mL streptomycin and 550 μg/mL sodium pyruvate at 4 °C for overnight. The following day and manually defolliculated before injection with cRNA (27.6 nL/oocyte). Amelα1, Amelα3, Amelα8 and ratβ2 cRNAs were injected at a ratio of 1:1 separately (Liu et al., 2006; Yao et al., 2008). The injected oocytes were incubated for approximately 48 h at 18 °C in Ringer's buffer before recordings. After two days, Two-electrode voltage clamp recordings were conducted. Currents were recorded with an Oocyte Clamp (OC-725C) and voltage clamped −70 mV. Oocytes, held in a RC-3Z bath, were perfused at 4.5 mL/min with modified Ringer's solution (NaCl 150 mM, KCl 2.8 mM, HEPES 10 mM, $MgCl_2$ 2 mM, atropine sulfate 0.5 mM, pH 7.4). Electrodes were pulled from Clark borosilicate glass by P-1000 and filled with 3 M KCl. The electrode resistance was 0.5–1 MΩ on the current-passing side. Experiments were terminated if the total holding current exceeded 2 μA in order to reduce the effect of series resistance errors. The data were acquired and analyzed with DIGIDATA 1440A and PCLAMP10.0 software. To assess sensitivities to ACh, *S*-(+)-dinotefuran, *R*-(−)-dinotefuran and *rac*-(±)-dinotefuran, the oocytes were applied via bath perfusion in Ringer's solution containing different concentrations of the chemicals for 40 s. After recording, the oocytes were washed in Ringer's solution buffer clean to remove the chemicals until a steady baseline was reached.

2.5. Homology modeling and molecular docking

The three-dimensional structure modeling of the functional subunit Amelα8/ratβ2 was generated with a template of the crystal structure of *Ac*-AChBP complexed with imidacloprid (3C79) by the On-line SWISS-MODEL (Talley et al., 2008). The Surflex-Dock algorithm of SYBYL 7.3 (Tripos Associates, St. Louis, MO) was employed for the molecular docking study (Jain, 2003). The original molecular conformations of *S*-dinotefuran and *R*-dinotefuran were constructed by the Sketch mode and then optimized using the Tripos force field combined with the Gasteiger-Hückel charge. The binding cavity of the obtained modeling for Amelα8/ratβ2 was set at a "ligand mode" referred to imidacloprid complexed with the template *Ac*-AChBP and the Total Score was usually used to evaluate the binding affinity between ligand and protein (Jain, 1996). All molecular modeling between the putative modeling of Amelα8/ratβ2 and the ligand for *S*-dinotefuran or *R*-dinotefuran were conducted on the Silicon Graphics® Fuel Workstation (Silicon Graphics International, CA, USA).

2.6. Statistical analyses

The median lethal doses (LD_{50}, μg/bee) and the median lethal concentrations (LC_{50}, mg/L) were used to evaluate the enantioselective lethality of *S*-dinotefuran and *R*-dinotefuran, and their equal-proportional mixture *rac*-dinotefuran. The LD_{50} and LC_{50} were both determined by probit analysis using SPSS 20.0 (IBM, Beijing, China). One-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference test was applied to examine significant differences among the three forms of dinotefuran. Electrorheological data were analyzed by GraphPad Prism 5, one-way ANOVA with at least three repeats (different batches of oocytes from different frogs). Differences between values were analyzed using an LSD pair wise comparison of means.

3. Results and discussion

3.1. Chiral preparation and identification of dinotefuran enantiomers

Individual enantiomers of dinotefuran were prepared on a Chiralpak IC chiral column (2.0 cm I.D. × 25 cm L, 5 μm particle size, Daicel) by SFC MultiGram II system. Baseline resolution of 3.45 was achieved using CO_2/ethanol (65/35, v/v) as the mobile phase at 35 °C under a 254 nm UV absorption wavelength (Fig. 1). The first peak eluted at 7.03 min was (+)-dinotefuran with (−)-dinotefuran following at 8.26 min under the same chromatographic conditions in the study. In addition, the relationship between the optical rotation and the absolute configuration was clarified as *S*-(+)-dinotefuran and *R*-

Table 1
The enantiomeric lethality of dinotefuran enantiomers for *Apis mellifera*, *Aphis gossypii* and *Apolygus lucorum*.[1]

Insects	Assay mode	*R*-(−)-dinotefuran	*S*-(+)-dinotefuran	*rac*-(±)-dinotefuran	Fold change[2]
Apis mellifera	Physical contact (LD_{50}, μg/bee)	2.997 ± 0.107[a]	0.023 ± 0.002[b]	0.041 ± 0.001[c]	128.4
	Oral ingestion (LC_{50}, mg/L)	5.127 ± 0.107[a]	0.125 ± 0.002[b]	0.328 ± 0.006[c]	41.1
Aphis gossypii	Physical contact (LC_{50}, mg/L)	1.244 ± 0.028[a]	0.460 ± 0.022[b]	0.726 ± 0.066[c]	2.7
	Oral ingestion (LC_{50}, mg/L)	1.206 ± 0.050[a]	0.375 ± 0.073[b]	0.592 ± 0.044[bc]	3.2
Apolygus lucorum	Physical contact (LC_{50}, mg/L)	84.984 ± 5.174[a]	25.225 ± 0.559[b]	35.298 ± 1.435[bc]	3.4
	Oral ingestion (LC_{50}, mg/L)	59.718 ± 3.40[a]	18.334 ± 3.182[b]	27.031 ± 3.347[bc]	3.3

[1] Data are represented as mean ± standard error of the mean (SEM) and the different superscript letters in the same row differ significantly ($P < 0.01$).
[2] Fold change is calculated between the enantiomers.

(−)-dinotefuran (Kiriyama et al., 2003). Therefore, the order of the eluted peaks was *S*-(+)-dinotefuran and *R*-(−)-dinotefuran, respectively, in the study. As shown in Fig. 1, the enantiomeric ratio of *S*-(+)-dinotefuran and *R*-(−)-dinotefuran were 1224.74 and 0.00, indicating the optical pure individual dinotefuran enantiomers were achieved. The resolution, plate number and tailing factor of *S*-(+)-dinotefuran were 3.57, 7398.85 and 1.05, whereas they were 3.56, 7534.07 and 1.15, respectively, for *R*-(−)-dinotefuran. Chiral configuration stability of the two enantiomers was also confirmed at the beginning of the study (Z. Chen et al., 2015a). Then the prepared *S*-(+)-dinotefuran and *R*-(−)-dinotefuran, as well as *rac*-(±)-dinotefuran, was used for the following enantioselective lethality evaluation.

3.2. Enantioselective lethality evaluation for dinotefuran enantiomers

3.2.1. Chiralspecific toxicity of dinotefuran enantiomers to Apis mellifera

As documented in Table 1, the *S*-(+)-dinotefuran was of 128.4- and 1.8-fold more toxicity than *R*-(−)-dinotefuran and *rac*-(±)-dinotefuran to *Apis mellifera* by contact mode ($P < 0.01$), expressed as LD_{50} values of 0.023, 2.997 and 0.041 μg/bee for *S*-(+)-dinotefuran, *R*-(−)-dinotefuran and *rac*-(±)-dinotefuran, respectively. As for the oral mode, the toxicity of *S*-(+)-dinotefuran was of 41.1- and 2.6-fold than its antipode and racemate with $P < 0.01$, indicated as LC_{50} values of 0.125, 5.127 and 0.328 mg/L for *S*-(+)-dinotefuran, *R*-(−)-dinotefuran and *rac*-(±)-dinotefuran, respectively. The findings showed that a consistent order between contact and oral toxicities was clarified to *Apis mellifera* as *S*-(+)-dinotefuran > *rac*-(±)-dinotefuran ≫ *R*-(−)-dinotefuran.

3.2.2. Chiralspecific toxicity of dinotefuran enantiomers to Aphis gossypii and Apolygus lucorum

The toxicity of *S*-(+)-dinotefuran exhibited approximately 2.7- and 3.2-fold higher than *R*-(−)-dinotefuran on *Aphis gossypii* by physical contact and oral ingestion tests, respectively ($P < 0.01$, Table 1). Meanwhile, the *S* form also experienced highly toxicity to *Apolygus lucorum* with of 3.3–3.4 times more than the *R* form of dinotefuran ($P < 0.01$). So the potency of *S*-(+)-dinotefuran was slightly greater than *R*-(−)-dinotefuran to *Aphis gossypii* and *Apolygus lucorum*. However, we further evaluated the fold changes of insecticidal efficiency between *R*-(−)-dinotefuran and *rac*-(±)-dinotefuran. There were just 1.7–2.0 for *Aphis gossypii* and 2.2–2.4 for *Apolygus lucorum* between *R* form and racemate of dinotefuran with $P < 0.01$. Therefore, the *R*-(−)-dinotefuran exhibited comparative insecticidal activities to the target insects, implying the only use of *R* form of dinotefuran could still have a good control to target pests.

Therefore, strong chiralspecificity of dinotefuran enantiomers to *Apis mellifera* (41.1–128.4) was of particular interest as a non-target species, which was much greater than that to the target pests *Aphis gossypii* (2.7–3.2) and *Apolygus lucorum* (3.3–3.4) (Table 1). We further compared with those of sanitary pests that have been studied previously, and it also indicated the chiralspecificity was weak in *Periplaneta americana* (2.2–7.3) and *Musca domestica* (3.0–3.4) (Kiriyama et al., 2003; Mori et al., 2002). More importantly, according to the toxicity classification of pesticides to bees (OECD, 1998), the *S*-(+)-dinotefuran and *rac*-(±)-dinotefuran belong to high toxicity whereas the *R*-(−)-dinotefuran pertains to moderate toxicity. *R*-(−)-dinotefuran can not only significantly reduce the toxicity to honeybee but also exhibit comparable potency to target pests. These findings lay a solid foundation for the development of chiral neonicotinoid products with a high proportion or a full scale of *R*-dinotefuran.

3.3. Phylogenetic and electrophysiological studies on nAChR of Apis mellifera

3.3.1. Sequence and phylogenetic analysis of Apis mellifera nAChR

Whether the chiralspecific toxicity of dinotefuran to honeybee is dependent on binding specific nAChR subunit(s)? Based on the current genome analysis data (Dupuis et al., 2011; Jones et al., 2006; Louis et al., 2012), the brains of *Apis mellifera* were isolated to extract total RNA for synthesis first-strand cDNAs. Ten full-length coding sequences of the nAChR subunits were identified in *Apis mellifera* Linnaeus, including nine α subunits and one β subunit. It was reported that the honeybee genome contained 21 candidate cys-loop superfamily subunits but 11 orthologs were known *Drosophila* nAChR subunits-9α and 2β (Jones and Sattelle, 2006). The reported honeybee subunits had unclear vertebrate homologs and therefore their subunits had been followed *Drosophila* counterparts (Jones et al., 2006). A high similarity was observed for the relationship between the corresponding nAChR subunits of *Apis mellifera* and those of the same number nAChR subunits of other insects such as *Drosophila melanogaster* Meigen, *Bombyx mori* and *Anopheles gambiae* Giles by the phylogenetic tree. The *Nilaparvata lugens* trehalase-1 (Nltre-1) was used as outgroup (Fig. S1). Protein sequence alignment of the nAChR subunits from *Apis mellifera* and a reference sequence from the α1 subunit of *Drosophila melanogaster* Meigen, allowed for determination of functional motifs within nAChR. These functional motifs consisted of: six conserved Loops A-F at the N-terminal domains involved in ligand binding, transmembrane motifs (TM1–TM4) at the C-terminal domain forming the ion channel, sites of Cys residues involved in a Cys-loop, and the vicinal Cys residues characteristic of α-type subunits (Fig. S2).

3.3.2. Potency of heterologously expressed hybrid nAChR of Apis mellifera

The nAChR subunits played function as homopentamer (five copies of one subunit) or heteropentamer (different types of subunits). It was reported that nAChRs α1 and α2 exhibited distinct molecular when exposure to different classes of insecticides (Christen and Fent, 2017). Therefore, the insecticides and nAChRs expression patterns both affect nAChRs function and pharmacology. Different receptor recombinations were tried, but as shown in Fig. 2a, only the Amelα1, Amelα3 and Amelα8 evoked inward currents in response to the application of ACh when the cRNA of subunits was injected separately with rat β2 cRNA into *Xenopus* oocytes at a ratio of 1:1. The other subunits, including Amelα2, Amelα4, Amelα5, Amelα6, Amelα7 and Amelα9, were not observed the inward currents in voltage-clamp electrophysiological recording. Then the two concentration levels of 10 and 500 μM of dinotefuran forms were further applied to verify the response of the three

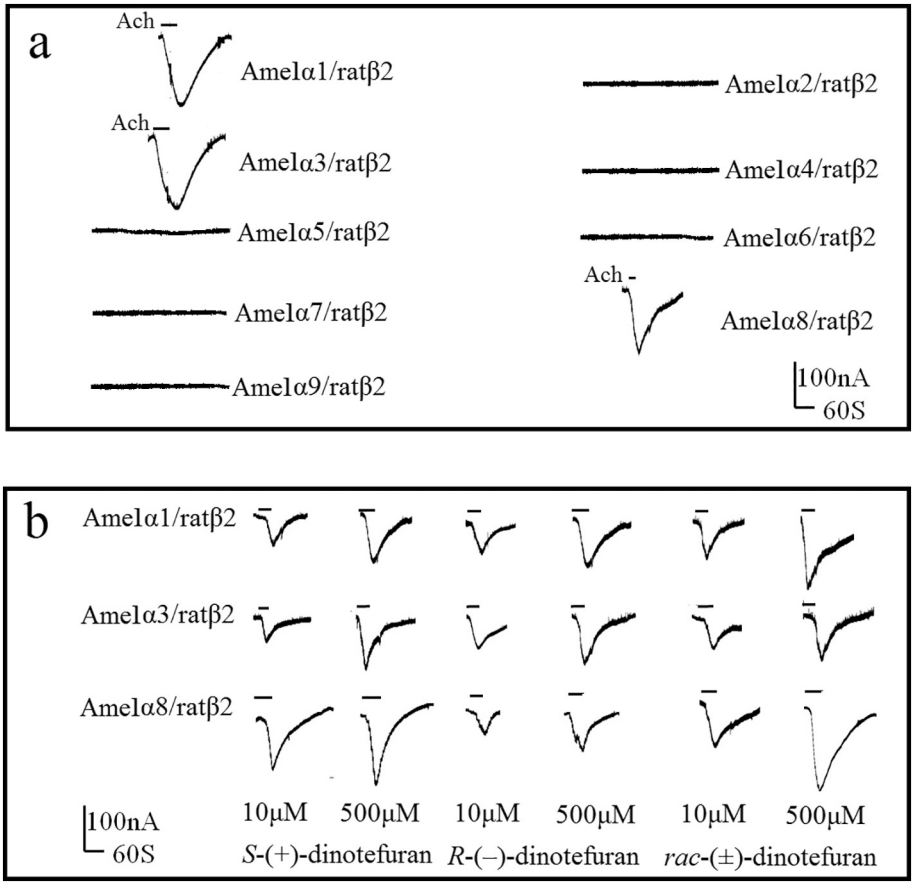

Fig. 2. Representative whole-cell agonist responses of hybrid nAChR to 500 μM ACh (a) and to 10 and 500 μM dinotefuran enantiomers for Amelα1/ratβ2, Amelα3/ratβ2, and Amelα8/ratβ2 (b), respectively.

Table 2
The pEC_{50} of dinotefuran enantiomers and ACh for Amelα1/ratβ2, Amelα3/ratβ2 and Amelα8/ratβ2 subunits expressed in *Xenopus* oocytes.[1]

Subunits	*R*-(−)dinotefuran	*S*-(+)-dinotefuran	*rac*-(±)-Dinotefuran	ACh
Amelα1/ratβ2	6.78 ± 0.15[a]	6.63 ± 0.13[a]	7.10 ± 0.11[a]	6.78 ± 0.14
Amelα3/ratβ2	7.00 ± 0.18[a]	5.71 ± 0.13[a]	6.80 ± 0.26[a]	6.31 ± 0.10
Amelα8/ratβ2	3.80 ± 0.17[a]	5.69 ± 0.27[b]	6.12 ± 0.34[bc]	5.83 ± 0.11

[1] Data shown are the result of a fit of the concentration–response data (mean ± SEM, n = 6–8) and the different superscript letters in the same row differ significantly ($P < 0.01$).

subunits in *Xenopus* oocytes expression system. As shown in whole-cell agonist responses of the hybrid nAChR, none of notable changes were found in Amelα1 and Amelα3 with any one of the *S*-(+)-dinotefuran, and *R*-(−)-dinotefuran and *rac*-(±)-dinotefuran (Fig. 2b). No obvious right or left shifts in median effective concentration (EC_{50}) between *S*-(+)-dinotefuran and *R*-(−)-dinotefuran were observed in the agonist dose–response curves of Amelα1 (Fig. S3a) and Amelα3 (Fig. S3b). Also no significant difference was obtained from the negative logarithm of the EC_{50} (pEC_{50}) of dinotefuran enantiomers in response to both Amelα1 and Amelα3 (Table 2). Notably, the significant differences were solely observed in the Amelα8 subunit between *S*-(+)-dinotefuran and *R*-(−)-dinotefuran under both concentrations. An obvious right shift in the EC_{50} for *R*-(−)-dinotefuran was observed in Amelα8 (Fig. S3c) with the lowest pEC_{50} of 3.80 ± 0.17 (n = 6–8), which is approximately 77.6 times greater than that of *S*-(+)-dinotefuran with a significant *P* value < 0.01 (Table 2).

Consequently, the findings concluded that the enantioselectivity was largely dependent on fit at the binding nAChR subunit. *S*-dinotefuran as the high-toxic enantiomer of dinotefuran was principally associated with nAChR α8 of *Apis mellifera*. Meanwhile, a minor inconsistency observed between the electrophysiological recording and lethality evaluation to *Apis mellifera*. The toxicity of *S*-(+)-dinotefuran is larger than that of *rac*-(±)-dinotefuran under both exposure modes ($P < 0.01$), whereas non-significant differences were obtained in the *Xenopus* oocyte expression system (Table 2). No obvious differences were observed in the whole stage between *S*-(+)-dinotefuran and *rac*-(±)-dinotefuran and even in the maximum current of the three forms of dinotefuran (Fig. S3c). This may because of the limitation of the current heterologous expression systems. The nAChR subunits played function as pentamer, and some chaperones also played an important role in heterogenous expression system, but only few hybrid nAChRs were successfully constructed with insect subunits. It was extremely difficult to efficiently express functional proteins in the heterologous expression system (Millar, 2003). Immunocytochemical and electrophysiological studies have shown that in the honeybee *Apis mellifera*, nAChRs were expressed in diverse structures including the primary olfactory centers of the brain, the antennal lobes (ALs) and the mushroom bodies (MBs) (Dederer et al., 2011). The functional nAChRs were different between adult mushroom bodies Kenyon cells and the antennal lobes cells in both their molecular composition and

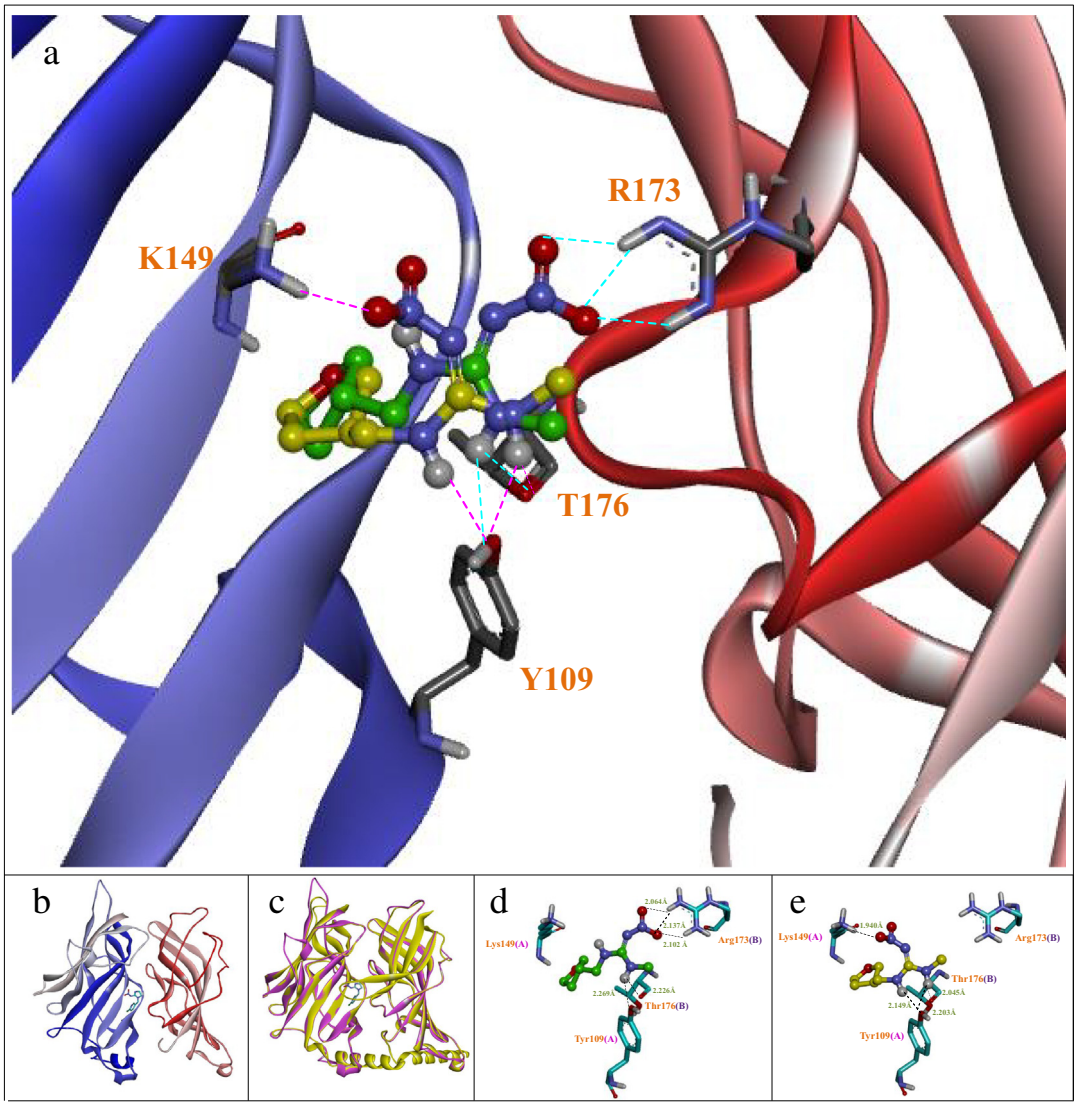

Fig. 3. (a) The alignment plot of *S*-(+)-dinotefuran (green) and *R*-(−)-dinotefuran (yellow) in Amelα8/ratβ2 of *Apis mellifera*; (b) the Amelα8 subunit (left) and the ratβ2 subunit (right) in Amelα8/ratβ2 modeling complexed with a ligand imidacloprid (cyan blue); (c) the alignment plot between the Amelα8/ratβ2 (pink) and the template protein *Ac*-AChBP (yellow); d–e refer to the binding mode of *S*-(+)-dinotefuran (green) and *R*-(−)-dinotefuran (yellow) with Amelα8/ratβ2, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

pharmacological properties. The nicotinic subunit α2, α7, α8 and β1 response to ACh was significantly different in adult bee olfactory neuropils because of their different expression patterns (Dederer et al., 2011). But in our study, nicotinic subunits α2 and α7 were no response to ACh, and we suspect that some crucial components may be needed or the nAChR subunits variants have been not found. Moreover, each of insects has their own specific enzyme system or functional areas for pesticide detoxification, laying the material basis for the different toxicities of the dinotefuran enantiomers among *Apis mellifera*, *Aphis gossypii* and *Apolygus lucorum*. There are still other molecular mechanisms that we will keep tracing in the follow-up studies.

3.4. Homology modeling on honeybee nAChR and the binding mode to dinotefuran enantiomers

3.4.1. Three-dimensional model of the functional subunit Amelα8/ratβ2

To better reveal the potential molecular toxicity mechanism on honeybee of dinotefuran enantiomers, the homology modeling study on honeybee nAChR, namely target subunit α8 of *Apis mellifera*, were performed in this study. As known-well to us, the *Aplysia californica* acetylcholine binding protein (*Ac*-AChBP) was a good structure template to generate the molecular model of different nAChR (Toshima et al., 2009; Yuan and Petukhov, 2006). The sequence identity of the *Ac*-AChBP was 32% and 25% to the target subunit Amelα8 and ratβ2, respectively, which is high enough as a suitable template for generating a reasonable target model. As reported on homology modeling of different nAChR in previous work (Dilna et al., 2018; Saladino et al., 2005), the three-dimensional model of the functional subunit Amelα8 and ratβ2 was firstly constructed based on the crystal structure of *Ac*-AChBP complexed with imidacloprid, respectively (Fig. 3b). The two target subunits Amelα8 and ratβ2 were merged together to form a functional subunit model for Amelα8/ratβ2, where imidacloprid was located into the same binding site of the complex structure of *Ac*-AChBP. It was found that the three-dimensional model of the functional subunit Amelα8/ratβ2 aligned well with the template protein *Ac*-AChBP (Fig. 3c). As a result, the putative model of the functional subunit Amelα8/ratβ2 was successful to be used to explore the binding mode between dinotefuran enantiomers and Amelα8/ratβ2 to further clarify its possible molecular toxicity mechanism.

3.4.2. Binding interaction between functional subunit Amelα8/ratβ2 and dinotefuran enantiomers

The docking score of dinotefuran enantiomers into the functional subunit Amelα8/ratβ2 was usually used to evaluate its binding affinity to honeybee nAChR. It was found that the docking score of *S*-(+)-dinotefuran (7.15) was higher by one order of magnitude to Amelα8/ratβ2 than that of *R*-(−)-dinotefuran (6.09), which indicated to a much higher binding ability of *S*-(+)-dinotefuran for honeybee nAChR. More H-bond interactions were showed to contribute to the binding affinity of *S*-(+)-dinotefuran for Amelα8/ratβ2 than that of *R*-(−)-dinotefuran (Fig. 3a). Specifically, a triple H-bond network was found between the nitro group of *S*-(+)-dinotefuran and a unique residue Arg173 of chain β in Amelα8/ratβ2. Moreover, one of the imino group also formed a double H-bond with a distance of about 2.2 Å to a nearby residue Thr176 in chain β and the other one Tyr109 in the complementary chain α (Fig. 3d). It was obvious that the double-originated effect induces a higher binding ability of *S*-(+)-dinotefuran to honeybee nAChR to further show a higher toxicity to bee. However, because of a different molecular orientation in dinotefuran enantiomers, only one H-bond interaction was formed with a distance of 1.940 Å (Baker and Hubbard, 1984; Zhou et al., 2012) between the nitro group of *R*-(−)-dinotefuran and a key residue Lys149 in chain α, not chain β above-described for *S*-(+)-dinotefuran (Fig. 3e). Meanwhile, two imino groups also formed a triple H-bond network with the same residues Tyr109 of chain α and Thr176 of chain β, like that of *S*-(+)-dinotefuran, with distance of about 2.0–2.2 Å. It was clear that a less H-bond network was observed between *R*-(−)-dinotefuran and the functional subunit Amelα8/ratβ2 than that of *S*-(+)-dinotefuran, especially only one H-bond interaction for *R*-(−)-dinotefuran to a special residue Lys149 of chain α, not multiple H-bond interaction for *S*-(+)-dinotefuran to a unique residue Arg173 of chain β. It was just the weaker interaction between *R*-(−)-dinotefuran and the functional subunit Amelα8/ratβ2 possibly resulted in its lower toxicity to bee. The binding mode results above-described for dinotefuran enantiomers to honeybee nAChR were also proved by not only its binding affinity to the functional subunit Amelα8/ratβ2 in the electrophysiological study but also its toxicity bioassays to bee.

4. Conclusion

In summary, *S*-dinotefuran is the high-toxic enantiomer of dinotefuran to honeybee by binding to nAChR α8 via a more stable and functional binding cavity. Notably, the *R*-dinotefuran greatly reduces the ecotoxicity to nontarget honeybee and maintains comparable potency to target pests. Moreover, *R*-dinotefuran was preferentially degraded in soil than its antipode (X. Chen et al., 2015). Therefore, new chiral neonicotinoid products with a high proportion or a full scale of *R*-dinotefuran could be a suitable alternative to achieve biological pests control and honeybee services protection simultaneously in actual agricultural production. First work was done on the relationship between the functional properties of nAChR subunits of honeybee and chiral neonicotinoid pesticides, providing a novel insight into the toxicity reduction of neonicotinoids to pollinators.

Declaration of Competing Interest

The authors declare no competing financial interests.

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Appendix A. Supplementary data

The supplementary information regarding details on the chiral separation and preparation and the procedures of lethality evaluation can be found in Supplementary material. Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2019.05.048.

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