RESEARCH ARTICLE

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Cite this: *Med. Chem. Commun.*,
2018, 9, 1630

Discovery of new FXR agonists based on 6-ECDCA binding properties by virtual screening and molecular docking

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FXR is a member of the nuclear receptor superfamily, which regulates the expression of various genes involved in bile acid, lipid and glucose metabolism. Targeting FXR with small molecules has been exploited to treat lipid-related disorders and diseases such as cholestasis, gallstones and hepatic disorders. In this work, we expand the existing pool of known FXR agonists using a fast hit-to-lead structure-based pharmacophore and docking screening protocol. A set of 25 molecules was selected after screening a large database of commercial chemicals, and experimental tests were carried out to demonstrate their ability to activate FXR. Three novel FXR agonists are reported, namely, one full agonist, more efficient than the endogenous ligand chenodeoxycholic acid, and two partial agonists.

Received 28th May 2018,
Accepted 28th June 2018

DOI: 10.1039/c8md00272j

rsc.li/medchemcomm

Introduction

The farnesoid X receptor (FXR) has been identified as a nuclear receptor for bile acids, which could regulate lipid and glucose metabolism through modulation of the gut–liver–adipose axis, along with other nuclear receptors, such as the peroxisome proliferator activated receptor (PPAR) and liver X receptor (LXR).¹ In recent years, many modulators of these receptors have been studied, with the aim of obtaining new lead compounds to be used in a multitude of pathological conditions, ranging from metabolic diseases to cancer.²

In mammals, FXR is predominantly expressed in the liver, kidney, and intestine, with lower expression in the heart, lung and spleen. This nuclear receptor is activated by bile acids (BAs), like chenodeoxycholic acid (CDCA) and cholic acid (CA), which are the final products of cholesterol metabolism.³ FXR binds to DNA either as a monomer or as a heterodimer with the retinoid X receptor (RXR), regulating the expression of genes involved in bile acid homeostasis. Recent studies also demonstrated the effects of BAs, through the activation of FXR, on lipoprotein and glucose metabolism and inflammatory responses.⁴ Multiple preclinical and clinical trials suggest that FXR agonists are an attractive treatment option for different metabolic and chronic liver diseases, such

as cholestasis, gallstones and hepatic disorders.⁵ FXR is also under discussion as a potential target for novel pharmacotherapies that address liver and intestine cancer.⁶

In the past two decades, several activating compounds of FXR were discovered, and efforts have been made to improve the potency and efficacy of the agonists, for pharmacological applications.⁷ Some fine examples include: fexaramine,⁸ GW4064,⁹ MFA-1 (ref. 10), XL335,¹¹ and 6 α -ethyl-chenodeoxycholic acid (6-ECDCA)¹² (Fig. 1). At the moment, only 6-ECDCA, also named obeticholic acid, a synthetic CDCA analogue about 100-fold more potent than CDCA, is approved by the FDA for the treatment of patients with primary biliary cirrhosis (PBC).¹² Furthermore, clinical studies suggest that 6-ECDCA possesses beneficial effects in the treatment of non-alcoholic steatohepatitis (NASH).¹³

The identification of some partial or full agonists of FXR has been accomplished by means of virtual screening techniques; for example, pyrazolidine-3,5-dione derivatives,¹⁴ 1-(4-methylpiperazin-1-yl)-3-phenoxypropan-2-ols,¹⁵ pyrazole-[3,4-*e*][1,4]thiazepin-7-ones,¹⁶ 3-amino-imidazo[1,2-*a*]pyridine derivatives¹⁷ and anthranilic acid derivatives¹⁸ were selected from chemical libraries of compounds (Fig. 2).

Here, we report the use of 6-ECDCA, a potent selective FXR agonist, to create four pharmacophore models employed in the virtual screening of the CoCoCo database composed of 7m compounds,¹⁹ which mostly contains compounds of synthetic origin with unknown biological characterization, for the discovery of new FXR agonists. Thus, we have integrated the concept of fast hit-to-lead discovery with a combined multi-query pharmacophore and molecular docking protocol, in order to

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Fig. 1 Structures of FXR activating compounds.

Fig. 2 Structures of FXR ligands identified by virtual screening.

expand the chemical space of the existing FXR agonists, aiming to deliver novel scaffolds with distinctive chemical properties.

Results and discussion

In silico screening

The identification of new scaffolds able to modulate FXR through an *in silico* screening was realized using the following workflow: i) a pharmacophore search, based on the active agonist 6-ECDCA co-crystallized in the FXR ligand binding domain (LBD), ii) a pharmacophoric virtual screening, using the obtained four pharmacophores against the CoCoCo database, and iii) a molecular docking protocol, resulting in the

selection of 25 compounds as potential agonists of FXR. This method was chosen to compensate for potential issues regarding the modelling of the induced fit of FXR upon binding of ligands, due to the described structural flexibility of the FXR LBD.²⁰ Indeed, it is commonly accepted that at the early-stage of new chemical scaffold identification, induced fit calculations are unfeasible with large chemical databases.

Pharmacophore modeling

The use of multiple pharmacophores has been demonstrated to be useful in a number of cases. For instance, Schuster *et al.* reported a work where six pharmacophores were helpful

to filter the ChEMBL database to discover new FXR agonists;¹⁵ in the multiple pharmacophore approach performed by Grienke *et al.*,²¹ two pharmacophore hypotheses from two different initial protein structures were used, FXR bound with fexaramine and MFA-1; meanwhile Achenbach *et al.*¹⁷ described the use of two clusters of superimposed FXR-agonist complexes based on GW4064 and benzimidazoles. In our study, the crystal structure of the rat FXR bound to the active agonist 6-ECDCA was selected to generate four pharmacophores, representing the chemical features of the 6-ECDCA binding mode. The pharmacophores created in this work shared only a few features in comparison with previous works, purporting the idea that the use of multiple pharmacophores extracted from the 1OSV structure, together with the screening of a large database of commercial chemicals, can be a new way to uncover novel FXR agonist scaffolds.

To mimic native steroidal ligand binding, we relied on the use of only one structure representing a real binding mode for an agonist to FXR for the pharmacophore design, overcoming possible glitches when using very different structures to create an average pharmacophore that might not represent a real binding mode for an FXR agonist. We selected the crystal structure of the rat FXR bound to the active agonist 6-ECDCA, in the Protein Data Bank (PDB) with a resolution of 2.50 Å (PDB ID: 1OSV).²² This crystallographic structure was selected because it possesses the important residues and for the higher resolution of FXR-6ECDCA available structures. To confirm that the selected crystallographic template was suitable to infer the pharmacophore hypothesis, we analyzed the rat FXR by comparing it with entries from the PDB of the human FXR. We found that all residues involved in the binding of agonists in the rat FXR are identical and nicely superimposed to their human counterparts (data not shown). Additionally, a BLAST search of the FASTA rat sequence from the 1OSV reveals a 98% sequence similarity and 94% sequence identity with the human FXR, supporting the use of the rat FXR structure to perform the virtual screening studies.²³ The crystal structure of the rat FXR bound to 6-ECDCA presents: i) a negative charge feature representative of the 6-ECDCA carboxylate group in interaction with R328; ii) three hydrophobic features, two of which concern the steroid part of 6-ECDCA and one the ethyl group; iii) two possibilities for hydrogen bond features regarding the two hydroxyl groups of 6-ECDCA. For the latter, the possible combinations led to the creation of the four pharmacophore features, to cover representative ligand–protein interaction patterns: two hydrogen bond donors, two hydrogen bond acceptors, one donor and one acceptor, and *vice versa* (Fig. 3). Thus, the differences among the four pharmacophores are in the positions of the hydroxyl groups of 6-ECDCA that may form hydrogen bond acceptor and/or donor interactions with FXR.

Pharmacophoric screening

The generated pharmacophore models were used to search for FXR active compounds in the CoCoCo database.¹⁹ The

Fig. 3 Graphical depiction of the pharmacophore queries used to perform *in silico* screening. Pharmacophore features are color-coded as follows: green (H2), hydrophobic feature; blue (D2), hydrogen bond donor; light red (A2), hydrogen bond acceptor; dark red (N2), negative charge. Hypotheses were generated with a structure-based approach starting from the crystallographic structure PDB ID: 1OSV.

CoCoCo database was used as each chemical structure has pre-calculated conformers which greatly increase the speed of pharmacophore screenings. It is composed of 7 million molecules from different chemical vendors, consistent with the intention of broadening the chemical space of the new FXR agonists. The ready-to-use version CoCoCo-Phase was selected due to its compatibility with the used software, Phase. The pharmacophore screenings performed a filtering of the database, narrowing it down from its initial 7 million compounds to about 20 thousand hit molecules, representative of different chemotypes, bearing the chemical features of 6-ECDCA, which are essential for the binding to the LBD of FXR.

Molecular docking

It is well-known that the combination of different computational techniques may improve the discovery of bioactive compounds in virtual screening campaigns.²⁴ To refine the pharmacophore screening results by means of the 1OSV crystallographic structure of FXR, we took advantage of another *in silico* technique, structure-based molecular docking. Therefore, the pharmacophore filtered subset was subjected to a docking screening to the FXR structure 1OSV using Glide software.²⁵ The ligand–protein complexes resulting from the docking protocol were ranked according to their docking score. The first one thousand compounds were visually inspected by taking into account several structural and physicochemical characteristics, such as stereochemistry complexity, availability for ordering, chemical diversity, versatility of the chemical scaffold toward putative chemical optimizations and fitness within the LBD of FXR. As a matter of fact, a particular advantage of using molecular docking techniques is the possibility to analyze the binding modes of the best ranked molecules by dissecting their

ligand–receptor interactions as depicted below. This combined *in silico* workflow led to the selection of 25 molecules

(1–25, Fig. 4) that were purchased and tested *in vitro* for their ability to activate FXR.

Fig. 4 Selected 25 molecules by the *in silico* workflow.

Fig. 5 Efficacy values of FXR agonistic activity for selected compounds 1–25. The compounds were tested in at least two separate experiments at 100 μ M. Luciferase activity was determined as fold activation relative to untreated cells. The results are expressed with $\pm$ SEM. Efficacies are relative to CDCA (10 μ M) set at 100%.

FXR luciferase reporting assay

Compounds 1–25 were screened for *in vitro* FXR activity by means of a transient transfection assay using human embryonic kidney 293 (HEK293) cells, co-transfected with the expression plasmid for full-length FXR and FXRE-driven luciferase reporter plasmid.²⁶ The CDCA, a physiological FXR ligand with micromolar activity, was used as the reference compound.^{12a} In order to obtain preliminary indications of the effect of the selected compounds on FXR, they were first tested at a concentration of 100 μ M, and their activation efficacy values were calculated as a percentage of the maximum fold induction obtained with the endogenous reference ligand CDCA (Fig. 5). When no FXR plasmid was transfected in the HEK293 cells, no activity of the compounds was revealed.

Among the 25 selected molecules, compounds 2 and 5 presented good efficacy values (55.1% and 60.3%, respectively), although lower than that of CDCA. Compound 10 was the molecule with the best agonistic behavior for FXR amongst the test set, with an efficacy superior to that of CDCA (120%). All the other compounds did not demonstrate sufficient activation of FXR and were not considered for further investigations.

Compounds 2, 5, and 10 were then investigated by carrying out dose-response studies at seven concentrations between 0.1 and 250 μ M (Fig. 6). This range of concentrations did not reduce the Renilla luminescence indicating that no global impact on the cell or cell population was achieved. The three compounds demonstrated a concentration-dependent agonistic activity with EC_{50} values of 5.99 μ M (compound 2), 14.40 μ M (compound 5), and 4.33 μ M (compound 10). The best EC_{50} , similar to that of CDCA (7.01), was achieved by compounds 10 and 2.

Analysis of ligand–receptor interactions

The binding poses obtained from molecular docking calculations can be conveniently used to explain the interactions

that, at the molecular level, occur between a ligand and FXR, leading to a possible rationalization of the experimental results (Fig. 7).

For compound 2, the binding pose suggests the presence of three hydrogen bonds between this ligand and the LBD. The molecule forms two charge-assisted hydrogen bonds with Y358 and H444 side chains and an additional hydrogen bond with the side chain of R328. It is worth noting that such interactions have been identified in other previous works as key role players for the binding of FXR.²⁷ In particular, Mi *et al.* have reported that H444 is a critical residue for FXR activation²² suggesting that a π -cation activation trigger, between the indole ring of W456 and $N\epsilon$ (cation) of the H444 side chain, is the possible mechanism for activating FXR. As a result, the interaction of the agonists with H444 and Y358 leads to the preservation of the characteristic perpendicularity of the π -cation interaction between H444 and W456, possibly resulting in the FXR activation. Furthermore, with Y358 being close to H444, a further hydrogen bond with this tyrosine stabilizes the interaction with H444.

The docking pose of compound 5 shows the presence of two hydrogen bonds between the amidic carbonyl group and the side chains of Y358 and H444. The heterocyclic nitrogen forms an additional hydrogen bond with S329, which may assist the stabilization of compound 5 in the LBD. For this compound, there is a good match between its overall hydrophobicity and the FXR ligand-binding cavity.

Compound 10 showed the most relevant bioactivity in the luciferase assay as the results indicate: a potent agonistic activity with an activation efficacy of 120% compared to that of CDCA and an EC_{50} of 4.33 μ M. Similar to compounds 2 and 5, the binding pose of compound 10 emphasizes two hydrogen bonds between the carbonyl group and the side chain of H444 and Y358 that may explain the efficacy and potency of this compound. Another interaction is revealed with S329, which may stabilize the ligand–receptor complex. Finally, the binding pose of compound 10 shows a charge-assisted hydrogen bond interaction between its aryl-nitro group and the

Fig. 6 Dose-response curve for selected FXR activators. The compounds were tested in at least three separate experiments at five concentrations ranging from 0.1 to 250 μM . The results are expressed with $\pm$ SEM.

Fig. 7 Binding modes and main ligand-receptor interactions of three active agonists of FXR in the LBD.

guanidine side chain of R328. Overall, compound **10** is capable of forming a total of four hydrogen bonds and hydrophobic interactions within the LBD of FXR, matching the superior behavior of this compound, a putative new lead for FXR agonism. Notably, induced fit docking (IFD), performed with hit compounds **2**, **5**, and **10**, recapitulated the binding pose consistently with the structure-based pharmacophore hypothesis depicted in Fig. 3.

Experimental

Computational details

The crystal structure of the nuclear receptor FXR bound to 6-ECDCA (PDB ID: 1OSV)²² was minimized and used to create four structure-based pharmacophores with excluded volumes, applying the out of the box parameters of the software LigandScout v3 (Inte:Ligand). The CoCoCo-Phase database was used to carry out the pharmacophore-based virtual

screening, and Phase v3.1211 (Schrodinger) was used to perform ligand searches matching at least 5 of the 6 pharmacophore features. The molecules that suited the pharmacophore filtering were subsequently subjected to a standard precision (SP) molecular docking protocol with the software Glide v5.5211 (Schrodinger). To this purpose, using the above-mentioned crystal structure, we generated a docking grid centered on the 6-ECDCA ligand. The ligand-protein complexes resulting from the docking protocol were ranked according to their docking score and visually inspected. This resulted in a set of 25 molecules that were purchased in milligram quantities from commercial vendors to test their bioactivity on FXR. IFD was performed using the Schrodinger suite with standard settings.

Commercial compounds

All compounds tested for FXR activation were purchased from Asinex (ASN) and Enamine (ENM) with a purity of

≥95%, as declared by commercial vendors. The list of compounds with their docking scores (DS) (kcal mol⁻¹) is: 1 ASN BAS 03018860, DS -9905 141; 2 ASN BAS 07129816, DS -9418 322; 3 ASN BAS 01077577, DS -9598 352; 4 ENM T6266234, DS -11085 264; 5 ENM T6237087, DS -10302 330; 6 ENM T6426266, DS -10252 510; 7 ENM T6319025, DS -10248 960; 8 ENM T5930951, DS -10231-786; 9 ENM T6027461, DS -10140 257; 10 ENM T5918102, DS -10108 596; 11 ENM T6108664, DS -9863 298; 12 ENM T5667291, DS -9853 707; 13 ENM T0505-6433, DS -9834 118; 14 ENM T6145973, DS -9778 985; 15 ENM T6242557, DS -9720 763; 16 ENM T5361198, DS -9555 945; 17 ENM T6381717, DS -9540 497; 18 ENM T6026297, DS -9467 805; 19 ENM T6064014, DS -9466 063; 20 ENM T0511-4680, DS -9454 446; 21 ENM T5618005, DS -9384 883; 22 ENM T5599235, DS -9369 795; 23 ENM T6418727, DS -9314 839; 24 ENM T5417516, DS -9290 494; 25 ENM T6102438, DS -9213 162.

Cell culture and co-transfection assay

HEK293 cells were cultured in Dulbecco's Modified Eagle's medium (Sigma) with 10% fetal bovine serum (Gibco), 1% penicillin/streptomycin (Sigma), 1% sodium pyruvate (Sigma) and 1% nonessential amino acids (Sigma). Cells were grown at 37 °C in a humidified atmosphere of 5% CO₂ in air. The day before the experiment, HEK293 cells were seeded in 96-well plates to give a confluence of 50–80% at transfection. The following day, the medium was replaced with fresh serum-free medium and the cells were co-transfected by the calcium phosphate co-precipitation method with the reporter vector TK-FXRE-luc (which contains the firefly luciferase gene under control of FXRE), an expression vector encoding full-length FXR and an expression vector encoding Renilla. The test compounds were added 6 h after transfection, the cells were harvested 18 h after the treatment, and the firefly and Renilla luciferase activities were assayed using the Dual-Glo™ Luciferase Assay System (Promega) according to the manufacturer's protocol. Luminescence was measured with a microplate luminometer (Labsystem Ascent Luminoskan Reader). Luciferase data were normalized to the internal Renilla control. Each reported value is the average of triplicate assays. DNA co-transfection experiments were done using 50 ng of the reporter plasmid, 20 ng of the Renilla luciferase normalization vector, 30 ng of the receptor expression plasmid, and pGEM carrier DNA to make a total of 140 ng DNA per well in a 96-well plate. Luciferase activity was determined as fold activation relative to untreated cells. It is worth noting that, since the assay utilizes a dual reporter system in which firefly and Renilla luciferases are used as co-reporters, they provide an internal control to normalize the results. The test compounds in the range of concentrations 0.1–250 μM did not reduce the Renilla luminescence indicating that the test compounds do not determine the global impact on the cell or cell population (*i.e.*, cell death, inhibition of cell growth).

Conclusions

In this work, four pharmacophore hypotheses were able to solve computational difficulties that arise when complicated pharmacophore feature combinations are used. By comparison, the pharmacophores created in this work shared only a few features with previous works, purporting the idea that the use of multiple pharmacophores extracted from the 1OSV structure, together with the screening of a large database of commercial chemicals, can be a new way to uncover novel FXR agonist scaffolds. These results show that the workflow allowed the identification of three agonists of FXR, compound 10, with 120.5% efficacy and an EC₅₀ of 4.40 μM, compounds 2 with 55.1% efficacy and an EC₅₀ of 5.89 μM, and 5, with 60.3% efficacy and an EC₅₀ of 14.94 μM.

Abbreviations

FXR	Farnesoid X receptor
PPAR	Peroxisome proliferator activated receptor
LXR	Liver X receptor
CDCA	Chenodeoxycholic acid
CA	Cholic acid
RXR	Retinoid X receptor
6-ECDC	6α-Ethyl-chenodeoxycholic acid
PBC	Primary biliary cirrhosis
NASH	Nonalcoholic steatohepatitis
LBD	Ligand binding domain
IFD	Induced fit docking
LBD	Ligand binding domain
PDB	Protein data bank
HEK293	Human embryonic kidney 293 cells

Conflicts of interest

The authors declare no competing interests.

Acknowledgements

The study was supported by FAR funds from the Ministry of Education, University and Research (MIUR) of Italy. A. J. M. Barbosa was supported by the Post-Doc fellowship SFRH/BPD/112543/2015, FCT/MCTES, Portugal. Thanks are due to Dr. Alberto Del Rio for helpful discussions and to prof. Antonio Moschetta for the FXR plasmids.

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