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Identifying new liver X receptor alpha modulators and distinguishing between agonists and antagonists by crystal ligand pocket screening

Jia-Hau Lee^{‡,1,2}, Chien-Fu Huang^{‡,3}, Yi-Jing Chuang¹, Chang-Yin Lee^{4,5}, Wen-Hao Yu¹, Chin-Chung Wu^{2,6} & Ying-Ting Lin^{*,1,6}

¹Department of Biotechnology, College of Life Science, Kaohsiung Medical University, Kaohsiung, Taiwan

²Graduate Institute of Natural Products, College of Pharmacy, Kaohsiung Medical University, Kaohsiung, Taiwan

³Department of Biological Science & Technology, I-Shou University, Kaohsiung, Taiwan

⁴Department of Chinese Medicine, E-DA Cancer Hospital, Kaohsiung, Taiwan

⁵College of Medicine, The School of Chinese Medicine for Post Baccalaureate, I-Shou University, Kaohsiung, Taiwan

⁶Drug Development & Value Creation Research Center, Kaohsiung Medical University, Kaohsiung, Taiwan

*Author for correspondence: Tel.: +886 7322 1514; Fax: +886 7312 5339; ytlin@kmu.edu.tw

‡Authors contributed equally

Background: Modulators of LXR α are of high pharmacological interest as LXR α regulates fatty acid metabolism, inflammatory processes and cancer. We aim to identify new LXR α modulators and to recognize a distinguishable feature of agonists. **Results & methodology:** The ligand self-dock and largest-cavity-size searching purposely located two appropriate ligand-binding sites to reach the two aims. One is identifying the new modulators from Maybridge library. 20 new compounds are confirmed by the *in vitro* reporter gene assay. The other is denoting an agonist by at least one best docking pose having one hydrogen bond to LXR α Helix12 His421. **Conclusion:** Based on the quality x-ray binding pocket, we can identify new LXR α modulators and distinguish between agonists and antagonists by molecular docking.

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Liver X receptor alpha (LXR α) is one of the LXR subtypes belonging to the nuclear receptor superfamily. As a ligand-activated transcription factor, LXR α contains a highly conserved DNA-binding domain and ligand-binding domain (LBD), regulating the expression of genes through binding to the region of the so-called LXR response element of the downstream genes. LXR α participates in the regulation of lipid metabolism and inflammation, and also cancer. Nonalcoholic fatty liver disease is a common liver disease associated with LXR α [1,2]. LXR α also showed oncogenic properties in cancer cells, such as gastric and ovarian cancer [3]. Thus, LXR α can be an essential pharmaceutical target to alter the biological process for different diseases [4]. LXR α modulators, by their transcriptional activities, can be divided into agonists or antagonists. Depending on different expressions or organ heterogeneity of LXR α , the agonist in principle can relieve nonalcoholic fatty liver disease symptoms and the antagonist can be used to treat cancer. LXR α is an attractive target for its potential functions, and new LXR α modulators have a chance to be developed into new drugs [5,6]. To meet pharmaceutical demands, we need to identify more LXR α modulators and also to distinguish between the modulator types, especially for the early phase of drug discovery [7]. Here, we selected the appropriate crystal pockets as two binding site models for efficient molecular-docking screening to identify novel LXR α modulators and differentiate types of modulators.

To identify new LXR α modulators, we chose a suitable binding site model to conduct efficient structure-based molecular-docking screening. The molecular dockability [8,9] of the x-ray crystal ligand-binding pocket was examined by the self-docking of crystal complex ligands. If the ligand after self-docking can correctly find its crystal pose, it can indicate the dual facts that the native binding pocket and the methodology of molecular docking used are both reliable. The similarity between the best docking pose and experimental crystal pose is calculated using the root-mean-square deviation (RMSD) [10]. In primary molecular-docking screening, we also utilized two restrictions to reduce the number of purchased library compounds: consensus scoring [11] and Lipinski's rule [12]. The details of the methodology and procedure for efficient virtual screening are described in the Methods section. We screened 25 candidates and further analyzed them by LXR α transactivation luciferase assay [13]. 20 hits were classified as significantly either enhancing or reducing transactivation.

To differentiate types of modulators, we needed to choose another suitable binding site model to distinguish the agonism of the hits by structure-based molecular docking screening. According to the standard theory of nuclear receptor transactivation mechanisms, an agonist binding in the core of the LBD with contact to the AF2 function site induces a conformational change which can introduce a binding site for coactivators in a groove formed majorly by Helix12, leading to effective transactivation [14–16]. Molecular docking to see whether a hit can dock into the LBD and whether the hit can also show contact with LXR α Helix12 His421, which may denote its agonism. If not, lack of contact with His421 indicates antagonism. For this simulation, we used the largest binding pocket, which can accommodate the greatest variety of LXR α ligand structures. We found that the x-ray LXR α complex (Protein Data Bank [PDB] ID: 3FC6) has the largest ligand-binding pocket. The methods used to decipher the agonism of hits are described below. The docking poses of a hit tending to have the hydrogen bond binding to LXR α Helix12 His421 strongly imply that the compound is an agonist, while lack of binding implies an antagonist. The best agonist hit and the best antagonist hit in this study were demonstrated to qualitatively illustrate such features.

Appropriate crystal-binding pockets as models of ligand-binding sites are crucial for the success of molecular-docking simulations. Each binding cavity of the x-ray crystal LXR α complex can reflect a snapshot of a true dynamic ligand-binding pocket. Instead of computationally altering the pocket residues, we kept it frozen with the two selected x-ray coordinates of the ligand-binding environment for hit screening and to distinguish agonism. These two approaches may provide more efficient and more correct results for structure-based molecular modeling for both purposes.

Computational & experimental methods

Molecular docking

LigandFit [17] is a molecular docking method for accurately docking ligands into protein-binding sites and also for high-throughput hit screening. All molecular docking experiments using Discovery Studio 2.5 [18] were performed on an Asus computer with an Intel Core i7 4.00 GHz processor, running Windows 7. The energy grids of set binding sites can be further calculated by three different force fields, Dreiding [19], consistent force field (CFF) [20] and PLP1 [18] as implemented in LigandFit. Before molecular docking, the ligand is removed from the active site,

leaving a binding site model for molecular simulation. We added hydrogen atoms to LXR α receptors and their ligands according to the default values of the CHARMM force field [21]. DockScore [17] was then used to estimate the interaction energy between calculated ligand poses and the LXR α ligand-binding pocket. We used the same methodology and molecular docking procedure as a common base to inspect the quality of x-ray crystal-binding pockets [9]. Ligand self-docking is an important metric for evaluating docking methods [22,23] by correctly simulating the crystal ligand pose. We use self-docking to examine the intrinsic quality of x-ray binding sites since our docking method and procedure were the same. We emphasize the validation of the resulting RMSD for measuring the accuracy of the docking methodology and the quality of the x-ray ligand pocket. Ligand self-docking has the same procedure as mentioned [9].

Utility-based selection of x-ray binding sites

Binding site for molecular docking was defined by the occupied space of the cocrystal ligand, which was set by expanding the ligand hydrogen radius to 2.0 Å and heavy ligand atom to 2.5 Å (default settings) until reaching residues. We collected 19 x-ray crystal protein chains of human LXR α cocomplexed with ligands [14,24–29] deposited in the PDB (more than one decade) [30]. The same definition can also calculate the size of each binding site. The best x-ray binding site for new modulator screening was examined by the self-docking strategy, while the x-ray binding site for distinguishing agonism used the largest one.

New modulator screening & consensus scoring

To be as close to reality as possible, we set all the crystal protein residues as still. All crystal waters were also removed since the orientation of water is unknown. A maximum of ten poses was set to be saved after molecular docking. Then, for each docking pose, 11 scoring functions, LigScore1 [17], LigScore2 [17], DockScore [17], PMF [31], PMF04 [32], Ludi1 [33,34], Ludi2 [33,34], Ludi3 [35], PLP1 [18,36], PLP2 [18,36] and Jain [37], were used for consensus scoring. Consensus scoring was applied as follows: each library compound can have up to ten after-dock poses, and each pose has 11 scores. If a pose's score in one of the 11 scoring functions was within the top 6% of total library docking poses, by definition, the pose gained one consensus point. An in-house Ruby (www.ruby-lang.org) programming script for docking-pose-based consensus scoring was used to calculate consensus points.

Lipinski's rule of five

In drug discovery, desired molecules can fulfill particular criteria for further drug development. Lipinski's rule of five (ROV) [12] is a traditional way to address the drug-likeness of the organic compounds. ROV rules include no more than ten hydrogen bond acceptors, no more than five hydrogen bond donors, molecular weight (mass) lower than 500 Da and partition coefficient (AlogP) smaller than five. As improvements for the drug-likeness of organic compounds, Veber and coworkers [38] showed that the polar surface area (PSA) [39] and the number of rotatable bonds (RBs) can be discriminating molecular descriptors. Two additional criteria can be $PSA < 140 \text{ Å}^2$ and $RBs \leq 10$. Those compounds fulfilling Veber's rules are expected to have good oral availability. To further lower time and cost, we wanted to identify the best hits successfully passing ROV and the two additional criteria. The related molecular properties were calculated by the Discovery Studio 2.5 module for molecular properties.

Compound library

The Maybridge Screening collection, with >57,000 compounds, was the compound library used for screening. All Maybridge compounds were optimized within Pybel [40,41] using the MMFF94 forcefield until the structure's energy reached the RMSD gradient of 0.05 kcal/mol/Å [42]. The partial charges and the optimized structures of chemical compounds were automatically set and saved in mol file format for further hit screening of molecular docking.

LXR α transactivation luciferase assay

LXR α transactivation tests including Huh7 cells were used for the luciferase assay. Transfections were done in Huh7 cells using Lipofectamine Plus (Life Technologies, Inc., MD, USA) according to the manufacturer's instructions. In each transactivation assay, the following plasmids were involved: a reporter containing five Gal4 binding sites (0.2 µg), an expression plasmid for LXR α LBD fusion protein (0.6 µg) and 60 ng SV40-Relina plasmid. Pure compounds were added 12 h after cell transfection was done for 1 day. Cells were collected and lysed for luciferase assays following the manufacturer's instructions. L783483, being a pan-agonist for the nuclear receptors, was used

Table 1. List of 19 binding sites and root-mean-square deviation values of docked poses and crystal poses.

PDB ID (chain)	Resolution (Å)	Binding site size (Å ³)	RMSD (Å)		
			Dreiding	CFF	PLP1
1UHL (B)	2.90	416.75	0.92	1.02	2.73
2ACL (B)	2.80	368.75	0.73	0.78	0.79
2ACL (D)	2.80	336.38	0.82	0.87	0.69
2ACL (F)	2.80	376.50	0.84	0.71	1.18
2ACL (H)	2.80	373.25	0.59	0.60	0.59
3FAL (B)	2.36	369.38	0.82	2.55	2.07
3FAL (D)	2.36	393.13	2.57	2.57	2.05
3FC6 (B)	2.06	472.50	0.65	0.72	0.76
3FC6 (D)	2.06	473.88	0.50	0.57	0.66
3IPQ (A)	2.00	451.63	0.46	0.48	0.58
3IPS (A)	2.26	432.50	0.54	1.09	0.94
3IPS (B)	2.26	434.38	1.73	1.65	10.34
3IPU (A)	2.40	443.63	0.37	0.40	0.59
3IPU (B)	2.40	436.75	0.37	0.46	12.66
5AVI (A)	2.70	372.38	0.47	0.46	0.49
5AVI (C)	2.70	375.00	0.47	0.47	0.47
5AVL (A)	2.80	401.25	1.09	0.69	1.22
5HJS (A)	1.72	421.00	0.55	0.63	1.55
5HJS (B)	1.72	422.38	0.80	0.83	0.74
Average	2.42	409.02	0.80	0.92	2.16

All binding sites sizes and RMSD values were calculated after the ligand self-docking procedure. The best-docked poses for each crystal structure were spatially compared with their native binding sites. All the RMSD values were calculated with three energy grids of forcefield, including Dreiding, CFF and PLP1.

CFF: Consistent force field; PDB: Protein data bank; RMSD: Root-mean-square-deviation.

as an agonist standard. The LXR α ligand transactivation activities of 25 screened Maybridge compounds were tested with 10 μ M as the primary screening assay.

Results & discussions

Best x-ray binding site for molecular docking screening

The quality of the binding sites was our first concern for highly accurate molecular docking virtual screening for LXR α . X-ray crystallography can resolve high-resolution structures at the atomic level and structural details, but the intrinsic resolution of x-ray crystallography is still not enough for accurate structure-based drug design. Theoretically, a 2 Å deviation from reality at a critical binding-site residue can lead to failures in drug design, since the length of a single bond is <2 Å. We can see that the average resolution of the collected LXR α PDB complexes is 2.42 Å (Table 1, resolution column) [14,24–29]. Such a departure from reality, if it happens in the binding site, can severely decrease the accuracy of molecular docking, and thus, the efficiency of virtual screening. Therefore, a further inspection of the ‘dockable nature’ [8,9] of the ligand pockets of each crystal complex is essential for molecular docking. To raise our confidence for the crystal-binding pockets of LXR α for molecular docking, we used an additional strategy, self-docking, in which a geometry-randomized crystal ligand docks back into the natural crystal-binding cavity. The degree of regaining the ligand-bound pose is initially one of the central criteria for the correctness of the docking method [43,44], but here we used it to check the reliability and suitability of crystal-binding sites for hit screening. The shape overlap between the x-ray pose and the best ligand-docking pose was calculated by RMSD. The 19 results of self-docking are listed by the energy grids of the three different force fields, Dreiding, CFF and PLP1, in Table 1, where the RMSD ranged from 0.37 to 2.57 Å, from 0.40 to 2.57 Å and from 0.47 to 12.66 Å, respectively, as shown in the three violin distribution graphs (Figure 1A). We can see that better crystal protein resolution did not ensure lower RMSD values after self-docking. The best docking pose of the 3IPU ligand (Dreiding) was similar to the native bound crystal pose, with an RMSD of 0.37 Å (Figure 1B). Thus, the 3IPU ligand-binding site was considered to be the best x-ray binding site for the new molecular docking modulator screening of LXR α . Lastly, we stress here that all the protein residue side chains were not allowed to move to remain close to reality as possible, and also that crystal waters were also removed since the orientation of

Figure 1. Ligand self-docking. (A) The three violin distribution graphs show that all RMSD values of 19 liver X receptor alpha ligands ranged from 0.37 to 2.57 Å, from 0.40 to 2.57 Å and from 0.47 to 12.66 Å, on the energy grids of three different force fields, Dreiding, CFF and PLP1, respectively. All docked poses were compared spatially with their native crystal poses after the ligand self-docking. The smallest one is represented by the dark red spot. (B) The best spatial RMSD, 0.37 Å, occurring in the 3IPU crystal ligand-binding pocket, illustrated with zoom-out, middle and zoom-in pictures. The critical residue, His421, is shown. CFF: Consistent force field; RMSD: Root-mean-square-deviation.

water is unknown in x-ray crystallography. In this case, there is no water existing in this binding pocket. We believe that each x-ray binding site reflects a snapshot of dynamic ligand–pocket interactions. Thus, we left the selected x-ray coordinates unchanged, rather than computationally altering the ligand-binding surroundings of the pocket. So, in this study, we excluded the method of molecular dynamics.

Possible LXR α modulators by screening & consensus scoring

The best 25 hit compounds were purchased for *in vitro* reporter gene assays. The chemical structures are shown in Figure 2. Each hit was picked out by the following sequence: when the best x-ray binding site was located, we did molecular docking to each of >57,000 library compounds. This step generated about 167,000 docking poses with 11 scoring functions, of which 228 screening compounds had consensus points >7. Ligand–receptor binding may involve a variety of mathematical forms for noncovalent interactions, presenting differently in diverse ligand–receptor interactions [45–47]. As the literature shows [22,23], no single scoring function can handle all types of binding pockets equally well. To ensure finding the best hit, we did consensus scoring [45–47] for all docking poses. If the specific score for docking poses was within the top 6% of total docking poses it gained one consensus point. We took all docking poses as the total population rather than only the best docking poses from the library compound, and thus, the consensus scoring was based on docking poses.

Moreover, the selected compounds have different structures than the parent crystal ligand. Scaffold hopping [48] seemed to be easily achieved in this molecular docking screening. Note that we chose hits by the prediction of ligand–receptor interaction for our concept validation. However, to reduce the time and cost of the luciferase assay, we further considered the ROV, PSA and number of RBs for the possible drug development of these hits. The related ROV values are listed in Supplemental Table 1. At last, we purchased 25 compounds for the *in vitro* reporter gene assay.

Novel LXR α modulators by *in vitro* reporter gene assay

The best 25 hit compounds were evaluated by *in vitro* LXR α luciferase reporter gene assay. The luciferase activities for all hit structures are shown in Figure 3. Luciferase assays are a typical cell-based experimental method used to measure the transactivation activity of LXR α . The 25 hits and L783483 [49] were assayed in transfected Huh7 cells at 10 μ M. The control was the normal luciferase activity of the system when no compound was added. L783483 is a pan-agonist for nuclear receptors, here used as an agonist standard for comparison. The outcome of luciferase chemiluminescence reveals the degree of LXR α transactivation, showing whether a hit activates or inhibits it. More than half of 20 hits show a significant influence on LXR α transactivation. Then we further classified the identified

Figure 2. Chemical structures of compounds 1–25 were screened using the best selected 3IPU ligand-binding pocket. Significantly different structures other than the parent ligand are shown. Scaffold hopping is observed in these compounds. The values of Lipinski's rule of five, polar surface area and number of rotatable bonds were also calculated and listed in Supplementary Table 1.

hits into five groups. In group 1, six agonists (**1**, **9**, **11**, **12**, **22** and **24**) showed higher LXR α transactivation activity of at least 20% more than control. Notably, among group 1, the four agonists (**1**, **9**, **11** and **22**) had LXR α transactivation higher than or similar to that of L783483. Group 2 had the 14 antagonists (**2–8**, **13**, **14**, **16**, **19**, **20**, **23** and **25**), showing the LXR α transactivation activity less than 80% of blank control. We also noticed that two antagonists (**2** and **5**) showed significant antagonism less than 20% of control and most antagonists show less than 50% of control. Group 3 had five hits (**10**, **15**, **17**, **18** and **21**) showing no significant alteration of LXR α transactivation activity and were similar ($\leq \pm 20\%$) to blank control. Overall, 80% of our purchased LXR α modulators demonstrated molecular docking of selected x-ray binding sites, suggesting that this method could provide an extremely efficient way of virtual screening.

Distinguishing agonism

According to the recognized concepts of nuclear receptor transactivation mechanisms, a ligand is necessary to bind to the AF2 function site for the activation of a specific nuclear receptor [14–16]. In LXR α (as well as other nuclear receptors), we can also see that a full agonist always exhibits a feature whereby the head of the ligand structure body has a direct hydrogen bond link with the AF2 Helix12 His421 in x-ray crystallography images. We used this criterion to see if our molecular docking simulation could differentiate the agonism of a virtual screening hit. We

Figure 3. LXR α ligand transactivation activities of 25 screened compounds are shown as primary screening by luciferase assays.

C: Control was the normal luciferase activity of the system when no compound was added; L: L783483 is a pan-agonist for the nuclear receptors used as an agonist standard.

Figure 4. X-ray crystal ligand-binding sites. (A) A violin distribution graph showing all size values of 19 collected x-ray crystal ligand-binding sites ranging from 336.38 to 473.88 Å³. The largest is represented by the dark red spot. (B) The biggest crystal ligand-binding site located on the 3FC6.D protein crystal has pocket size, 473.88 Å³, illustrated by yellow dots, while the smallest binding site located on the 2ACL.D crystal is 336.38 Å³ in size, represented by the blue dots.

believe any computational alteration of the protein backbone structure may cause some amount of uncertainty. A specific binding site can be seen as a snapshot of the dynamic reality of LXR α complex. We tried to avoid unnecessary modifications to the original structure coordinates revealed by x-ray crystallography. To allow the maximum docking poses, we took the largest binding pocket, which may accommodate the greatest variety of LXR α ligand structures. The volumes of different binding sites, as well as the largest-size ligand-binding pocket (PDB ID: 3FC6), are listed in Table 1 (binding site columns) and illustrated in Figure 4A and B. Our aim was to see if hits could dock into the LBD and whether the hit furthered show a hydrogen bond link with 3FC6 LXR α AF2 His421 [14–16] or not. As a result of all simulation observations, the best antagonist (compound 5) and the best agonist (compound 11) in our study were taken as examples. The docking pose of compound 5 showed multiple definite binding interactions: a strong π – π interaction with Trp443 (head of the ligand) and hydrogen bonds with Asn225 and Arg305 (tail of ligand) as shown in Figure 5A. The docking pose of compound 11 also demonstrated multiple definite binding interactions: strong π –cation interactions with Arg305 (tail of ligand) and hydrogen bonds with Ser264 (body of ligand) and, most importantly, with His421 (head of the ligand) as shown in Figure 5B. By observing plentiful docking poses of nuclear receptor ligands including LXR α ligands, we can safely draw a qualitative conclusion that either a potent binding nuclear receptor agonist or antagonist tends to have multiple significant interactions in the binding site and that the distinguishing feature is that the full agonist must have the hydrogen bond binding to Helix12 His421. Furthermore, the particular structural features of the strong LXR α agonists are having strong hydrogen acceptors on the heads of agonists, which can fit spatially into

Figure 4. X-ray crystal ligand-binding sites (cont.). (A) A violin distribution graph showing all size values of 19 collected x-ray crystal ligand-binding sites ranging from 336.38 to 473.88 Å³. The largest is represented by the dark red spot. (B) The biggest crystal ligand-binding site located on the 3FC6.D protein crystal has pocket size, 473.88 Å³, illustrated by yellow dots, while the smallest binding site located on the 2ACL.D crystal is 336.38 Å³ in size, represented by the blue dots.

Figure 5. Analysis of the docking poses of compound 5 and 11. (A) Docking pose of compound 5 shows multiple definite binding interactions: a strong π - π interaction with Trp443 (head of the ligand) and hydrogen bonds with Asn225 and Arg305 (tail of the ligand). (B) Docking pose of compound 11 also shows multiple definite binding interactions: strong π -cation interactions with Arg305 (tail of the ligand) and hydrogen bonds with Ser264 (body of the ligand) and, most importantly, a hydrogen bond with His421 (head of the ligand).

the Helix12 His421 vicinity surroundings. For example, the trifluoromethyl group in Y1, the carbonyl group in Y9 and Y22 and the carboxylic acid in Y11 serve as such hydrogen acceptors. In the molecular docking simulation, the greater frequency of this hydrogen bond link in hit docking poses indicates that the hit is a full agonist (a strong binder with Helix12 His421). In contrast, in a strong binding antagonist hit it is hard to see any such hydrogen bond link in any docking pose (a strong binder with no Helix12 His421 link).

Conclusion

Modulators of LXR α are of high pharmacological interests as LXR α regulates fatty acid metabolism, inflammatory processes and even related to cancer. Based on the suitably selected experimental native binding sites of x-ray receptor complexes for molecular docking, we aim both to identify the new LXR α modulators and to recognize

the distinguishable feature between agonists and antagonists. Our two approaches, ligand self-dock and largest-cavity-size searching, purposely located two appropriate ligand-binding site models to reach the following two aims: for identifying new modulators, after sorting all the docking poses of a chemical library of 57,000 compounds, out of 25 purchased compounds, *in vitro* reporter gene assay confirmed up to 20 agonists or antagonists; and for distinguishing types of the modulators, the results of docking pose indicates a feature that at least one pose, out of ten poses, having one hydrogen bond to LXR α Helix12 His421 denotes its agonism. Thus, the main structural features for the LXR α agonists are having strong hydrogen acceptors on the heads of agonists fitting to the Helix12 His421 vicinity surroundings. Overall, the utility-based selections of x-ray binding sites showed that molecular docking not only can screen new active modulators but also can differentiate between agonism and antagonism.

Future perspective

In the future, new LXR α modulators will have a chance to be developed into new drugs. Appropriate crystal-binding pockets as models of ligand-binding sites are important for the success of molecular docking simulations. Each binding cavity of the x-ray crystal LXR α complex can reflect only a snapshot of a true dynamic ligand-binding pocket. The two selected x-ray coordinates of the ligand-binding environment were used for hit screening and to distinguish agonism. These two approaches may provide more efficient and more correct results for structure-based molecular modeling for both purposes. The full agonist must have the hydrogen bond binding to Helix12 His421, is also an important judgment for virtual LXR α modulators development. We conclude that computationally assessed qualities of crystal pockets are an important checkpoint that can help discover structurally novel nuclear receptor modulators and cost less.

Summary points

***In silico* approaches of molecular docking screening**

- We can see that better crystal protein resolution did not ensure lower root-mean-square-deviation values after self-docking.
- 3IPU ligand-binding site was considered to be the best x-ray binding site for the new molecular docking modulator screening of liver X receptor alpha (LXR α).

***In vitro* reporter gene assay**

- In *in vitro* reporter gene assay, the 20 agonists or antagonists were confirmed.
- Four agonists (1, 9, 11 and 22) had LXR α transactivation higher than or similar to the transactivation of L783483.
- Two antagonists (2 and 5) showed significant antagonism less than 20% more than control.

Distinguishing agonism

- To allow the maximum docking poses, we took the largest binding pocket, which may accommodate the greatest variety of LXR α ligand structures.
- 3FC6 has the largest-size ligand-binding pocket, might be a fit model to distinguish agonist and antagonist.
- We can safely draw a qualitative conclusion that the agonist must have the hydrogen bond binding to Helix12 His42.
- The main structural features for the LXR α agonists are having strong hydrogen acceptors on the heads of agonists.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.4155/fmc-2020-0069

Financial & competing interests disclosure

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