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ORIGINAL PAPER

Modeling the interaction of fipronil-related non-competitive antagonists with the GABA β3-receptor

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Abstract A three-dimensional model of the β3-homopentamer of the γ-aminobutyric acid (GABA) receptor/chloride ionophore complex was developed by homology modeling using the cyro-electron microscopy structure of nicotinic acetylcholine as a template. Interactions between the β3-homopentamer and two classes of fipronil-related non-competitive antagonists were investigated using docking studies. The phenyl groups of these compounds were stabilized by strong hydrophobic and hydrophilic interactions with the rings formed by Thr256 and Ala252. Leu253 and Ile255 were involved mainly in hydrophobic contact with the pyrazole moiety. Different substitution at positions 15, 16 and 17 of the pyrazole ring of fipronil resulted in weakening of the hydrogen bonds and hydrophobic interactions between the β3-receptor and fipronil-related heterocyclic compounds, which maybe the principal cause of the decreased affinities reported in vitro. Moreover, a good correlation between total binding energies calculated by AutoDock and experimentally determined IC_{50} values proved our models to be reasonable in predicting the interaction mode of the antagonist with the GABA β3-receptor.

Keywords Homology modeling · β3-homopentamer · Fipronil-related non-competitive antagonist · Docking

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Introduction

The γ-aminobutyric acid (GABA) receptor/chloride ionophore complex is the primary site of action for the botanical toxicant picrotoxinin (PTX) and several major insecticides in current use, including lindane, α-endosulfan and fipronil [1]. All of these compounds act as noncompetitive antagonists (NCAs) by interacting within the GABA receptor chloride channel and stabilizing non-conducting conformations of the chloride channel [2, 3]. Blockage of the GABA-gated chloride channel reduces neuronal inhibition, which leads to hyperexcitation of the central nervous system, convulsions and death. The action of the above compounds as chloride-channel blockers can be measured directly by binding studies using [^{35}S]t-butylbicyclophosphorothionate([^{35}S]TBPS) and [^{3}H]ethynylbicycloorthobenzoate([^{3}H] EBOB) as the radioligand [4].

The mammalian $GABA_A$ pentameric receptor consists primarily of heterooligomeric assemblies of α (1–6), β (1–4), and γ (1–4) subunits. In insects, three GABA receptor subunits have been cloned to date, of which only the RDL subunit (resistance to dieldrin) forms a functional GABA-gated chloride channel [5]. Although the subunit composition of the native insect receptor is not defined, the drosophila RDL subunit has a high sequence homology to that of the human β3-subunit [6–8]. [^{3}H]EBOB binding assays with human and housefly GABA receptors showed the GABAergic insecticide target to be conserved in sensitivity and specificity between insects and the β3-subunit of mammals. The binding potency of 25 insecticides and related compounds at the human β3-homooligomer correlates well with that at the housefly receptor (r=0.88, n=25) [7]. The human β3-homopentamer and housefly receptors are more sensitive than other types of GABA receptor to α-endosulfan, lindane and fipronil [7, 9].

Thus, it is proposed that the human β3-homooligomer can be used as a model to study the interaction modes of noncompetitive antagonists with the insect GABA receptor. This model had been used by Casida, who proved by mutagenesis and molecular modeling that widely diverse noncompetitive antagonist structures fit the same binding site of the β3-homopentamer [10]. The fipronil-based photoaffinity probe for drosophila and human β3-GABA receptors, which have almost the same affinities, provided a more direct test for this model [11].

Fipronil is a widely used phenylpyrazole insecticide with high selectivity and potency for insect chlorine channels, low mammalian toxicity and low persistence in the environment. In addition, fipronil-related phenyl heterocyclic compounds were also effective as noncompetitive antagonists to the housefly GABA receptor [12].

To guide the design of novel compounds with high activity with the insect GABA receptor, it is valuable to understand the precise location of the noncompetitive antagonists within the β3-homopentamer model. Several observations suggest that the non-competitive antagonists interact with the same position of the M2 transmembrane segment region on the cytoplasmic side [10, 13]. To investigate, at the molecular level, the interaction of fipronil-related non-competitive antagonists with the ion channel associated with the β3-homopentamer, a three-dimensional model of the β3-homopentamer was constructed and validated. The molecular docking of two classes of fipronil-related heterocyclic compounds with our model was used to help explore the architecture of the binding site. The interaction-mode analysis of six fipronil-related compounds with the β3-homopentamer will also provide insights into how these compounds interact with the β3-receptor, and why different substitutions of the pyrazole moiety of fipronil result in low affinity.

Materials and methods

Sequence alignment

The human $GABA_A$ receptor β3-subunit sequence was obtained from the Swiss-Prot/TrEMBL database (accession numbers P28472). The sequence and structure of the nicotinic acetylcholine receptor (nAChR) were obtained from the RSCB protein data bank at 4 Å resolution (PDB ID 2BG9) [14]. The amino-acid sequence of the β3-subunit was edited to remove the extracellular region and residues in the loop between transmembrane (TM) domains 3 and 4 (TM3–TM4 loop). Sequence alignment was carried out using the FUGUE program, which uses environment-specific substitution tables and structure-dependent gap penalties, where scores for amino-acid matching and insertions/deletions are evaluated depending on the local environment of each amino-acid residue in a known structure [15].

Model of the β3-homopentamer

Using the above sequence alignment, three dimensional models of the transmembrane region of the β3-subunit were built by homology modeling using MODELLER8v2 software with the default parameters that proposed loop conformations [16]. The α-subunit of nAChR was used as the structural template. In the model building, we employed an optimization method involving conjugate gradients and molecular dynamics to minimize violations of the spatial restraints. In all, 100 structures were constructed and the best model was determined by the lowest value of the MODELLER objective function. For the models chosen, MODELLER was used to calculate the discrete optimized potential energy (DOPE) score for each residue. The scores were graphed and the area with the highest DOPE score was then refined using the loopmodel module of MOD ELLER. The best model was the one with the lowest MODELLER objective function number after loop refinement. The β3-homopentamer receptor was generated by duplicating the best model four times and rotating each copy an additional 72°. The pentamer model thus generated was then energy-minimized with the GROMOS96 implementation of SPDBV [17]. Computations were done in vacuo with the GROMOS96 43B1 parameters set without reaction field. A 10 Å cut-off was adopted for non-bonded interactions. The minimization protocol included 500 steps of steepest descent, followed by 500 steps of conjugate gradients. The final model was evaluated with PROCHECK [18].

Docking study

To investigate how the insecticide anchored at the putative binding cavity, fipronil-related non-competitive antagonists were docked into the ion channel pore formed by the second transmembrane segments of the β3-homooligomeric receptor using the AUTODOCK3.05 program [19]. This program starts with a ligand molecule in an arbitrary conformation, orientation, and position, and finds favorable dockings in a ligand-binding site, using both simulated annealing and genetic algorithms. The non-competitive antagonist structures were built with standard bond lengths and angles using the molecular modeling package SYBYL, and were then optimized with an energy minimization with the Tripos force field [20]. The computations were processed on an SGI Octane 2 graphics workstation. The protein and the antagonists were prepared using the program SYBYL. For macromolecules, polar hydrogens

were added, and Kollman united atom charges and atomic salvation parameters were then assigned to the protein atoms. For small molecules, all hydrogens were added before computing Gasteiger charges, and non-polar hydrogen was then merged. The grid maps around the protein binding site were calculated using AUTOGRID3.05 with 40×40×40 points, and a grid spacing of 0.375 Å. The GALS method was adopted using the default settings. A final docked representation of the potential binding mode of the antagonist was chosen based on selection of the compound that possessed the lowest docked energy within the most populated cluster of lowest possible energy. The models generated by AUTODOCK were used by SPDBV to extract all amino-acid residues within 5.0 Å of the antagonist's surface. The receptor-antagonist complex was then analyzed by Ligplot 4.22 to identify hydrophobic contacts [21].

Results and discussion

Sequence alignment

For this study, our target was focused on the transmembrane domains of the human $GABA_A$ receptor β3-subunit sequence. Subunit equivalence to the nAChR was based on having homologous residues involved in ligand-binding, and this equivalence gave the same subunit arrangement as that of the extracellular domain model [22]. Figure 1a shows that GABA β2 corresponds to nAChR α, GABA α1 to both nAChR γ and δ, and GABA γ2 to nAChR β. The final sequence alignment of the human $GABA_A$ receptor β3-subunit to the sequence of the α-subunit of nAChR is shown in Fig. 1b; the sequence identity is more than 60%.

Homology modeling of β3 homopentamer

Based on the sequence alignment in Fig. 1b and the cyro-electron microscopy structure of nAChR, which has recently been refined to a resolution of 4 Å, a three-dimensional model of the transmembrane region of the GABA β3-subunit was built using MODELLER software. in all, 100 models were constructed and the best was selected by picking the model with the lowest value of the MODELLER objective function. The model chosen was then refined by using the loopmodel module of MOD ELLER. DOPE score profiles before and after loop refinement are shown in Fig. 2. Evaluation of the initial model with the DOPE potential in MODELLER shows that the TM1–TM2 and TM3–TM4 loops have relative positive energies. The original TM3–TM4 loop is approximately 100 residues, and there is recent evidence that it is involved in clustering during receptor expression in the membrane [23]. Because no residue in TM3–TM4 was found to contribute to the binding of non-competitive antagonists, and no structural information was available about this domain in the original template, this domain was removed before homology modeling. Considering the above, it is easy to understand why a relatively high energy peak appeared in this region. Analysis of the refined DOPE score profile, as compared to the profile of the initial model, shows overall improvement at TM1–TM2 loop. At almost every residue of

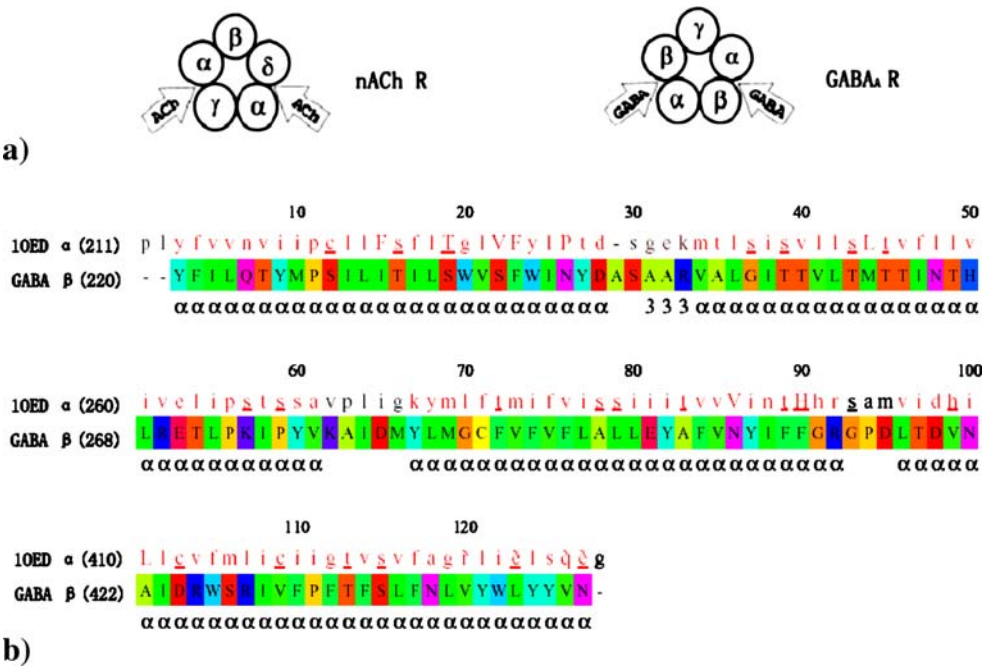


Fig. 1 **a** Subunit correspondence between nicotinic acetylcholine receptor (nAChR) and γ-aminobutyric acid receptor ($GABA_A$ R) is indicated by the schematic *pentamers*. *Arrows* Agonist binding site location. **b** Alignment of the amino-acid sequences of the transmembrane domain of the $GABA_A$ receptor β3-subunit with that of the nAChR α-subunit annotated using the program JOY [29]. Key to alignment: *red* a-helix, *blue* β-strand, *maroon* 3_{10} helix, *lower case* solvent accessible, *upper case* solvent inaccessible, *bold* hydrogen bond to main-chain amide, *underlined* hydrogen bond to main-chain carbonyl, *cedilla c* disulfide bond, *italicised* positive f-torsion angle

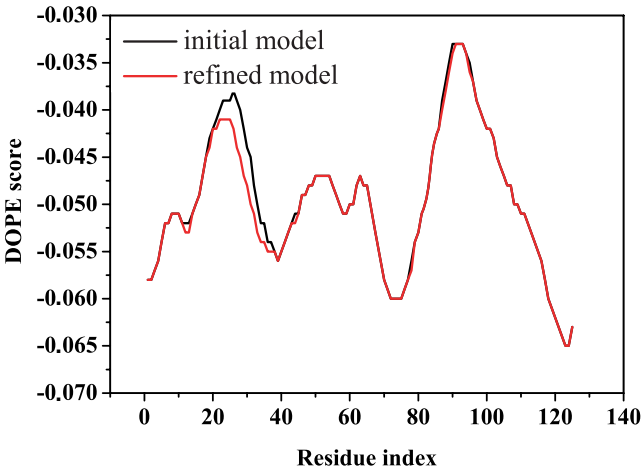


Fig. 2 Discrete optimized potential energy (DOPE) score profiles before and after loop refinement

the TM1–TM2 loop, the score is lower than that of the corresponding residue of the unrefined model.

A pentameric $GABA_A$ receptor was created by duplicating the best model four times and rotating each copy an additional 72°, followed by subsequent energy minimization to remove steric clashes at subunit interfaces. The backbone conformation of the constructed model was evaluated by inspection of the Psi/Phi Ramachandran plot obtained from a PROCHECK analysis. As shown in Fig. 3, 97.4% of the residues are in most favored regions, and no residues have disallowed conformations. The results indicate that the homology model is reliable. The final refined β3-subunit and the pentamer are shown in Fig. 4.

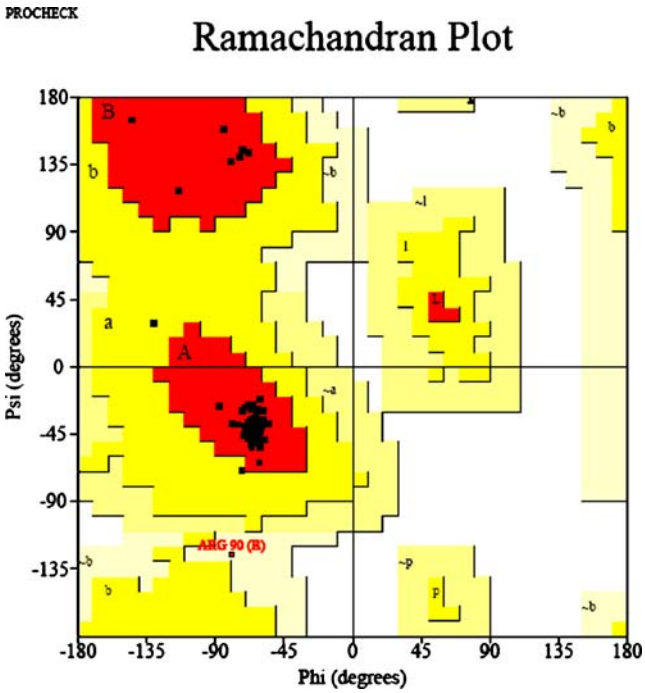


Fig. 3 Ramachandran plot of the β3-homooligomeric receptor. The most favored regions are colored red, additional allowed, generously allowed, and disallowed regions are indicated as yellow, light yellow and white fields, respectively

Locating the putative binding site

The molecular localization of the binding site for non-competitive antagonists was indicated by previous mutagenesis studies to include Ala252, Leu253, Thr256 and Leu259 in the cytoplasmic half of the TM2 domain of the channel. In drosophila, a single point mutation (A302S in the Rdl subunit) greatly reduced the binding affinity for the major GABAergic insecticides, suggesting that this residue was at or near the insecticide-binding site [8, 24]. Among the amino acids from the presumed membrane-spanning segment M2 of different GABA receptor subunits, Ala252 in the human β3-subunit(which occupies a cytoplasmic position) is equivalent to A302 in the drosophila Rdl subunit. Substitution of β3A252 and β3L253 resulted in reduced PTX affinity, which indicated the importance of Ala252 and Leu253 for PTX binding. [25] Site-directed mutagenesis and two-electrode voltage-clamp techniques showed that the single mutant β2 (T256F or L259T), in combination with wild type α and γ-subunits, conferred PTX-insensitivity or led to spontaneous channel openings that were blocked by PTX [26, 27].

Thr256 was positioned at the center of all important residues that led to high antagonist-insensitivity in site-directed mutagenesis. Thus, placing the grid center at the center of the ring formed by five Thr256, thus creating a three-dimensional box that covered all the residues mentioned above, was an ideal model with which to investigate the binding mode of fipronil-related non-competitive antagonists with the β3-homopentamer receptor in our docking study.

Binding-mode analysis

Two classes of fipronil-related heterocyclic compounds were docked into the β3-homopentamer receptor (Table 1).

Fipronil was chosen as the leading antagonist to be docked in the first series of fipronil-related compounds, because it binds to the insect GABA receptor with the highest affinity. The reported IC_{50} of fipronil to the housefly GABA receptor was ~2.3–6.3 nM [28]. Figure 5a shows the docking result of fipronil in the lumen of the chloride channel, with the pyrazole group towards the cytoplasmic domain. The surface of docked fipronil is 5 Å distant from the five rings formed by Ala252, Leu253, Ile255, Thr256 and Leu259 (Fig. 6a); the side chains of five residues approximately face toward the central axis of the pore after rotation of the α-helix. Figure 6a shows that two fluorines of the trifluoromethyl group of the phenyl and pyrazole moieties are predicted to form six H-bonds simultaneously: four to the three hydroxyl groups from

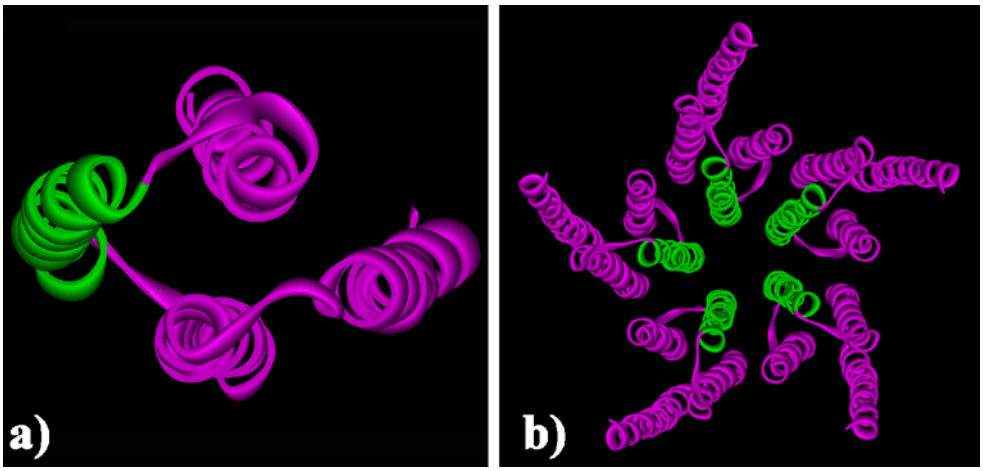
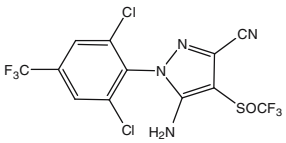
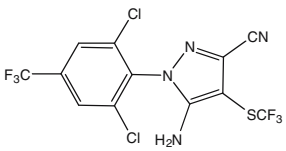
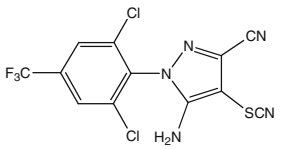
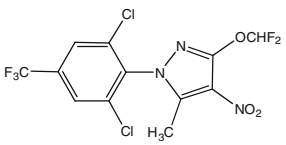
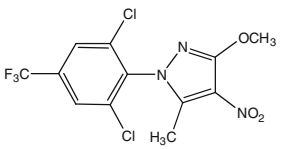
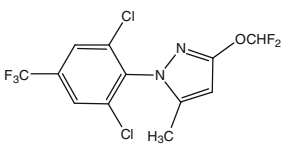


Fig. 4 The final refined transmembrane domain (TMD) of the β3-subunit (**a**) and β3-homopentamer (**b**) shown in ribbon representation. *Pink* TM1, TM3 and TM4; *green* TM2

Table 1 Predicted docking energy and experimentally determined IC_{50} for fipronil-related non-competitive antagonists

Antagonist	Structure	The lowest $E_{docking}$ (kcal mol^{-1})	IC_{50}(nM)	pIC_{50}	Total cluster number
fipronil		-9.59	2.30	8.63	21
A1		-8.37	7.55	8.12	54
A2		-3.65	48.60	7.31	14
B		-5.19	14.60	7.84	11
B1		-3.45	79.20	7.10	21
B2		-3.29	265.00	6.58	27

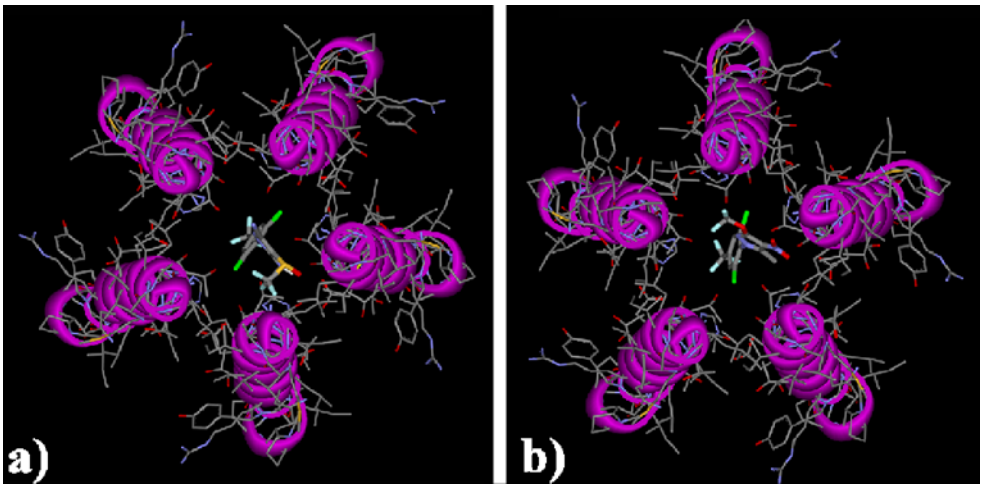


Fig. 5 Stereo pictures show the docking orientation of fipronil (**a**) and compound B (**b**) in the lumen of the chloride channel of the β3-pentamer

three Thr256s, and two to the backbone amino group of one Lue253. Another important hydrogen bond is found between the carbonyl group of one Ala252 and the oxygen of the S(O)CF3. All H-bond distances between the atom pairs are below 3.2 Å. Hydrophobic interactions are indicated in Fig. 6b. The aromatic ring and S(O)CF3 of fipronil are surrounded by two Ala252, two Leu253 and four Thr256 residues. Favorable hydrophobic contacts are observed for CB of Ala1 and CA of Leu253, with the trifluoromethylsulfinyl and CB or CG2 of Thr256 associated with the phenyl group and chloro substituents.

Chen et al. also reported docking results for six non-competitive antagonists with the β3-homopentamer, including EBOB, TBPS, PTX, fipronil, lindane and α-endosulfan

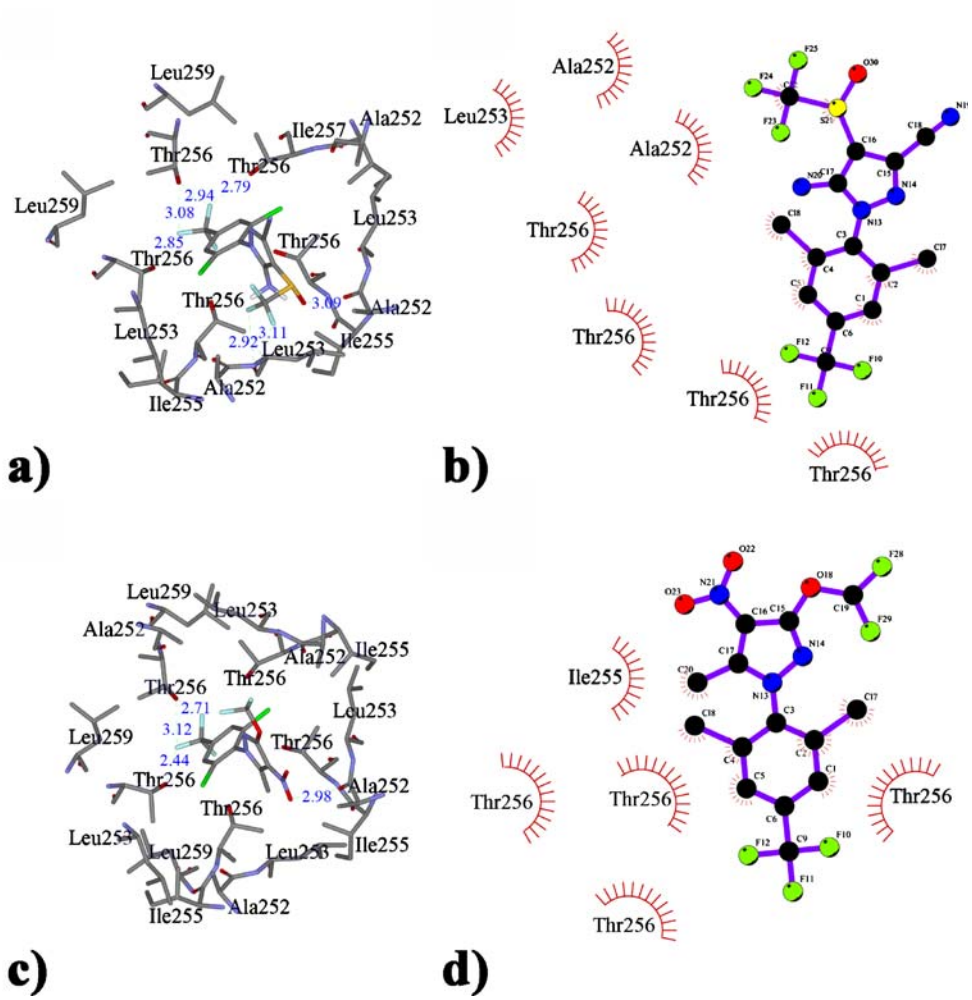


Fig. 6 The predicted hydrogen-bonding interaction of Fipronil (**a**) and compound B (**c**) with residues within 5 Å of the antagonist. Two-dimensional representation of the hydrophobic interaction of Fipronil (**b**) and compound B (**d**) with the residues from the chloride channel of the β3-pentamer. *Dotted lines* Hydrogen bonds, *spokes* hydrophobic interactions (Ligplot 4.22)

[10]. These compounds fit the pore region from Ala252 to Leu259, forming hydrogen bonds with the hydroxyl group of Thr256 and hydrophobic interactions with Ala252, Thr256 and Leu259. This docking result was basically consistent with our results. The minor differences between the two models occur primarily in the hydrophobic interactions. In our model, Leu253 may play a more important role than Leu259, which, at within 5 Å of the surface of docked fipronil, perhaps only modulates the function of fipronil, without participating direct binding of the antagonist.

Compound A1 was generated with an SCF_3 group in place of the S(O)CF3 group of fipronil, and substitution of the SCF_3 group of compound A1 with an SCN group produced compound A2. These two compounds have very similar structures to fipronil, and have similar hydrophobic contact with the transmembrane ion pore of the β3-homopentamer. However, the removal of sulfur and fluorine atoms in the S(O)CF3 of fipronil led to the disappearance of a hydrogen-bond acceptor at a suitable distance from the hydrogen-bond donor of the antagonist. Substitution of SCF_3 and SCN at position 16 of the pyrazole moiety of the fipronil was also associated with an increase in predicted docking energy. These findings suggest that substitution at this position may potentially weaken stability within the pore when compared with unchanged fipronil. This was supported by the finding of Ozoe et al. [12] who reported a decrease in binding affinity to housefly head membrane for compounds A1 and A2, with IC_{50}=5.32–10.70 nM (Edocking=−8.37 kcal mol^{-1}) and IC_{50}=36.6–64.6 nM (Edocking=−3.65 kcal mol^{-1}), respectively, compared to unsubstituted fipronil, IC_{50}=2.3–6.3 (Edocking=−9.59 kcal mol^{-1}) (Table 1).

Taken together, the above results indicate that the sulfoxide moiety of fipronil plays an important role in exerting high binding affinity within the channel to stabilize the closed conformation of the channel and consequently cause excitation in animals. A CoMFA study of fipronil-related heterocyclic compounds for housefly receptors showed negative electrostatic-potential regions surrounding the SCF3 group, suggesting that the negative charges of the three fluorine atoms of this group contributed positively to the high activity [13]. Changing S(O)CF3 to SCF_3 or SCN reduced the binding affinity to the ion pore of the insect GABA receptor.

For the second series of antagonists, the lead compound B involved substitution of CH_3, NO_2 and $OCHF_2$ groups at positions 15, 16 and 17 of fipronil. Our studies predicted an orientation similar to that of fipronil in the ion channel (Fig. 5b), and the formation of four hydrogen bonds when compound B was docked into the channel pore of the β3-homopentamer (Fig. 6c). Two fluorine atoms of the 9-CF_3 group are predicted to be involved in hydrogen bonding, interacting with side chain hydroxyl groups from two Thr256 residues. The backbone NH-group of Ala252 can form an H-bond to one oxygen of the nitro-group, stabilizing its position. The OCHF2 group of compound B is located facing the backbone NH-group of Leu253, but the distance is large (4.38 Å and 5.33 Å) and does not favor the formation of an H-bond. The benzene ring fitted well into the lipophilic binding cavity created by CB, CA or CG2 of Thr256. Moreover; CG2 of Ile255 is predicted to undergo hydrophobic interaction to the pyrazole methyl of compound B (Fig. 6d). Conversion of the $SOCF_3$ of fipronil to NO_2 was associated with a small increase in predicted docking energy and a decrease in the extent of hydrogen bonding, suggesting a decrease in potential binding affinity at the insect GABA receptor. These findings were consistent with increasing IC_{50} for compound B compared to fipronil, i.e., compound B 9.8–21.9 nM; fipronil 2.3–6.3 nM [12, 28].

The replacement of the OCHF2 group with an OCH3 group and deletion of the NO2 group of compound B produced compounds B1 and B2, leading to a 0.2-fold and 0.63-fold increase in predicted docking energy, respectively (Table 1). These results were also consistent with the decrease in binding affinity measured in vitro using housefly membranes [12]. These data confirmed the conclusion that the NO_2 group was essential to the high affinity in the second series of antagonists. No definite interaction was found between the receptor and the OCHF2 group of compound B, which may have produced steric hindrance.

Having studied fipronil-related heterocyclic compounds, a potential interaction mode to residues within the second transmembrane domain was proposed. Our docking studies highlighted the importance of Thr256 in the receptor-antagonist interaction. Phenyl and CF3 groups in position 6 had strong hydrophobic contacts and hydrophilic inter-

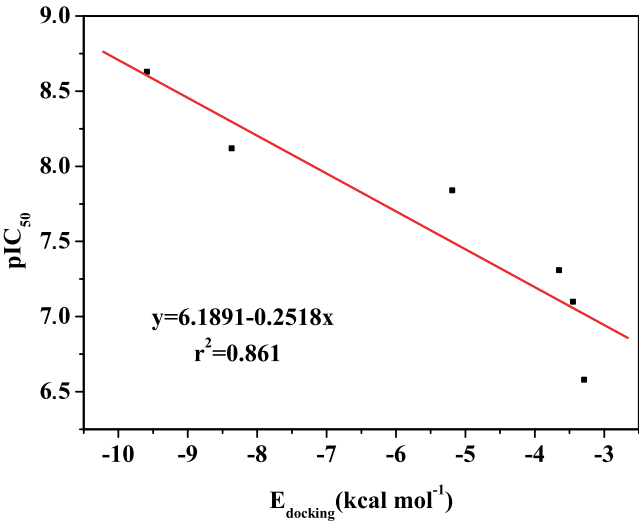


Fig. 7 Relationship between binding energy in the receptor-antagonist complex as calculated by AutoDock and experimentally determined extent of inhibition (pIC_{50}) (values plotted are those in Table 1)

actions through the alkyl and hydroxyl groups of Thr256. The docking calculations underlined the fact that the different affinities of these compounds to the insect GABA receptor were influenced mainly by different substitutions at positions 15, 16 and 17 of the pyrazole ring.

Six fipronil-related heterocyclic compounds were used to derive a relationship between predicted final docked energy and in vitro IC_{50} values (Table 1). The correlation between total binding energies calculated by AutoDock and experimentally determined IC_{50} values is shown in Fig. 7. The straight line was drawn using the least squares method. The slope of the line is 0.861, which was a definite positive value and shows that there is a fairly good correlation between the total energy values provided by AutoDock and the experimental results. This linear dependency could be used to make rough predictions of candidate compounds at early stages of drug screening to identify compounds with possible activity towards the receptor. Prediction of the extent of inhibition as described above can be made only within series of compounds belonging to the same structural class.

Conclusions

A three-dimensional model of the β3-homopentamer was generated using the cyro-electron microscopy structure of the nicotinic acetylcholine receptor as a structural template. The potential binding modes of fipronil-related heterocyclic compounds were illustrated for the first time by means of docking studies. In our model, Ala252, Leu253, Ile255 and Thr256 showed strong hydrophobic and hydrophilic interactions with heterocyclic compounds—in agreement with mutagenesis studies. Docking studies highlighted different substitutions at positions 15, 16 and 17 of the pyrazole ring as key factors responsible for the different activity of the compounds tested. The results obtained from docking studies also illustrated a good correlation between predicted docking energy and experimentally determined IC_{50} values. A better understanding of the interaction mode of fipronil-related heterocyclic compounds with the β3-homopentamer may allow the rational development of insecticide-related compounds and increase the efficiency of computational-based drug design.

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