Review Article

A chemogenomics based approach for deorphanization of testicular receptor 4: An orphan receptor of nuclear receptor superfamily

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Abstract

Orphan Receptor of Nuclear Receptor superfamily is the one with no known endogenous ligands. Many of these orphan receptors are associated with different types of diseases and therefore deserve special attention to find the potential ligands they would be associated with. The major task of molecular pharmacology is the deorphanization of the large number of nuclear receptors with unidentified endogenous agonists. The deorphanization provides a promising research for new therapeutics. The Testicular Receptor 4 being negative modulator to other members of the nuclear receptor superfamily, is one of the Orphan members of this family and is associated with prostate cancer, breast cancer, sickle cell anemia and joint diseases. The knowledge that related receptors of the same family often have ligands with similar structural features has helped us to utilize the chemogenomic approach to deorphanize the orphan receptor. *Chemogenomics* approach involves screening of known ligands of a protein family having analogous domain architecture for identification of new leads for existing protein family members. The deorphanization involved the database homology searching, followed by domain identification, active site prediction, sequence and structure comparative studies. A ligand library set was prepared based on these studies and was used to deorphanize the receptor. The molecular docking study conducted using PyRx revealed that estradiol and tretinion as a potential ligand for Testicular Receptor 4.

Key words: Chemogenomics, deorphanization, estradiol, orphan nuclear receptor, testicular receptor 4, tretinion

INTRODUCTION

Proteins of the Nuclear Receptor Superfamily are the transcription factors involved in many physiological, as well as in pathological processes.[1,2] They mediate transcriptional responses to hormones and other metabolic ligands through co-activators and co-repressors activity. The ligand induced transcription involves recruitment of co-activators complexes whereas orphan nuclear receptor involves recruitment of co-repressor complexes. The members of the nuclear receptor superfamily can be grouped into 3 broad categories based on the involvement of the ligands in their activity.[3-5] They are endocrine receptors (with identified endogenous ligands), adopted receptors (with ligands identified in recent years) and orphan receptors (with no known ligands identified till now).[6] Since many of these orphan receptors are associated with variety of physiological and pathological processes, identification of potential ligands would be of great therapeutic value.

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The process of finding the new ligands for the orphan receptors is termed as Deorphanization. Many methods are available for deorphanization like cell based assay[7,8]; direct binding method followed by mass spectroscopy[9] and bioinformatics[10] etc., Computational techniques has helped to speed up the deorphanization studies since many tools are available - to model the tertiary structure for which no RCSB-PDB data is available, servers which help in sequence and structure comparative studies, tools for active site comparison studies and the virtual screening using molecular compound libraries has enabled large number of compounds to be screened with good accuracy and speed.[8]

Among more than 20 orphan receptors of the nuclear receptor superfamily, the Testicular Receptor 4 (TR4) also known as *NR2C2* (nuclear receptor subfamily 2, group C, member 2) is an orphan receptor encoded by the *NR2C2* gene in humans [http://www.iuphar-db.org]. This family also involves hepatocyte nuclear factors (HNFs), Retinoic X receptors (RXRs), Tailless like receptors and Coup TF like receptors.[11] It functions as negative modulators of Androgen Receptor (AR) in prostate, Estrogen Receptor (ER) α in the mammary gland or Peroxisome proliferator Activated Receptor (PPAR) α in keratinocytes and RXR.[12] TR4 is ubiquitous and expressed in tissues in all major physiological systems central nervous system (CNS), endocrine, metabolic, gastrointestinal, immune, reproductive, cardiovascular, respiratory and structural with particularly high levels in the cerebrum, kidney, thymus and testis[13] [http://www.nursa.org]. Recent gene knockout studies have reported its importance in reproduction, coordination and in Animal behavior.[14-16] The TR4 is being associated with diseases like prostate cancer, breast cancer, sickle cell anemia and joint diseases.[17,18].

Here in our study, we have used a combinatorial approach by integrating bioinformatics with Chemogenomics to deorphanize the orphan TR4. Chemogenomics involves the investigating ligand libraries against families of functionally related proteins. This approach is reliable, faster and quicker when compared to cell based assay, reverse pharmacology and mass spectroscopic methods. Chemogenomics is a novel method to identify drug to a target by combining the genomics with chemistry.[9,19,20]

MATERIALS AND METHODS

The overall methodology followed can be summarized as in the Figure 1.

Retrieval of sequence and structure data for TR4

The protein sequence of the testicular receptor 4 (TR4) was retrieved from the SWISS-PROT database with ID P49116. The structure of TR4 was retrieved from RCSB PDB database with ID 3P0U_A.

Database homology searching

The sequence retrieved was then used to search for sequence homologs against SWISS-PROT database using blastp. The structure homologs were searched using PDB 3D similarity search.

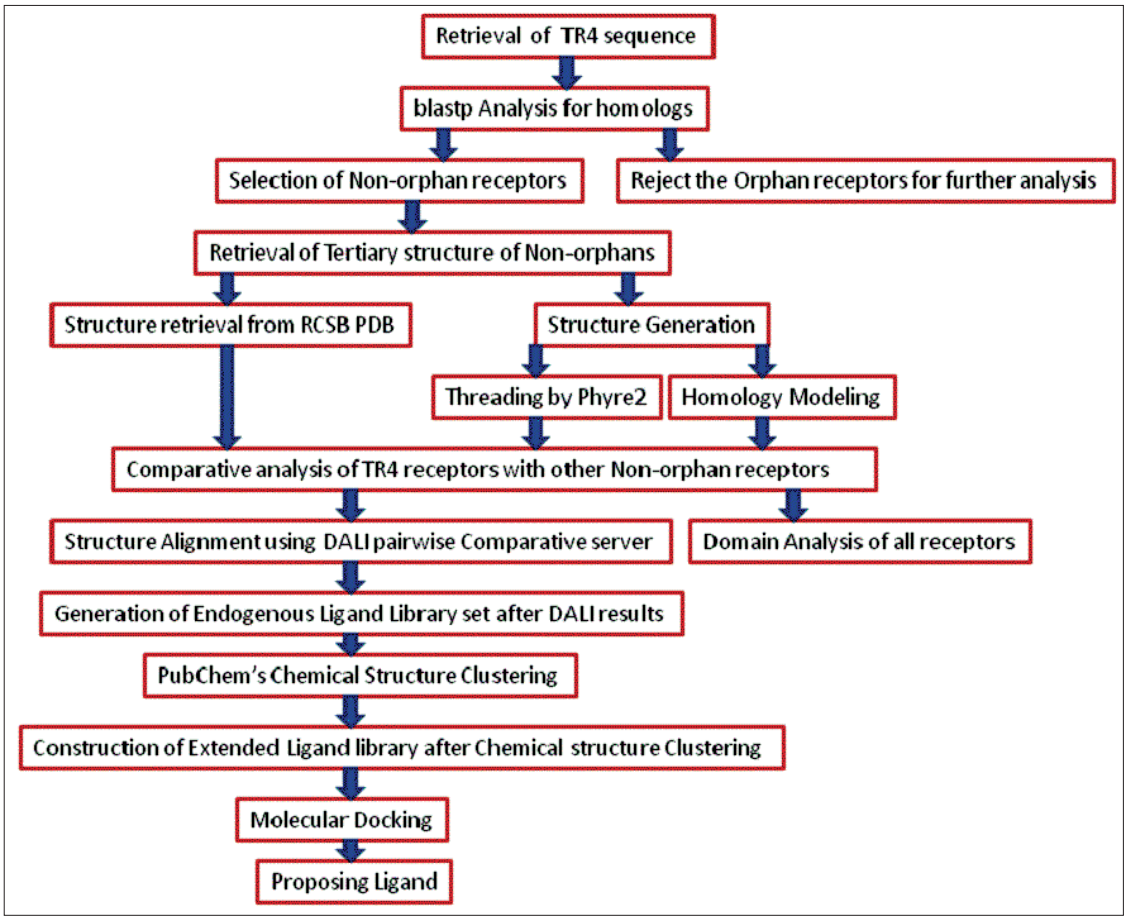


Figure 1: Overall methodology followed for the Deorphanization of Testicular Receptor 4

Selection of homolog receptor sets

The receptors which had sequence similarity >65% and had endogenous ligands were taken for further analysis. Similar procedure was followed for structure homologs too. Totally 30 receptors were obtained in both sequence and structure homolog search.

Structure retrieval of homologs receptor sets

The structures of all the receptors which had endogenous ligands were retrieved from RCSB PDB database. At the ends of this step out of 30 only 20 receptors with endogenous ligands were listed since orphans receptors were not taken for further analysis.

Comparative analysis of TR4 with structural homologs

Pairwise structural comparison studies was done with TR4 against all other homologs using DALI structure comparative server (http://ekhidna.biocenter.helsinki.fi/dali_lite/start). The Z score and the RMSD values further helped to concentrate on more related homolog's to the TR4.

Active site determination of TR4 and other structural homologs and its analysis

Since the functionally similar proteins bind to similar ligands, the active sites of all those receptors after DALI server results were compared. This was done by submitting the PDB structures to Q-site finder, which predicts the active site of a protein. The data so obtained was converted into single letter amino acid codes and was analyzed using Multiple Sequence Alignment using ClustalW2.[21] The pairwise analysis of the active site was also carried out using EMBOSS NEEDLE Global Alignment [http://www.ebi.ac.uk/Tools/psa/emboss_needle/].

Domain analysis of TR4 and other structural homologs

According to the central idea of chemogenomics, that protein of the same family share similar domain architecture therefore, should also share similar ligands. For this reason, domain analysis of TR4 and all other proteins was carried out using PROSITE to see the domain similarity among the proteins after active site determination of the receptors. Only those receptors which showed the domain similarity were retained for further study.

Endogenous ligand library generation

A small ligand library was developed by listing all those ligands which were the endogenous ligands of homologous receptors after DALI Pairwise structure comparative study, active site and domain analysis study. This set consisted of totally 12 ligands for 11 receptors which were the ligands natural receptors.

Chemical structure clustering for generation of extended ligand library

The ligands listed were then used to build a large library using PubChem's Chemical Structure Clustering (http://pubchem.ncbi.nlm.nih.gov) which reveals the structurally similar ligands based on the Tanimoto score. The library so developed had 120 ligands which were similar in structure to the 12 endogenous ligands.

Molecular docking study against large ligand library set

The Protein-Ligand docking study was carried out using PyRx which gives the binding energy of a ligand to its active site. Multi-receptor docking was performed in which endogenous ligand library and extended ligand library was docked with TR4 and their respective receptors. The docking of endogenous ligand with their respective receptor was performed so as to get a reference value for an optimal protein-ligand bound complex.

RESULTS AND DISCUSSION

The protein sequence retrieved from the SWISS-PROT (ID P49116) was given to BLASTp analysis against SWISS-PROT database. Only those proteins which had >65% identity and had endogenous ligands were taken for further analysis. Overall 10 such receptors were listed [Table 1].

The receptors obtained after sequence homology were searched for structural homologs in the RCSB PDB

Table 1: Sequence homologs of TR4 and their endogenous ligands

Uniprot ID	Name of the protein	% identity	Ligand
Q6PH 18.1	COUP-TF alpha-B	65	Retinoic acid
P16375.1	Steroid receptor seven-up, isoforms B/C	70	Steroid hormones
Q24142.2	Nuclear hormone receptor HR78; (dHR78)	69	Mediate ecdysteroid signaling
P16376.3	Steroid receptor seven-up, isoform A	70	Similar to RXR and ecdysone receptor
Q06726.1	Steroid receptor homolog SVP 46	68	Steroid hormones
P51128.1	Retinoic acid receptor RXR-alpha	67	Retinoic acids
Q8T5C6.1	Retinoic acid receptor RXR	67	Retinoic acids
P54779.1	Protein ultraspiracle homolog;	67	Similar to RXRs A docking study done against 9C-RC and juvenile hormone
P49743.1	Retinoic acid receptor RXR-beta	66	Retinoic acids
P20153.1	Protein ultraspiracle	68	Ecdysone

RXR: Retinoic X receptor, TR4: Testicular receptor 4, SVP: SevenUP, HR78: Hormone Receptor, COUP-TF: chicken ovalbumin upstream transcription factor (COUP-TF)

database. The structure of proteins which were sequence homologs to TR4 were obtained in the PDB database. The RCSB PDB 3D similarity was used to search for structural homologs. The structures of 29 proteins [Table 2] were retrieved and were then used for Structure comparative analysis using DALI server.

In DALI structure, comparative study only those receptors which showed higher structural similarity, a good Z-score and low RMSD, were taken for further analysis [Table 3]. The active site prediction was done with the help of Q site finder. The amino acid code given by it were converted to single letter code and was submitted to EBI Clustal omega for multiple sequence study and also EMBOSS NEEDLE for pairwise comparison. The domain analysis was done using PROSITE to see the domain similarity among the proteins after active site determination of the receptors [Figure 2]. This revealed a very high conservation among all the proteins except for 3KB4 (Alr8543 protein). Of 21 receptors except Alr8543 protein (Domain ID-PF05019.8) all other proteins showed the same domain (PF00104.25).

At the end of the DALI server and active site analysis the number of receptors for further analysis was only11, for which an endogenous ligand library of 12 ligands was developed. The reduced number of ligands was because some receptors shared the similar ligands as endogenous ligands. This endogenous ligand library was then used to develop an extended ligand library based on the Tanimoto score of the chemical using PubChem's substructure clustering. The substructure clustering

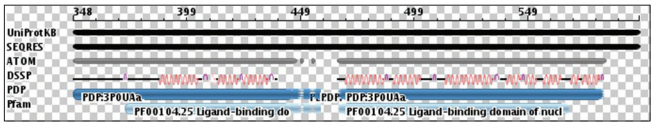


Figure 2: Ligand binding domain of the homologous receptors of 3P0U

Table 2: 3D structure similarity with TR4 against PDB after removing orphans

Protein	PDB ID	% ID	Ligand
Oxysterols receptor LXR-alpha	PDP: 3IPQAa	17	Oxysterols
Retinoic acid receptor RXR-alpha	PDP: 3DZUAb	26	9-cis-retinoic acid
Bile acid	d3beja1	18	bile acids
Vitamin D receptor	PDP: 2HC4Aa	15	1,25-dihydroxyvitamin D3
Estrogen receptor	PDP: 3LTXAa	15	17β-estradiol
Nuclear receptor ROR-Beta	d1nq7a_	12	All-trans-retinoic acid
Peroxisome proliferator-activated receptor gamma	PDP: 3U9QAa	13	Fatty acids, prostaglandin J2 and eisosanoids
Retinoic acid receptor gamma-	d1fcya_	15	All-trans-retinoic acid
Vitamin D3	d2o4ja1	16	1,25-dihydroxyvitamin D3
Retinoic acid receptor, beta	d1xdkb_	15	All-trans-retinoic acid
Thyroid hormone receptor, alpha isoform 1 variant	PDP: 3ILZAa	13	T3 T4
Estrogen receptor beta	PDP: 3OLLAa	16	17β-estradiol
Ecdysone receptor	PDP: 1Z5XEa	19	Ecdysone
Estrogen receptor	PDP: 2OCFAa	16	17β-estradiol
Vitamin D3 receptor	PDP: 3B0TAa	16	1,25-dihydroxyvitamin D3
Mineralocorticoid receptor	PDP: 3VHVAa	13	Aldosterone, corticosterone cortisol, progesterone
Nuclear hormone receptor HR38	d1pdua_	18	Ecdysteroid sensor
AceDAF-12	PDP: 3UP3Aa	15	Dafachronic acids, cholestenoic acid
Pregnane X receptor, linker, steroid receptor coactivator 1	PDP: 3CTBAa	12	Xenobiotics, steroids and benzoates
Alr8543 protein	PDP: 3KB4Aa	4	GeranylGeranyl Monophosphate

TR4: Testicular receptor 4, ROR: Related Orphan Receptor, PDB: Protein Data Bank

Table 3: DALI server results of TR4 (3P0U_A) against other receptors

PDB ID	Protein	Z score	RMSD	%ID
1NQ7	Nuclear receptor ROR beta;	16.4	2.9	17
1PDU	Nuclear hormone receptor HR38	16.4	2.9	21
1XDK	Retinoic acid receptor RXR-alpha	17.5	2.7	29
1Z5X	Ultraspiracle protein a homologue of RXR	18.2	2.3	32
2OCF	Estrogen receptor	15.6	3.2	19
3B0T	Vitamin D3 receptor	16.1	2.6	20
3BEJ	Bile acid receptor	17.5	2.6	23
3ILZ	Thyroid hormone receptor, alpha isoform 1 variant	15.3	2.7	18
3IPQ	Oxysterols receptor LXR-alpha	17.8	2.7	21
3LTX	Estrogen receptor	16.4	2.6	19
3VHV	Mineralocorticoid receptor	14.5	3.0	17

TR4: Testicular receptor 4 , RXR: Retinoic X receptor, PDB: Protein Data Bank, LXR: Liver-X-Receptor, ROR: Related Orphan Receptor, RMSD: Root Mean Square Deviation

helped us to develop an extended library having 120 ligands with Oxysterols, All trans retinoic acid, bile acids, 17-beta-estradiol, 1,25-VitD3, triiodothyronine, tetraiodothyronine, aldosterone, cortisol, progesterone, corticosterone and ecdysone. The ligands which were available as 3D sdf files were used whereas the one that were in the 2D form were converted to least energy conformer (LEC) using Marvin view and were named as LEC in front of their names. The Protein-Ligand docking using PyRx was carried out, which gives the binding energy of a ligand to its active site. The ligands were docked with orphan receptor TR4, as well as with their natural receptors to know the binding energy similarity. Some receptors like 1PDU and 1Z5X shared the same ligands. Of 120 ligands that were docked two ligands 17-Beta estradiol and tretinion (All Trans Retinoic Acid) had almost similar result with 3P0U (Testicular Receptor 4) and to its natural receptor 3LTX (Estrogen Receptor) and 1XDK (RXR alpha) respectively. The docked poses of 3P0U with tretinoin and estradiol are shown in the Figure 3a and b. The detailed view of ligand binding cavity of 3P0U with Tretinion and Estradiol has been shown in the Figure 4. Figure 4a shows the binding cavity of 3P0U with Tretinion which forms two hydrogen bonds (green dotted lines) one with Arg367 with a bond length of 3.13A° and another with Lys503 with a bond length of 2.76A°. The figure 4b shows single hydrogen bond of estradiol with Arg561 with a bong length of 2.97A°.

The docking score of the ligands which showed better results with the receptor TR4 along with their natural receptor are given in the Table 4. The binding energy of 17 beta estradiol with 3LTX, its natural receptor is −5.24 Kcal and for 3P0U is −5.87 Kcal. 1NQ7 and 1XDK have Tretinion as their endogenous ligand. 3P0U

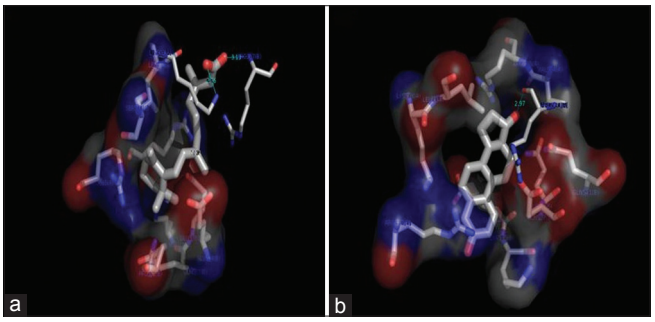


Figure 3: (a) Docked pose of 3P0U (Testicular Receptor4) with Tretinion (All trans retinoic Acid). (b) Docked pose of 3P0U with 17 beta Estradiol

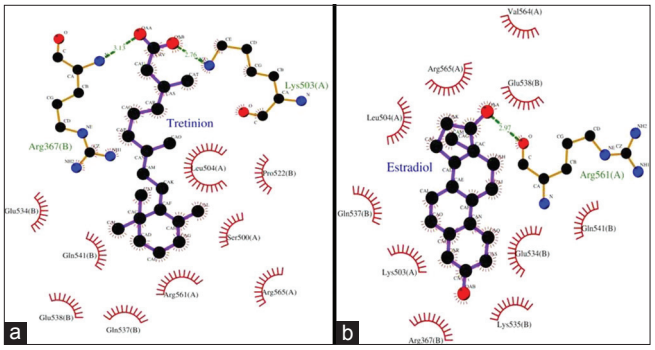
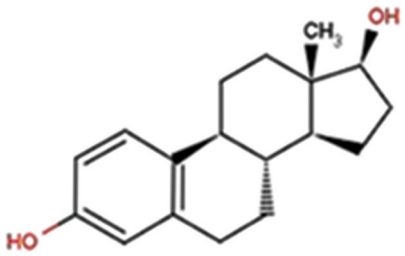
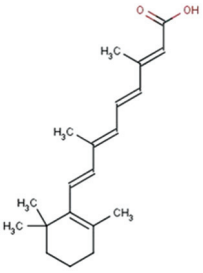


Figure 4: (a) Binding cavity of 3P0U showing 2 hydrogen bonds with Tretinion. (b) Binding cavity of 3P0U showing single hydrogen bonding with 17 beta Estradiol

Table 4: Molecular docking results of 3P0U against estradiol and tretinion, and their natural receptors using PyRx

Ligand	Target	Binding energy	Non binding energy	Structure of the ligand
17 beta estradiol	3LTX	–5.24	0.07	
	3P0U	–5.87	0.07	
Tretinion (all-trans-retinoic acid)	1NQ7	74.17	0.63	
	1XDK	–4.27	-0.58	
	3P0U	–4.88	-0.47	

showed binding energy of −4.88 Kcal as similar to 1XDK with −4.27 Kcal.

CONCLUSION

Combinatorial approaches of chemoinformatics and bioinformatics have led to the novel methods to drug discovery in the recent past. The chemogenomics approach that we have discussed above identifies new drug targets for pre-existing ligand library developed by the endogenous ligands of the homologous proteins. The ligand library would help in finding the new ligands for an orphan receptor through virtual screening methods. In this work we have found through chemogenomics that the estradiol and the tretinion may act as endogenous ligand for TR4. The binding affinity of TR4 with estradiol and tretinion may lead to reduced activity of estrogen receptor (ER) and retinoic acid receptor alpha (RXRalpha) which are the endogenous receptors for these ligands. Since this is an *in-silico* approach the identified ligands affinity for TR4 have to be established with *in vivo* studies.

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