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Atsushi Kasuya · Yoshihiro Sawada
Yoshihisa Tsukamoto · Keiji Tanaka · Tetsuya Toya
Mikio Yanagi

Binding mode of ecdysone agonists to the receptor: comparative modeling and docking studies

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Abstract Three-dimensional structure models of the ligand-binding domain of the ecdysone receptor of *Heliothis virescens* were built by the homology modeling technique from the crystal structures of nuclear receptors. Two models were created based both on known ligand-binding domain structures of the receptors with the highest sequence identity to the ecdysone receptor, and on those of steroid hormone receptors. The latter model, which was found to have better stereochemical quality and be in good agreement with the binding of the steroidal framework of the endogenous agonist 20-hydroxyecdyosone, was used for docking studies. The docking of 20-hydroxyecdysone to the receptor model revealed that the ligand molecule can interact with the receptor in a similar manner to other steroid hormone–receptor complexes. The docking of a dibenzoylhydrazine agonist, chromafenozide, was performed based on the correspondences between the molecule and 20-dydroxyecdysone expected by molecular comparison. The interactions of the ligands with the receptor in the complexes modeled were investigated and found to be consistent with known structure–activity relationships.

A. Kasuya (✉)
Exploratory Chemistry Research Laboratories,
Sankyo Co., Ltd., 2-58 Hiromachi 1-chome, Shinagawa-ku,
140-8710 Tokyo, Japan
e-mail: kasuya@shina.sankyo.co.jp
Tel.: +81-3-3492-3131, Fax: +81-3-5436-8570

Y. Sawada · Y. Tsukamoto · K. Tanaka
Crop Protection Department, Sankyo Co., Ltd.,
1041 Yasu, Yasu-cho, Yasu-gun, 520-2342 Shiga, Japan

T. Toya
Research and Development Laboratories,
Agro and Speciality Chemicals Group,
Nippon Kayaku Co., Ltd., 225-1, Koshikiya Horigome, Ageo,
362-0064 Saitama, Japan

M. Yanagi
Agro Group, Agro and Speciality Chemicals Group,
Nippon Kayaku Co., Ltd.,
1-11-2, Fujimi, Chiyoda-ku, 102-8172 Tokyo, Japan

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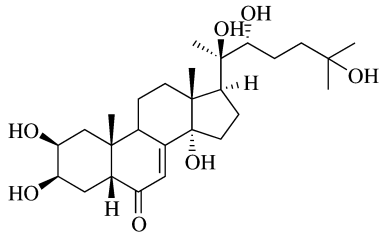
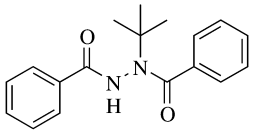
Introduction

Ecdysones are the steroid hormones released by the prothoracic glands of arthropods. 20-Hydroxyecdysone (20-HE, Fig. 1), the endogenous active form of ecdysones, binds to the ecdysone receptor (EcR), and through the activation of the receptor controls various physiological functions concerning the growth of insects and crustaceans, including molting and metamorphosis. [1, 2] The EcR is a member of the nuclear receptor family. Nuclear receptors are intracellular transcription factors that share common functional domains, namely, the N-terminal region, DNA-binding domain, hinge region, ligand-binding domain (LBD) and C-terminal region. [3, 4] To date, crystal structures of the LBDs of many nuclear receptors, including those complexed with agonists, partial agonists or antagonists, have been determined. [5] These structures have provided important information on the recognition of the ligands and the mechanism of activation of nuclear receptors, which is useful for designing ligands with the desired modulation activity. [5, 6] Recently, crystal structures of the LBD of ultraspiracle (USP), which forms a heterodimer with the EcR, have been determined. [7, 8] However, the three-dimensional structure of the EcR has yet to be elucidated.

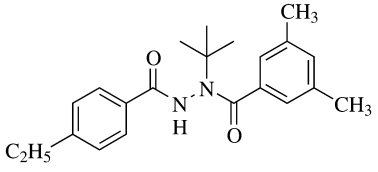
Since the first nonsteroidal ecdysone agonist, RH5849, and its larvicidal activity were reported, [9, 10] extensive explorations of its derivatives, so-called dibenzoylhydrazines, have been performed. [11, 12, 13] We reported that chromafenozide (ANS-118, Fig. 1), a new dibenzoylhydrazine with a chroman ring, has potent larvicidal activity for lepidoptera insects. [14] It is known that dibenzoylhydrazines show insecticidal activity by continuous activation of the EcR. [10] Both 20-hydroxyecdysone and dibenzoylhydrazines, hence, interact with the EcR and activate it, although their chemical scaffolds



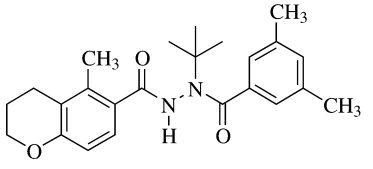
20-hydroxyecdysone (20-HE)



RH5849



tebufenozide



chromafenozide

Fig. 1 Chemical structures of ecdysone agonists

are quite different. The chemical structures of these ecdysone agonists are shown in Fig. 1. Elucidation of how these molecules interact with the receptor is very important for understanding the mechanism of their action and for designing new ligands.

In this study, three-dimensional structures of the ligand-binding domain of the EcR were modeled using the homology modeling technique. Two sets of reference structures, one the structures of the receptors with the highest sequence identities to the EcR and the other those of the steroid receptors, were used, and from the latter reference set A a more relevant model was obtained. Docking studies of 20-hydroxyecdysone and chromafenozide to the model were performed in order to elucidate the interactions between the receptor and the agonists and the correspondence between the two classes of molecules.

Methods

Homology modeling

A three-dimensional structure of the LBD of the EcR of the tobacco budworm *Heliothis virescens* (HvEcR) was modeled from known three-dimensional structures of nuclear receptors. We used two sets of reference molecules for the homology modeling, each set consisting of two molecules. The two receptors with the highest sequence identities to the EcR were selected as set A: the LBD structures of human retinoic acid receptor γ (RARγ) in a complex with all-*trans* retinoic acid (PDB ID: 2LBD [15]) and human thyroid hormone receptor β (TRβ) in a complex with 3,5,3′-triiodothyronine (1BSX [16]). Although the receptors of set A had the highest sequence similarity, their ligands were not steroids. In order to build ligand–receptor complex models of the EcR, we considered that it might be necessary to use structures of steroid hormone receptors. Therefore, the crystal structures of the human estrogen receptor α (ERα)–17β-estradiol complex (1ERE [17]) and the human progesterone receptor (PR)–progesterone complex (1A28 [18]) were also used as set B for the homology modeling. A homology model was created for each set using the Modeler module [19] of InsightII. [20] First, the sequences of the reference structures were aligned based on their three-dimensional structures, and structurally conserved regions (SCRs) were obtained for each set. The sequence of the HvEcR LBD was then aligned to the reference sequences. The resulting alignments are shown in Fig. 2. For each set, five models of the HvEcR LBD were obtained from the Modeler calculation. Each model consists of five structures with different loop conformations. Among those, the structure having the best probability density function (PDF) value was selected as the final model for each set. The geometries of the final models were evaluated using the Procheck program. [21] The statistics of the stereochemical parameters showed that the model from set B had better quality.

Docking of 20-HE

For the docking of 20-HE, the EcR LBD model from set B was used. The structure of 20-HE was placed within the binding pocket of the receptor, using the interactions of ERα and 17β-estradiol, and PR and progesterone in their respective complexes as reference models. The overall shape and size of the binding pocket of the EcR LBD model were similar to those of the estrogen receptor and progesterone receptor. Although the 5β-cholest-7-en-6-one structure of 20-HE has a conformation with its A-ring bent down from the average plane of the B, C and D rings, the β-configurations of the 2, 3-hydroxy groups have their oxygen atoms folded up nearer to the corresponding oxygen atoms at position 3 of 17β-estradiol and progesterone. Therefore, 20-HE could be placed in the binding pocket in a similar manner to the ligands in the receptor pockets of the reference structures without difficulty. The geometry of the ligand and side-chain atoms within 5 Å from the ligand was optimized by the molecular mechanics force field calculation using CHARMm. [22]

Comparative study of 20-HE and chromafenozide

Correspondences between the features of the three-dimensional structures of 20-HE and chromafenozide were searched for using the Catalyst program. [23] We assumed that the structure of chromafenozide should correspond to the steroid nucleus of 20-HE. In order to avoid unnecessary matches to the side chain of 20-HE, a model structure was created in which the side chain of 20-HE was truncated at C23, and this side chain-shortened 20-HE model was used in the following pharmacophore search. By the "best" conformer generation function in Catalyst with an energy threshold of 10 kcal mol^{-1}, seven and 14 conformers were obtained for the 20-HE model and chromafenozide, respectively. The conformers of the 20-HE model were searched for feature matches (hypotheses) to the chromafenozide conformers using the HipHop function of Catalyst. Ten hypotheses of the highest scores were analyzed. The best-scored hypothesis had five features, and the correspondences of six of the ten hypotheses, including the best-scored one, were similar to each other (correspondence I). Another correspon-

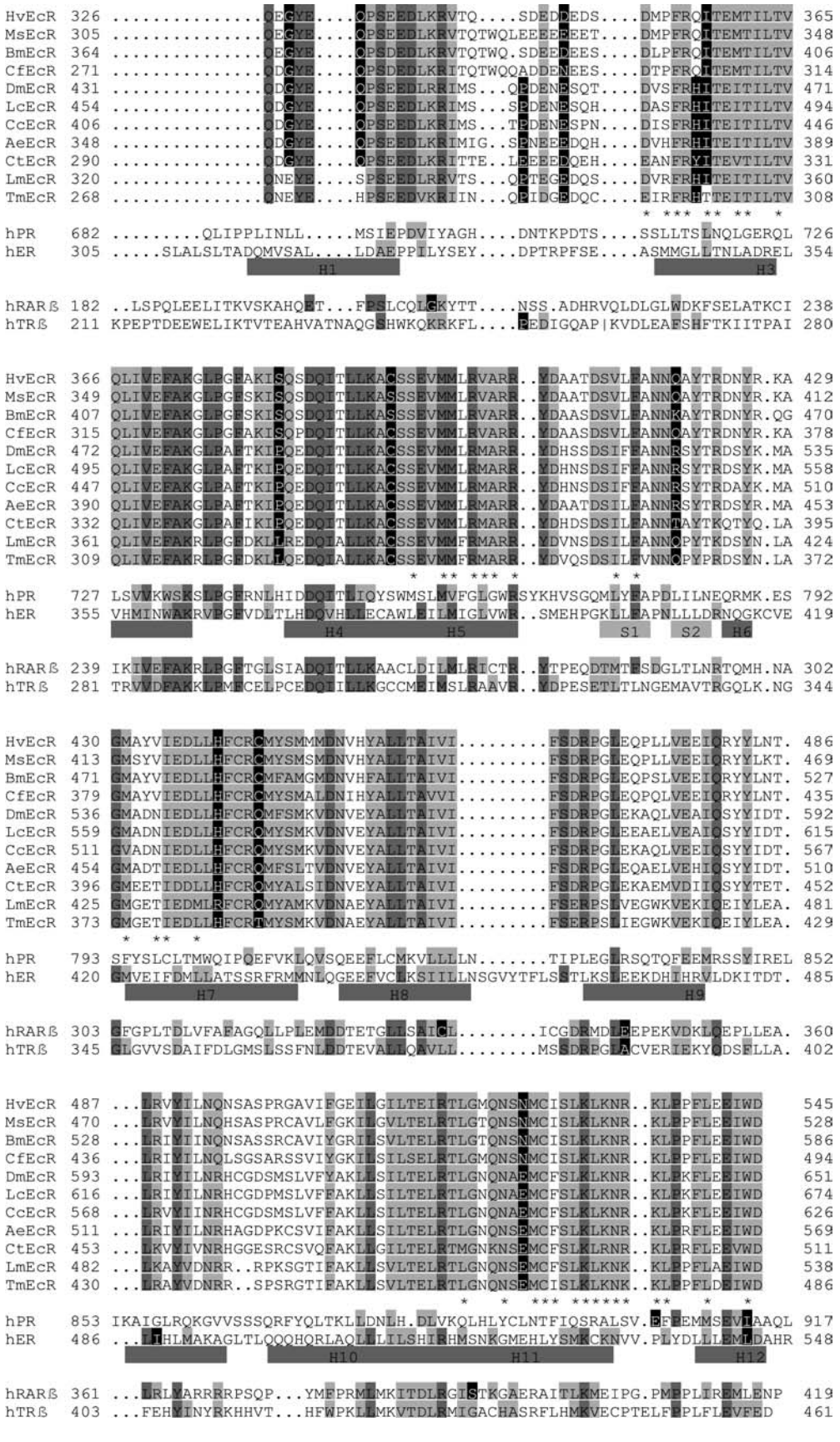


Fig. 2 Sequence alignment of 11 EcR LBDs and two sets of reference structures. The reference structures are human progesterone receptor (hPR) and human estrogen receptor α (hER) (set B), and human retinoic acid receptor γ (hRARγ) and human thyroid hormone receptor β (hTRβ) (set A). The sequence alignment of EcR LBDs was done using the ClustalW program. [32] EcR sequences consist of four lepidoptera sequences from *Heliothis virescens* (Hv), [25] *Manduca sexta* (Ms), [33] *Bombyx mori* (Bm) [34] and *Choristoneura fumiferana* (Cf), [35] five diptera sequences from *Drosophila melanogaster* (Dm), [36] *Lucilia cuprina* (Lc), [37] *Ceratitis capitata* (Cc), [38] *Aedes aegypti* (Aa) [39] and *Chironomus tentans* (Ct), [40] and one sequence each from orthoptera *Locusta migratoria* (Lm) [41] and coleoptera *Tenebrio molitor* (Tm). [42] The amino acids are shown in grayscale depending on their similarities. The amino acids involved in the ligand binding in receptor model B are marked by *asterisks*. The sequences of the reference structures are aligned based on three-dimensional structures using the Homology module of InsightII. [20]

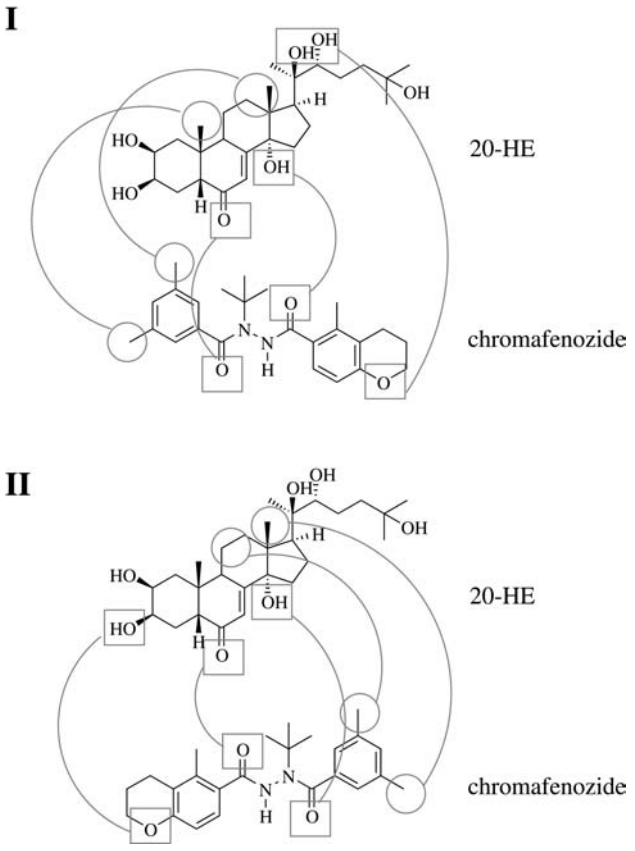


Fig. 3 Two correspondences (I and II) between 20-hydroxyecdysone (20-HE) and chromafenozide calculated by the Catalyst program [23] using the HipHop function. For each of the two correspondences, three hydrogen-bond acceptor functions (squares) and two hydrophobic functions (circles) were matched between the two molecules

dence of the highest score among the rest of the hypotheses was correspondence II (Fig. 3).

Docking of chromafenozide

Based on the modeled structure of the HvEcR-20-HE complex and the two correspondences between 20-HE and chromafenozide, shown in Fig. 3 as I and II, the docking of chromafenozide was performed. For each of the correspondences, the structure of chromafenozide could be placed in the binding pocket of the receptor without any steric hindrance with the receptor atoms, and two models, I and II, of the HvEcR–chromafenozide complex were obtained.

Results

Sequence alignment

The multiple alignments of amino acid sequences of the LBD of 11 insect EcRs obtained from GenBank [24] are shown in Fig. 2. The sequences were well conserved between the species with more than 60% amino acid identities. The conservation was even higher within related species. For example, the amino-acid identities of the sequences of the EcR LBD among four species of lepidoptera were 85–91%. The conservation was more distinct in the region including helixes 3 and 4, which form the core part of the receptor structure.

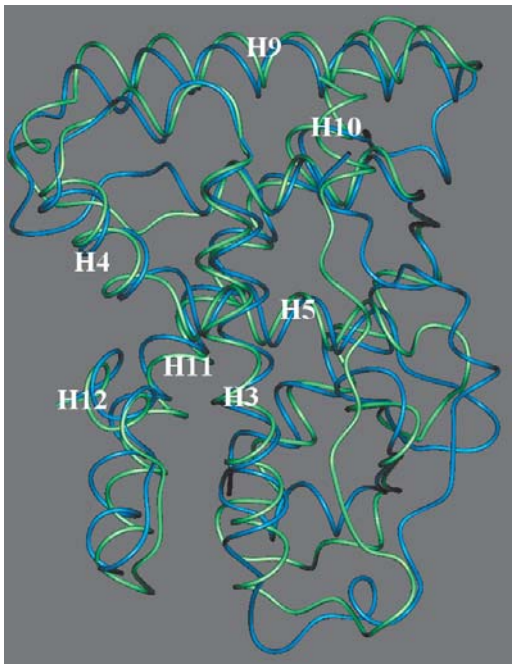


Fig. 4 Two EcR LBD structure models from set A (*blue*) and set B (*green*)

Using the sequence of the LBD of HvEcR (lepidoptera), [25] the sequences of the proteins of known three-dimensional structures were searched for sequence similarity. The sequence of the HvEcR LBD had similarity with the LBDs of other nuclear receptors of known three-dimensional structures. Those included RARγ (30% residue identity), TRβ (30%), ERs (22%) and PR (22%). The sequence alignments of the reference receptors in sets A and B were performed based on their three-dimensional structures. The alignment of the HvEcR LBD to the sequences of reference set B is shown in Fig. 2.

Homology modeling

Two models, A and B, were obtained from separate Modeler calculations based on reference sets A and B, respectively. The overall structures of these two models were very similar (Fig. 4). Their main-chain structures were almost identical except for the N- and C-terminal residues and loop regions. The most significant difference that could affect the ligand binding was the conformational change of the loop between the s1 and s2 β-strand structures. In model A, the side-chain conformation of Arg405 had changed and its guanidino group was located in a position where it could not interact with the ligand molecule. The interaction between the Arg405 side chain and the ligand is commonly observed in ste-

roid receptor–ligand complexes, and so we assumed that it is also essential for the recognition of agonists by the EcR. Therefore, we decided that model B was more plausible than model A. The geometry of the final model was evaluated using the Procheck program. The ratios of the main-chain phi and psi angles within the allowed region of the Ramachandran plot were 95% and 99% for models A and B, respectively. Thus, stereochemical evaluations gave slightly poorer results for model A than for model B. For these reasons, we used model B in the following docking study.

EcR–20-HE complex

The modeled structure of the ecdysone receptor–20-HE complex is shown in Fig. 5. The model was built so that the interactions of the ligand and the receptor would be similar to those of the reference structures. The 6-carbonyl group of 20-HE interacts with the side chain of Asn522. The A-ring of 20-HE is in a position where the 2- and 3-hydroxy groups can interact with Arg405 and Thr364 through a water molecule, in a similar manner to the oxygen atoms at position 3 of both 17β-estradiol and progesterone which interact with their respective receptors. The 20-hydroxy group of 20-HE forms a hydrogen bond with the Oγ of Thr358 and the main-chain carbonyl group of Phe354. The Oγ of Thr358 also interacts with the 22-hydroxy group of 20-HE. The terminal 25-hydroxy group of 20-HE is within hydrogen-bond distance of the main-chain carbonyl group of Lys535. These interactions of the modeled structure of the EcR, and comparison with the crystal structures of PR and ERα are shown in Fig. 5. Although interactions of the steroid nucleus of 20-HE could be modeled in accordance with the reference structures, the side-chain conformation of 20-HE could not be built only by similarity because the ligand molecules of the reference structures do not have side chains. The structure of the side chain was modeled manually and a conformation in which the ligand atoms and receptor atoms of the AF-2 region could interact with each other was selected. In the final model, the side chain of Trp544, which is one of the AF-2 helix residues, interacts with the aliphatic part of the 20-HE side chain. The residues involved in the ligand binding in this model are indicated in Fig. 2. Most of these residues are conserved well in the sequences from different species.

EcR–chromafenozide complex

Nakagawa et al. have proposed superpositions of 20-HE and dibenzoylhydrazines in which the two carbonyl

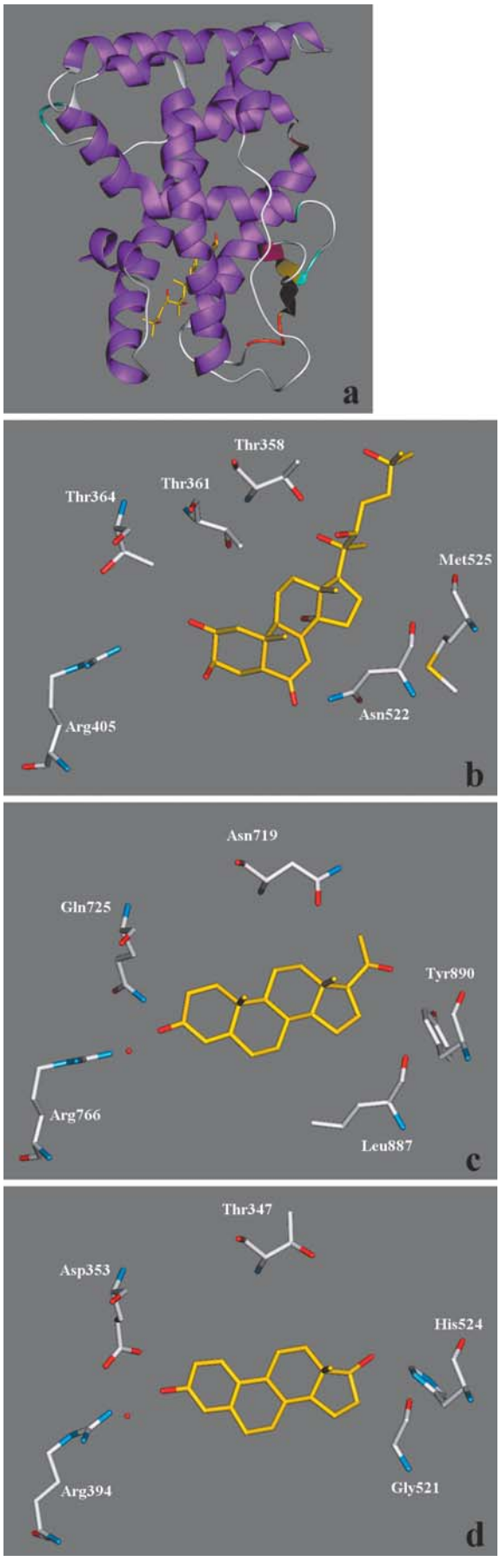


Fig. 5 Overall structure (**a**) and ligand–receptor interaction (**b**) of the HvEcR–20-HE complex. For comparison, interactions of progesterone with PR [18] (**c**) and 17β-estradiol with ERα [17] (**d**) in their crystal structures are also shown. The carbon atoms of the ligands are shown in *yellow*

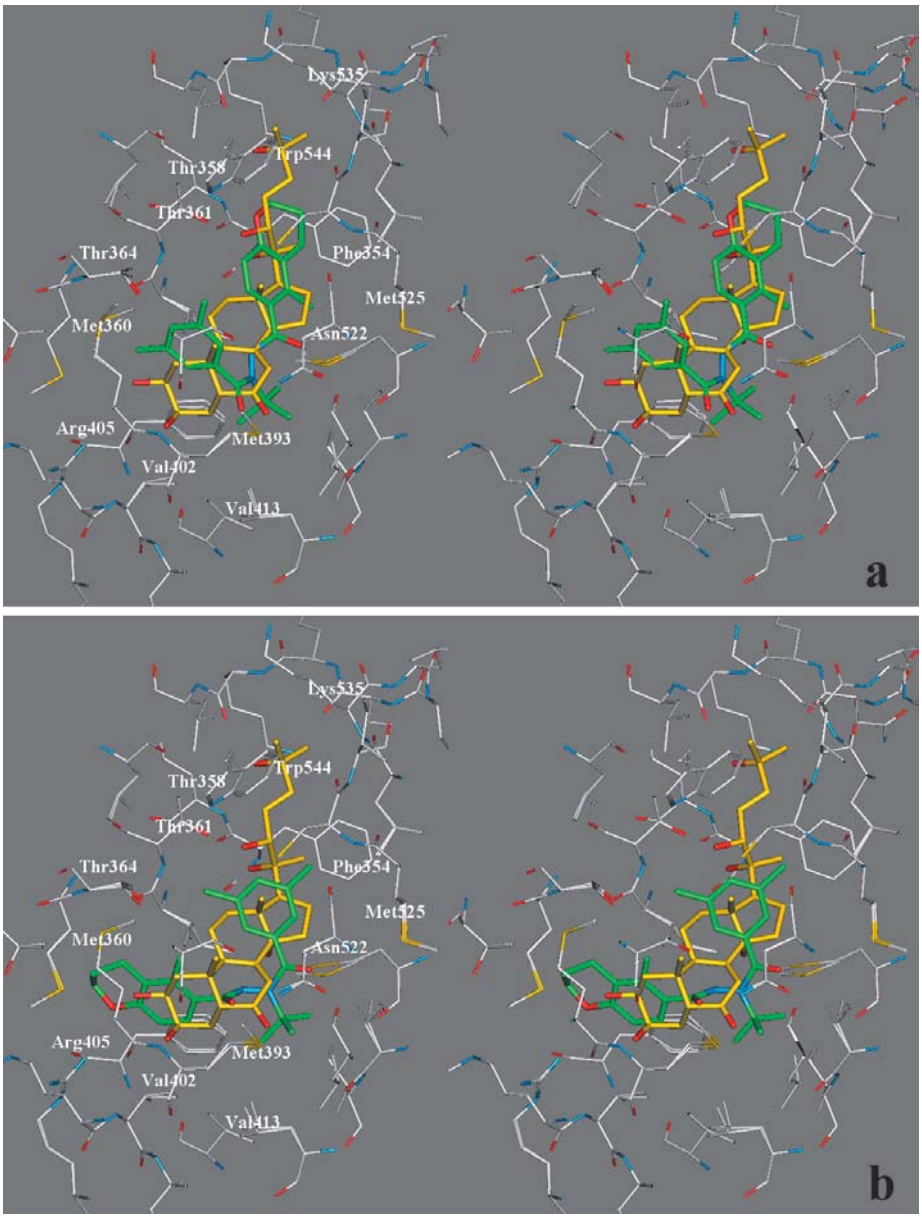


Fig. 6 Stereoview of the superposition of the structures of the EcR binding site complexed with chromafenozide (*green*) and 20-HE (*yellow*). Two models for chromafenozide, models I and II, are shown in **a** and **b**, respectively, together with the same 20-HE model

groups of dibenzoylhydrazines correspond either to the 14- and 20-hydroxy groups, or to the 20- and 22-hydroxy groups of 20-HE, based on structure–activity relationships and a CoMFA (comparative molecular field analysis) study. [26, 27] We used the HipHop function of Catalyst for the comparative analysis of 20-HE and chromafenozide. The program exhaustively searches feature matches of the input molecules, gives multiple pharmacophore models (hypotheses) and scores them by evaluating their accordance with the input molecules. The side chain-shortened 20-HE model used in the calculation had 20- and 22-hydroxy groups, and the possibilities of correspondences between these hydroxy groups to chromafenozide were, therefore, also considered in the search. In the best-scored hypotheses, however, no correspondences with matches between the 20- or 22-hydroxy groups and the carbonyl groups of chromafenozide were found. Instead, two correspondences (I and II in Fig. 3) between the two molecules were obtained, in which the carbonyl groups of chromafenozide corresponded to the 6- and 14-hydroxy groups of 20-HE. In correspondence I, the chroman ring of chromafenozide was matched to the A-ring of the 20-HE steroid nucleus, while in correspondence II, the oxygen atom of the chroman ring of chromafenozide corresponded to the 20-hydroxyl group of 20-HE. The conformations of the two amide bonds of chromfenozide were *cis* for the *N*-*tert*-butyl amide and *trans* for the other amide bond in both correspondences. The same amide conformations were also seen in the crystal structure of RH5849. [28]

Although correspondence I had a better score regarding fitness of the two molecules than correspondence II, we considered that evaluation by docking studies would be necessary. The docking of chromafenozide to the

HvEcR LBD was performed, therefore, based on each of these two correspondences obtained from comparative study of the ligands, and two model complexes, I and II, were obtained from correspondences I and II, respectively. The ligand–receptor interactions in these models are shown in Fig. 6. In each correspondence, the chromafenozide molecule was docked to the receptor without any serious hindrance. In model I, the oxygen atom of the chroman ring of chromafenozide was within hydrogen-bond distance of the Oγ of Thr358. The oxygen atom of *N-tert*-butyl amide interacted with Asn522. The orientation of the chromafenozide molecule was reversed in model II, and the oxygen atom of the chroman ring formed a hydrogen bond with the side-chain guanidino group of Arg405. Another hydrogen bond was formed by the carbonyl oxygen adjacent to the chroman ring with the side chain of Asn522 in this model. In both models, the *tert*-butyl group of chromafenozide was located in a similar position and interacted with the hydrophobic pocket formed by residues including Leu438, Met431, Val434 and Leu435. The side chain of Trp544 was located near the 3,5-dimethylphenyl group (model I) or the chroman ring (model II) of chromafenozide, and in each position it could make hydrophobic interactions with chromafenozide.

Discussion

Binding activities are known for only a small number of the ecdysone agonists. Displacing activities (EC_{50}) of [^{3}H]ponasterone (0.5 nM) measured with Drosophila Kc cell extracts have been reported for 20-HE (0.1 μM) and RH5849 (3 μM). [9] Recently, similar activities were measured using intact SF-9 cells for 20-HE, RH5849 and tebufenozide, and their pIC_{50} (M) values were 6.78, 6.44 and 8.81, respectively. [29] Although there are many other structure–activity data reported for ecdysone agonists, in most cases these are from in vivo or cellular level activation assays. Such activity data correspond only indirectly to the ligand–receptor interactions, because differences in the potencies of the ligands can be attributed, at least partly, to their different permeabilities and transportation. It should also be noted that activities measured in various species cannot be compared with each other directly. Furthermore, limited accuracy of the current HvEcR model prevents us from detailed evaluation of the ligand–receptor interactions. Therefore, we could investigate only a limited part of known structure–activity information, and found that our models of the HvECR–ligand complexes are consistent with them as follows.

From the comparison of the activities of α-ecdysone and 20-HE, it is well known that the 20-hydroxy group is essential for strong activity. In our model of the HvEcR–20-HE complex, this hydroxy group forms hydrogen bonds to the Oγ of Thr358 and the main-chain carbonyl group of Phe354. These hydrogen bonds will be lost if the 20-hydroxy group is absent. Polypodine B and turkesterone, which have an additional hydroxy group at positions 5 and 11, respectively, are also known to retain activity. [30] In the model, Ser395 and Thr358 are located at the positions where the 5- and 11-hydroxy groups, respectively, can interact with the side-chain hydroxy groups of these residues.

Chromafenozide is more potent than tebufenozide regarding insecticidal activity for *Spodoptera litura* larvae (LC_{50} values: 0.9 and 3.4 ppm for chromafenozide and tebufenozide, respectively). From the structure–activity relationships of chromafenozide derivatives, it is known that both the oxygen atom and the substituted methyl group of the chroman ring are necessary for strong activity. [14] In models I and II of the HvEcR–chromafenozide complex (Fig. 6), the oxygen atom interacts with the side chain of Thr358 and Arg405, respectively. These oxygen atom positions were achieved by rotation of the chroman ring with respect to the adjacent carboxy group, and the methyl group of the chroman ring was considered to stabilize these conformations.

Chromafenozide is biologically more active for lepidoptera than for diptera. [14] Several amino acid substitutions were found in the residues of the binding pocket when the sequence of the HvEcR LBD (a lepidopteran) was compared to that of a dipteran, the fruit fly *Drosophila melanogaster*. [31] These included Pro353Ser, Met360Ile, Val402Met, Val413Ile, Val434Asn and Ile527Phe. Among these substitutions, the replacement of Val402 of the HvEcR with methionine (Met508 in the numbering of the *Drosophila* EcR) was the only one in which the size of the side chain in contact with the ligand changed significantly. Only in model II was the substitution found to affect the binding of chromafenozide to the receptor. The side chain of Met508 of the *Drosophila* EcR would be in close contact with chromafenozide at position 8 of the chroman ring. In model I, the binding of chromafenozide to the receptor was not expected to change significantly by this substitution, because in this case, the ligand would not occupy the volume filled by the Met508 side chain. This volume is not occupied by 20-HE in its model complex either, and thus the substitution does not affect the binding of the endogenous agonist to the receptor. It could be speculated that the amino acid replacement of Val402 with methionine is responsible for the different potencies of chromafenozide between lepidoptera and diptera. From this point of view, model II is more likely to reflect the true binding mode.

Conclusion

We have developed a three-dimensional model of the HvEcR LBD by homology modeling and performed the docking of two agonists, 20-HE and chromafenozide, based on the similarity between 20-HE and other steroid hormones and the correspondences between 20-HE and chromafenozide obtained by molecular comparative study. The docking studies gave model structures of the

receptor–agonist complexes that are basically consistent with known structure–activity relationships of 20-HE and chromafenozide. The interactions of 20-HE and chromafenozide with the receptor proposed in this study provide important information for designing new agonist scaffolds from these two classes of compounds, ecdysones and dibenzoylhydrazines. These models can be used for hypothesizing the activation mechanism, as well as for further analysis of the structure–activity relationships of the EcR and its ligands.

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