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Homology-modeled ligand-binding domains of medaka estrogen receptors and androgen receptors: A model system for the study of reproduction

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ABSTRACT

Estrogen and androgen and their receptors play critical roles in physiological processes such as sexual differentiation and development. Using the available structural models for the human estrogen receptors alpha and beta and androgen receptor as templates, we designed *in silico* agonist and antagonist models of medaka estrogen receptor (meER) alpha, beta-1, and beta-2, and androgen receptor (meAR) alpha and beta. Using these models, we studied (1) the structural relationship between the ligand-binding domains (LBDs) of ERs and ARs of human and medaka, and (2) whether medaka ER and AR can be potential models for studying the ligand-binding activities of various agonists and antagonists of these receptors by docking analysis. A high level of conservation was observed between the sequences of the ligand-binding domains of meERα and huERα, meERβ1 and huERβ, meERβ2, and huERβ with 62.8%, 66.4%, and 65.1% identity, respectively. The sequence conservation between meARα and huAR, meARβ, and huAR was found with 70.1% and 61.0% of identity, respectively. Thirty-three selected endocrine disrupting chemicals (EDCs), including both agonists and antagonists, were docked into the LBD of ER and AR, and the corresponding docking score for medaka models and human templates were calculated. In order to confirm the conservation of the overall geometry and the binding pocket, the backbone root mean square deviation (RMSD) for Cα atoms was derived from the structure superposition of all 10 medaka homology models to the six human templates. Our results suggested conformational conservation between the ERs and ARs of medaka and human, Thus, medaka could be highly useful as a model system for studies involving estrogen and androgen interaction with their receptors.

The estrogen receptor (ER) and androgen receptor (AR) are members of the steroid/nuclear receptor superfamily of intracellular ligand-dependent transcription factors [1,2]. Estrogens are compounds that interact with endocrine receptors (ERs) in target tissues to stimulate the development of reproductive organs and secondary sex characteristics in females. Androgens interact with the androgen receptor (AR) to maintain sexual characteristics such as muscle and bone mass, strength, fat distribution, and spermatogenesis [3]. The role of AR is to modulate the biological effects of the endogenous androgens which play numerous roles during male fetal and pubertal development [4]. Besides endogenous hormone substances such as estrogen and androgen, endocrine-disrupting chemicals (EDCs) can also elicit a variety of adverse effects in animals. These effects include reproductive tract disorders, reduction in reproductive fitness, changes in immunity, and promotion of hormone-dependent cancers [5].

The Japanese medaka is a particularly attractive model system to study reproductive biology, including sex determination and sexual differentiation [6,7]. As for many other fish, the sex of medaka is XX:XY-coded and functionally reversible if androgens or estrogens are applied during a sensitive period of embryonic or juvenile development [8]. Medaka has been used as a model organism to screen for the intended biological effects of drugs as well as their undesired toxic side effects [9,10]. Moreover, medaka is also used to estimate the potential effect of chemicals, especially the effect derived from endocrine disruptors which play important role in reproductive study [11–16].

In order to address the endocrine disruptor problem, an interagency committee [Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) has published an initial set of 78 recommended test substances to study estrogen receptor and androgen receptor binding assays [17]. Our *in silico* docking studies with medaka ERs and ARs were therefore

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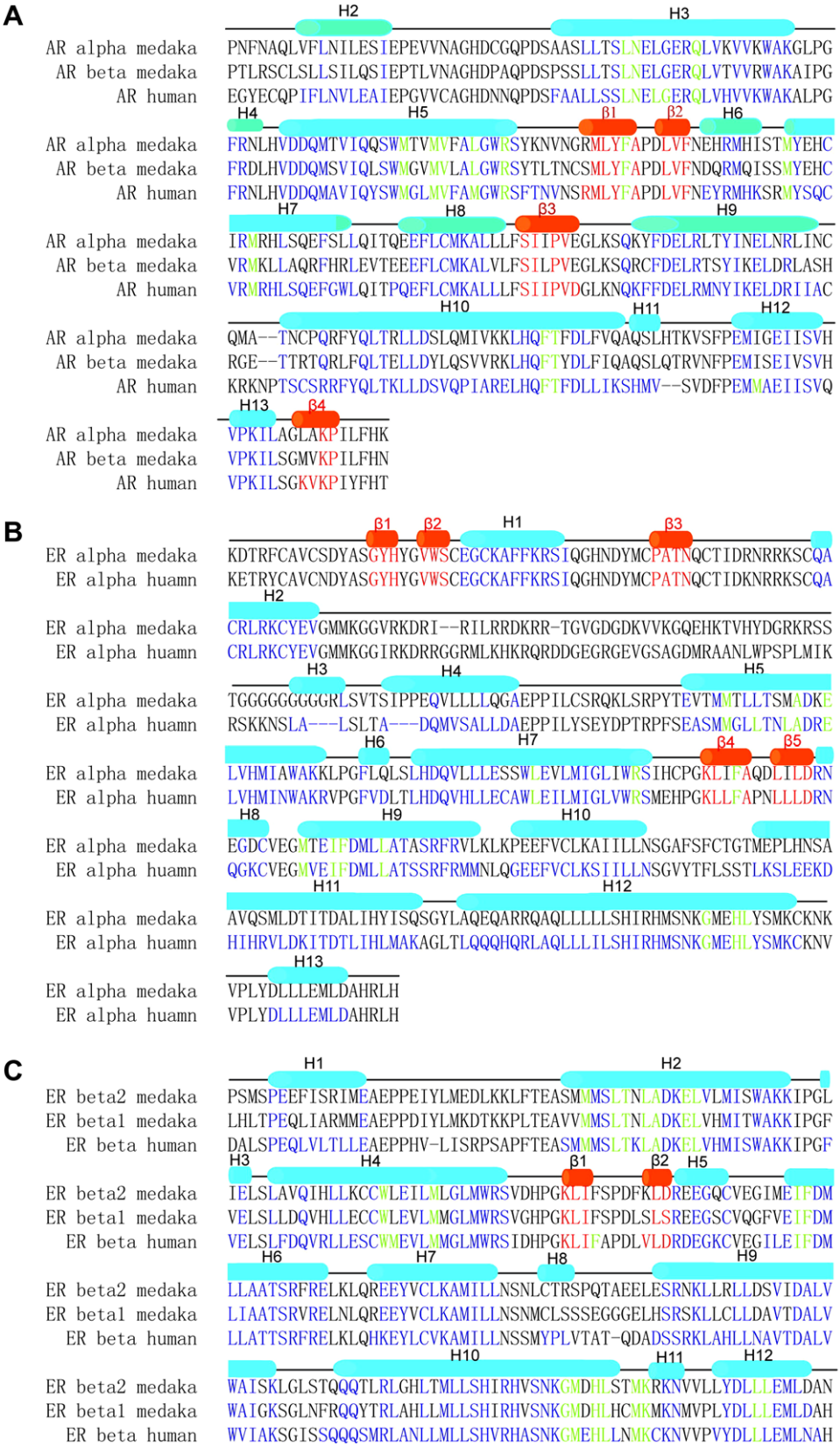

Fig. 1. Sequence alignments of AR and ER ligand-binding domains. Sequence identities are indicated with blue in α-helices and yellow in β-sheets, and residues involved in ligand interactions are highlighted in green. (A) Sequence alignment of medaka and human AR. The secondary structure of the medaka AR homology model is represented above the alignment as blue cylinders for α-helices and yellow cylinders for β sheets. (B) Sequence alignment of the medaka ERα isoform with human ERα, with secondary structures represented as described in (A) for the homology models. (C) Sequence alignment of the two medaka ERβ isoforms with human ERβ, with secondary structures represented as described in (A) for the homology models. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

performed with 33 recommended test substances, all well characterized as agonists or antagonists, binding with ER and AR, and potential endocrine disruption.

Despite the growing concern and the large amount of literature on the toxicological activity of EDCs, molecular data of the interaction of these compounds with ER and AR were still lacking [18–20]. Crystal structures of numerous human ERs and ARs are available, but no structures are available for medaka. In this study we present homology models of medaka ERs and ARs developed using human ER and AR crystal structures as templates. Although sequence identity among these structures was found to be 60–70%, the percent identity of binding site residues was even higher. In addition, potential endocrine disruptors to be tested in the ICCVAM assay had been identified based on *in silico* docking studies using 10 medaka ER and AR models.

Materials and methods

Preparation of the ligand for docking. Thirty-three EDCs were selected from the ICCVAM report based on their relative binding affinity (RBA) or from half maximal effective dose (EC50) data (EC50 value was >0.1 μM or the substance was positive in the single assay in which it was tested) [17]. Molecular structures of the EDCs ligands were built and energy minimized with Autodock 4.0 [21,22].

Sequences alignment. The meERs and meARs sequences were obtained from the NCBI http://www.ncbi.nlm.nih.gov/ with accession numbers P50241 (meERα), BAB79705.1 (meERβ1), AB428449 (meERβ2), BAC98301.1 (meARα) and ABV55993 (meARβ) respectively (Fig. 1). The accession numbers of the human receptors are P03372-1 (huERα), Q92731-1(huERβ) and P10275 (huAR). The alignment analysis was taken by BlastP online system.

Homology model construction. Homology models were constructed for all medaka ER and AR ligand-binding domains using Swiss-model. Medaka ERα was modeled using both human ERα agonist (1QKU) and antagonist (3ERT). Medaka ERβ1 and β2 were modeled using both human ERβ agonist (2JJ3) and antagonist (1L2J). Medaka ARα and ARβ were modeled using both human AR agonist (2AMB) and AR antagonist (1XNN). Homology models constructed based on templates that have greater than 50% sequence identity to the target sequence have previously been compared with crystal structure. Totally, 10 medaka models were constructed based on the six human templates mentioned above.

Docking simulations. The docking program used in this study was AutoDock 4.0 [21] in which the protein was embedded in a 3D-grid and a probe atom was placed at each grid point [22]. For docking analysis of the 33 compounds from the ICCVAM (supplemental Fig.1), all the 10 medaka and six human ER and AR models were used. Because most potential endocrine disruptors will have multiple rotatable bonds (unlike estradiol), flexible ligand docking was achieved using Autodock. The docking simulation consisted of 100 runs for each ligand and the backbone root mean square deviation (RMSD) for Cα atoms was derived from the structure superposition of all 10 medaka homology models and the six human templates. To predict the ligand-binding affinity for the ER and AR receptors, the docking score of each ligand/receptor complex was obtained from AutoDock 4.0.

Results

Analysis of meERs and meARs with homology-modeled structures

Alignments of medaka ER and AR isoforms with their human homologs were summarized in Fig. 1 (Fig. 1A for ARα and ARβ, Fig. 1B for ERα, Fig. 1C for ERβ1, ERβ2). Secondary structures from the each sequence were also showed in Fig. 1. Sequence identities between medaka ERs, ARs and their human homologs were always greater than 60%. Specifically, a high level of sequence conservation of the ligand-binding domain between meERα and huERα, meERβ1 and huERβ, meERβ2, and huERβ was found, with 62.8%, 66.4%, and 65.1% of identity, respectively. The sequence conservation between meARα and meARβ with huAR was found with 70.1% and 61.0% of identity, respectively (supplemental Fig. 2). The main regions of sequence variability between the human and medaka structures were between helices 3, 4, 10 in ER and helices 2 and 10 in AR. This variability was obvious in medaka ER sequences, which showed small insertions relative to human in helix 3.

The homology models of the ligand-binding domains of medaka were overlaid with the human homologs. The 3D structures derived from different sequence are shown in different color (purple for medaka and yellow for human) (supplemental Fig. 3). For the overlay of all 10 medaka homology models to the six human templates, the backbone root mean square deviation (RMSD) for Cα atoms is shown in Table 1. All agonist structures for ER and AR overlaid so closely that individual structures cannot be distinguished, as was the case for the antagonist structures. Among all the models and templates, the meERα and huERα had the best value in RMSN (0.09 and 0.07 for agonist and antagonist, respectively).

In silico docking of EDCs into medaka models and human templates

We selected 33 endocrine disruptors from 78 compounds in ICCVAM based on EC50 or relative binding affinity from experimental binding studies in human. All the selected endocrine-disrupting chemicals (EDCs) were docked into the LBD of ER and AR using Autodock [23], and the corresponding docking score for medaka models and human templates was calculated (Tables 2 and 3). 17α-Estradiol in the medaka ERα model and human ERα template had the highest docking score (8.40 and 8.36, respectively) and lowest RMSD (0.06) compared with other compounds in ER agonist column. Similar to 17α-estradiol in the ER agonist column, we found estrone had the best score in the ERβ1 and ERβ2 models and template in the ER agonist column. We also found several interesting EDCs such as raloxifene, 4-hydroxytamoxifen, testosterone, and bicalutamide, had the best score and lowest RMSD in ER antagonist, AR agonist and AR antagonist columns, respectively. The involved EDCs are labeled in bold in Tables 2 and 3.

Docked structures are given for six well-known ER ligands (17α-estradiol, raloxifene, 17β-estradiol, 4-hydroxytamoxifen, testosterone and bicalutamide; Fig. 2), which were oriented as expected, providing some validations for the docking calculations.

Table 1
RMSD for overlay of 10 medaka models to six human templates.

Medaka		Human					
		Human ERα		Human ERβ		Human AR	
		+	−	+	−	+	−
ERα	+	0.09					
	−		0.07				
ERβ1	+			1.85			
	−				0.30		
ERβ2	+			1.32			
	−				1.14		
ARa	+					0.50	
	−						0.83
ARβ	+					0.70	
	−						0.90

+, agonist; −, antagonist.

Table 2
Docking results of selected compounds in human templates and medaka models.

Compound name	Docking score for medaka ERα	Docking score for human ERα	RMSD between medaka ERα and human ERα	Docking score for medaka ERβ1	Docking score for medaka ERβ2	Docking score for Human ERβ	RMSD between medaka ERβ1 and human ERβ	RMSD between medaka ERβ2 and human ERβ
ER agonist								
Apigenin	7.55	7.73	0.26	7.30	6.87	6.93	3.05	2.48
Diethylstilbestrol	6.46	6.71	0.13	6.92	7.08	6.89	2.67	2.28
17α-Ethinyl estradiol	8.24	8.07	0.06	7.43	7.27	7.24	0.54	3.01
17β-Estradiol	8.40	8.36	0.07	7.94	7.99	8.36	1.06	0.38
Estrone	8.42	8.21	0.20	8.17	8.20	8.02	0.59	0.04
Meso-hexestrol	6.85	6.90	0.23	7.44	6.65	7.13	2.86	1.32
Bisphenol B	6.45	6.54	0.19	6.74	6.76	6.54	0.44	0.11
Coumestrol	7.86	7.83	0.19	7.36	7.10	7.82	0.54	0.32
5a-Dihydrotestos terone	7.92	7.77	0.05	6.96	7.02	8.27	2.89	0.13
17α-Estradiol	8.40	8.36	0.06	7.95	7.98	8.35	1.11	0.39
p-*n*-Nonylphenol	2.83	3.18	2.53	3.07	2.04	3.60	3.15	3.33
4-*tert*-Octylphenol	5.40	5.52	0.07	5.85	5.85	5.56	0.17	1.77
Butylbenzyl phthalate	5.29	5.34	1.20	4.90	4.92	4.46	2.97	5.46
Resveratrol	7.21	7.28	0.08	7.07	6.40	6.80	3.34	2.67
Methyl testosterone	8.37	8.18	0.37	7.82	7.09	7.21	3.07	0.38
Dexamethasone	6.56	7.15	7.21	6.02	6.68	5.96	0.75	1.23
Flavone	5.95	5.90	6.21	5.54	5.50	5.94	6.41	6.45
4-Hydroxytamoxifen	–	7.72	–	5.93	5.70	6.76	0.93	2.47
19-Nortestosterone	7.81	7.71	0.04	6.89	7.05	7.32	6.75	0.44
Progesterone	7.83	8.02	0.63	6.97	6.93	7.32	0.95	0.26
Tamoxifen	5.05	5.75	7.01	5.38	5.05	5.62	0.55	0.28
Testosterone	7.95	7.86	0.05	7.11	7.20	6.65	0.78	0.47
ER antagonist								
Flavone	5.31	5.32	3.46	6.45	6.47	5.08	6.70	6.71
Raloxifene	8.46	8.57	2.77	–	–	8.12	–	–
Tamoxifen	5.60	5.69	4.64	7.25	6.25	7.36	6.59	5.12
4-Hydroxytamoxifen	8.09	8.08	2.15	8.39	8.49	7.63	0.96	1.19
o,*p*′-DDT	5.60	5.59	0.03	6.34	6.46	5.83	1.28	1.28
Fenarimol	6.05	7.14	5.22	6.93	6.53	5.36	6.92	4.54
p-*n*-Nonylphenol	3.52	5.59	0.07	3.59	2.98	3.81	5.07	1.74
Resveratrol	6.93	7.14	4.40	6.48	6.45	7.08	1.66	7.22
Genistein	8.02	7.88	2.49	7.44	7.59	7.34	7.13	7.13

Table 3
Docking results of selected compounds in human templates and medaka models.

Compound name	Docking score for medaka ARα	Docking score for human AR	RMSD between medaka ARα and human ARα	Docking score to medaka ARβ	RMSD between medaka ARβ and Human ARβ
AR agonist					
5a-Dihydrotes tosterone	7.20	6.92	6.52	7.02	6.50
19-Nortestosterone	7.53	7.57	1.59	7.46	1.10
Testosterone	6.74	7.03	0.55	6.66	0.65
P,p′-DDE	5.19	4.68	2.26	4.96	3.79
17β-Estradiol	8.12	8.00	1.58	8.04	1.66
Estrone	7.26	7.98	6.52	7.20	6.52
Mifepristone	8.06	7.22	0.59	6.79	1.51
Methyl testosterone	6.40	6.62	1.61	6.42	1.57
AR antagonist					
p-n-Nonylphenol	3.11	2.90	2.26	4.14	2.70
Vinclozonlin	5.86	5.70	0.53	6.56	6.38
Bicalutamide	8.32	6.16	1.54	8.26	4.34
Cyproterone acetate	–	–	–	6.21	–
Hydroxyflutamide	6.94	5.70	6.89	7.27	6.73
Nilutamide	6.72	6.83	0.68	–	–
Spironolactone	–	–	–	6.06	–

The profile of 17α-estradiol docked into the medaka ERα agonist model and human crystal structure is shown in Fig. 2A and the detailed interaction site in Fig. 2A′. The docking structures and detailed binding sites of five other EDCs interacting with the medaka model and human templates are shown in Fig. 2B–F′. Taking 17α-estradiol (Fig. 2A′) as an example, all the key binding site residues (LEU79, ALA42, and GLY521) from human template (1QKU) are shown. Higher level structure superposition in the binding site between medaka and human was detected. The docking results show that all medaka models and human templates have higher similarity based upon the RMSD value or the docking geometry and the binding pocket.

Discussion

The ERs and ARs are ligand-activated transcription factors that participate in the regulation of many processes, including development and reproduction. Agonists cause ER and AR to adopt an activated state, whereas antagonists inhibit ligand-binding and cause the receptor to adopt an inactive state. Recent reports show that estrogen receptor is highly conserved between fish and human by sequence alignment and docking analysis [18,19]. Thus fish could be utilized as a model system to assist in drug screening as well as environmental detection of endocrine disruptors. In this study, we begin to examine medaka as a model organism to study endocrine disruption related to estrogen and androgen interactions with their receptor. Our results show that the only difference between the binding sites of medaka and human was minor replacement in the ligand-binding domain. For example, Leu was replaced by Met in the H5 region of the AR ligand-binding domain (Fig. 1). Although this is a relatively conservative substitution, it could effect binding of partial EDCs; however, for most AR ligands it is expected that they will bind in the same manner to both medaka and human AR. Our results suggest that the 10 3D models of medaka ER and AR show good overall conservation of structure with human templates.

The purpose of this work was to describe the molecular interactions between steroid receptors and some endocrine-disrupting pollutants. Most docking scores for selected EDCs are consistent with the relative binding affinity (RBA) data found in the review from ICCVAM [17]. Although, based on our docking analysis, we could not get the score value of the ER agonist 4-hydroxytamoxifen, or of the AR antagonists cyproterone acetate and spironolactone (Tables 2 and 3). The reason why there was a difference between the report data and our *silico* result may be that the background review concerning *in vitro* binding assays only emphasized the great variability of log (RBA). To get a deeper understanding of the difference, more thorough experimental data should be produced. Finally, the EDCs ligands did not display a unique mode of binding, probably due to their lipophilicity, flexibility and small volume, which conferred them a great adaptability in the hydrophobic and large binding pocket of steroid receptors [20].

The main regions of sequence difference between medaka models and human templates are found on several helices, for example, helices 3, 4, 10 in ER, and helices 2 and 10 in AR. These helices are either in a large loop or in an extended region of surface structure that is far from the binding site. Therefore, both of these variable regions are unlikely to play any direct role in binding EDCs. Although crystal structures for various members of the steroid hormone receptor class including ER and AR exist, no fully intact structure has yet been elucidated. However, the structures of the DNA binding domain (DBD) and ligand-binding domain (LBD) have been solved independently for some members [24]. The structural similarity among members of the steroid hormone receptor LBDs is well-known [25]. To understand the structure conservation relationship further, we constructed 10 medaka models based on existed human crystal structures (supplemental Fig. 3). The various helices of the receptors were differentially colored in order to provide contrast between helices and to allow intermolecular comparisons. It is apparent that each model and template exhibits the same triple-layered, helical sandwich, and that the spatial orientation of the helices was highly similar despite the small insertions in some regions revealed by initial alignment results (Fig. 1).

A wider exploration of the binding of ERs and ARs with other classes of EDC compounds needs detailed study in the future. Further, investigations of their binding properties at the molecular level using reproductive studies in medaka will provide useful information for toxicity prediction of compounds released into the environment, and also for the rational design and synthesis of new molecules with low impact on human health. Furthermore, our docking results (Tables 2 and 3) will be tested using *in vitro* binding assays with meERs and meARs. The ultimate goal of this research is to create a model system for studying reproductive biology and to use these data to construct three-dimensional

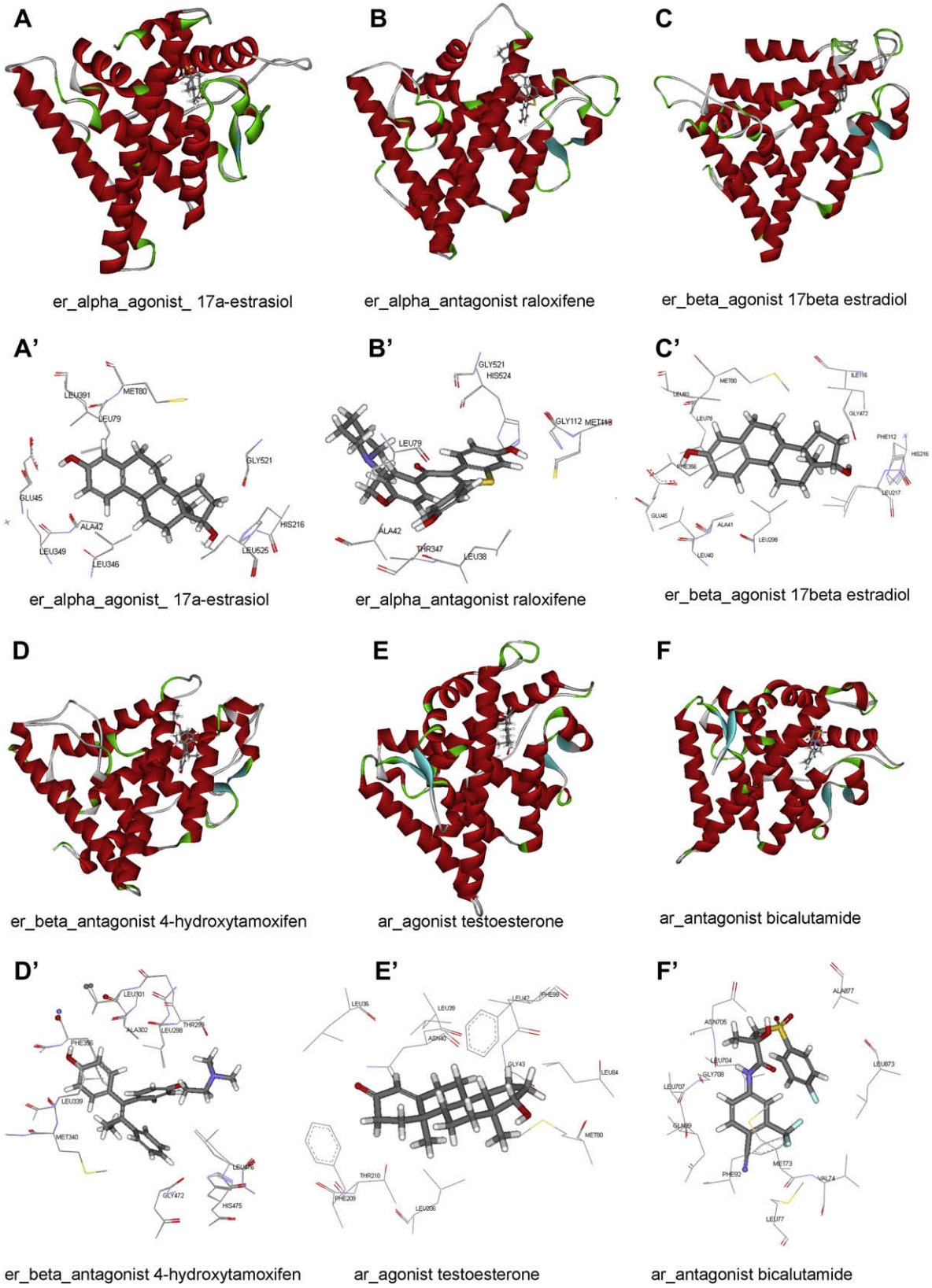

Fig. 2. *In silico* docking into medaka models compared with human structures. (A) Overlay of medaka ERα and human ERα by docking with 17α-estradiol. (A′) The detailed interaction relationship between binding residues and 17α-estradiol in medaka ERα model and human ERα template. (B–F′) The docking profile of Raloxifene, 17β-estradiol, 4-hydroxytamoxifen, testosterone, and bicalutamide in medaka and human and their detailed information in binding site.

quantitative structure–activity relationship models for predicting likely endocrine disruptor activity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.01.047.

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