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Identification of a lead pharmacophore for the development of potent nuclear receptor modulators as anticancer and X syndrome disease therapeutic agents

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Abstract—A series of tetrahydroisoquinoline-*N*-phenylamide derivatives were designed, synthesized, and tested for their relative binding affinity and antagonistic activity against androgen receptor (AR). Compound **1b** (relative binding affinity, RBA = 6.4) and **1h** (RBA = 12.6) showed higher binding affinity than flutamide (RBA = 1), a potent AR antagonist. These two compounds also exerted optimal antagonistic activity against AR in reporter assays. The derivatives were also tested for their activities against another nuclear receptor, farnesoid x receptor (FXR), with most compounds acting as weak antagonists, however, compound **1h** behaved as a FXR agonist with activity slightly less than that of chenodeoxycholic acid (CDCA), a natural FXR agonist.

Nuclear receptors act as transcription factors to modulate transcription actions of target genes involved in maintenance of cellular phenotypes, metabolism, and cell proliferation, through homodimerizing or heterodimerizing with other nuclear receptors. Currently, there are more than 30 members in the nuclear receptor superfamily.[1] Most of these receptors exert their functions by ligand-activation and they usually contains six functional domains (A–F), including the first transcription activation domain (AF-1, A/B), DNA binding domain (C), hinge domain (D), and the second transcription activation domain (AF-2, E/F), also referred to as the ligand binding domain. Because of their importance in many diseases, including breast cancer, prostate cancer, and diabetes, they are viewed as potential targets for the development of specific therapeutic agents.

Androgens are steroid hormones that are responsible for the cellular proliferation and differentiation of male sexual organs and secondary sexual characteristics, and their action is mainly exerted through a nuclear receptor, androgen receptor (AR).[2] Androgen exerts its effects via a genomic mechanism in which androgen passively enters the target cells and binds to AR in the cytoplasm.[3] The androgen/AR complex translocates into the nucleus. In the nucleus, the AR complex dimerizes and binds to the promoter region of the androgen-regulated gene to initiate the transcription action and enhance the production of androgen-regulated proteins such as PSA, Bcl-2, and maspin.[4–6] Prostate cancer is the most common type of non-skin cancer and the second leading cause of cancer death in American men. In the early stage of prostate cancer, its growth highly relies on androgen, and the use of androgen deprivation therapy can significantly slow down the tumor growth. Numerous compounds that act as AR agonists like R1881, or antagonists like flutamide, nilutamide, cyperoterone acetate(CPA), and bicalutamide, have been developed.[7] Although flutamide has been used as a first line adjuvant monotherapy against prostate cancer for more than two decades, most patients who took flutamide eventually encounter a resistant stage.[8] Thus, there is an urgent need to develop new antiandrogens.

Farnesoid x receptor (FXR) is also a member of the nuclear receptor superfamily whose endogenous ligands have been identified as numerous bile acids, including chenodeoxycholic acid (CDCA), CA, and DCA.[9] The

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action of FXR is through heterodimerizing with another novel nuclear receptor, retinoid x receptor (RXR), to regulate the transcription of many essential genes involved in bile acid metabolism, including small heterodimer partner 1 (SHP), cholesterol 7α hydroxylase (CYP7A1), and bile salt export pump (BSEP).[10–12] Because these genes, which are regulated by FXR, are involved in bile acid related diseases, FXR modulators are viewed as potential treatments for bile acid and cholesterol homeostasis diseases including cardiovascular and lipid metabolism.

Most nonsteroidal antiandrogens, such as hydroxyflutamide, contain two structural moieties, a nitro (or a cyano) group at the phenyl ring (A ring) of the core scaffold that mimics the 3-carbonyl group of steroidal androgen, like R1881, and a hydrogen-bond donor moiety, such as a hydroxyl group, that mimics the 17β-hydroxyl group of R1881 (Fig. 1a).

In this study, we report a new core scaffold, 1,2,3,4-tetrahydroisoquinoline-*N*-phenylamide, as potential AR/FXR modulating ligand. The hydroxymethyl substituent at the 3 position mimics the important moiety of flutamide as shown in Fig. 1b. The phenylamide substituent might locate within helices 5 and 11 of AR to make extra protein interactions. Based on the core scaffold, we have generated derivatives by substituting various hydrophobic or hydrophilic substituents on the additional phenylamide group. Additionally, our core scaffold also superimposes closely with the steroid core scaffold of FXR native ligands, such as CDCA. We tested these compounds for both AR and FXR modulation. Our goal is to determine whether any of the new compounds is capable of serving as a lead pharmacophore for the development of AR and FXR modulators.

Compounds **1a**–**2b** were synthesized by mixing commercial benzoic acid or phenyl acetic acid derivatives, (*S*)-(−)-1,2,3,4-tetrahydro-3-isoquinoline methanol, 1-hydroxybenzotriazole hydrate (HOBT), and 1-(3-(dimethylamino)propyl)-3-ethyl-carbodiimide) hydrochloride (DEC), and allowed to react overnight under nitrogen gas, as shown in Scheme 1. Table 1 summarizes the relative binding affinity of compounds **1a**–**2b**. The fluorescence-based competitive binding assay purchased from Panvera (Madison WI) utilizes a synthetic androgen with high fluorescence polarization property when it binds to AR and recombinant AR ligand binding domain fused with GST. After the individual binding affinity is determined, it is further calculated as relative binding affinity with flutamide as standard comparison and CPA as a reference. All the compounds exhibited higher binding affinity than flutamide with both **1h** and **2b** showing 12-fold higher binding affinity. However, all the compounds showed weaker binding affinity than CPA.

The ligand binding pocket of AR consists of three essential hydrogen bonding areas for interaction between a AR modulator and AR LBD. This includes Arg 752 of helix 5 and Gln 711 of helix 3; Asn 705 of helix 3 and Thr 877 of helix 11.[13] Apart from these residues, the bound modulator is surrounded by hydrophobic residues. Most antiandrogens like flutamide and bicalutamide bind to AR LBD with two hydrogen-bond interactions between the nitro or cyano group at the 4 position of the A ring with Arg 752 and Gln 711, and between the hydroxyl group mimicking 17β-hydroxyl group of R1881 and Asn 705. Interestingly, even though our compounds do not contain a hydrogen-bond acceptor on the A ring, they still bind to AR with moderate binding affinity. This is likely due to potential hydrogen-bond interaction between the 3-hydroxymethyl group and the side chain of Asn 705 or the amide backbone of Leu 704. This is consistent with the fact that our core scaffold and hydroxyflutamide superimpose well in the 3-hydroxymethyl-1,2,3,4-tetrahydroisoquinoline structural portion where the latter has been observed to interact with the protein. On the other hand, these compounds could adopt a different orientation such that the D ring may reside within helix 3 and helix 5, allowing the substituted hydrophilic groups on the D ring to interact with Arg 752 and Gln 711, similar to the A ring of flutamide. The structure–activity relationship of flutamide and bicalutamide derivatives demonstrates that compounds with cyano or nitro substitution on the A ring have optimal binding affinity, which is at least 10-fold higher than those with fluoro or hydroxyl substituent.[14] If our compounds adopt the latter orientation mentioned above, they should not be expected to exert better binding affinity than flutamide because of lack of a cyano or nitro substituent. In addition, the binding orientation would also abolish the potential hydrogen-bond interaction with Asn 705, which is known to be critical for increased binding affinity. However, since our compounds show higher binding affinity than flutamide, they are unlikely to assume the latter orientation. Crystallographic studies of the compounds/AR LBD complexes should throw light on the binding mode of this new scaffold.

To evaluate the agonist and antagonist activities of these compounds, a transient transfection reporter assay in HepG2 cells was employed. Generally, HepG2 cells were transfected with three plasmids by using Superfect transfection kit (Qiagen, CA) including hAR expression plasmid pcDNA-hAR, a luciferase reporter plasmid containing androgen receptor response element pGL3-ARE-E4, and a normalization control, β-galactosidase reporter plasmid, pCMVβ (Clontech, CA). For comparison, DMSO for androgenic activity and flutamide for antiandrogenic activity were used as standard reference. The antiandrogenic activity of compounds **1a**–**2b** is shown in Fig. 2. Essentially, the compounds did not exhibit obvious androgenic effects when compared with DMSO and R1881 (data not shown). However, compound, **1b** (relative luciferase activity, RLA = 0.61 of R1881 effects) and **1h** (RLA = 0.62 of R1881 effects) exerted optimal antiandrogenic activity against hAR, with activity slightly less than flutamide's (RLA = 0.55 of R1881 effects). For the rest of the compounds, **1c** and **1g** acted as weak antiandrogens. Based upon these results, it seems that enhancement of antiandrogenic activity could be achieved by substituting hydrophilic and hydrophobic groups on the D ring to increase the

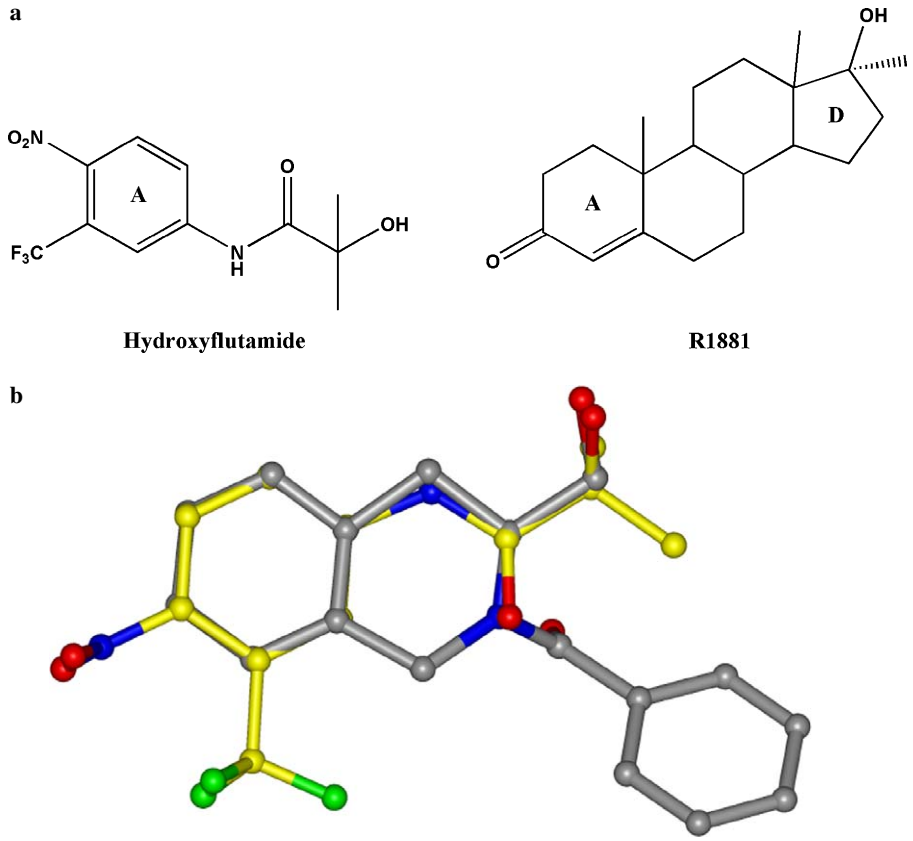
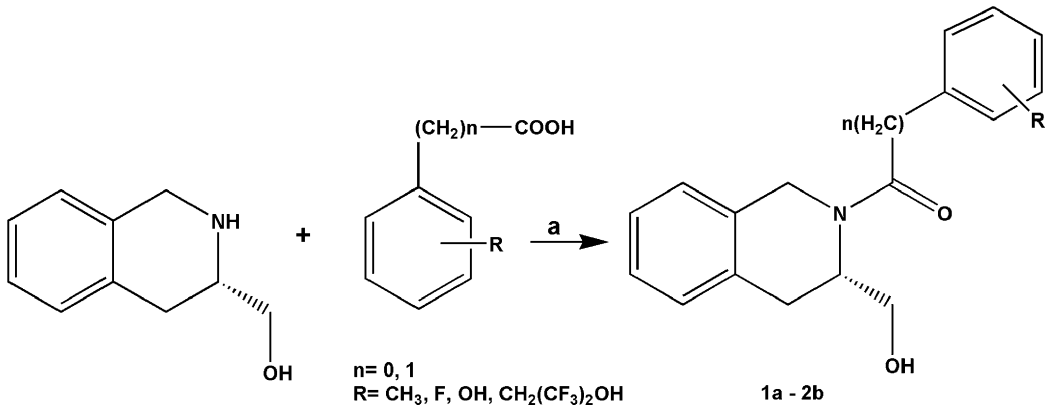


Figure 1. (a) Chemical structures of hydroxyflutamide and R1881. (b) Superimposition of 3-D structures of (*S*)-(3-(hydroxymethyl)-3,4-dihydroisoquinolin-2(1*H*)-yl)(phenyl)methanone and flutamide. The core structure of flutamide is shown in yellow. These structures and the figures were generated with Sybyl 7.0.

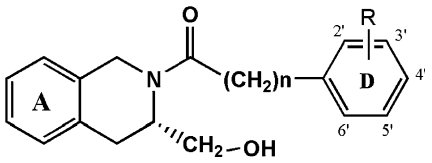


Scheme 1. Synthesis of compounds **1a**–**2b**, a: DEC, HOBT, DMF.

number of favorable interactions with helices 7 and 11 in the AR ligand binding pocket.

Since hAR mutation is one of the important factors for antiandrogen-resistant symptoms discovered in advanced prostate cancer patients, we also employed the transient transfection reporter assay in the prostate cancer cell line, LNCaP cells, expressing a novel mutant hART877A.[15] Several antiandrogens and hormones have been reported to exert agonistic effects against mutant hART877A in hydroxyflutamide-resistant prostate cancer patients. These compounds showed either antagonistic or null activity against wild type AR. Although compounds **1b** and **1h** showed moderate antagonistic without agonistic effects against wild type hAR, it is important to find out whether these compounds have potential to behave as androgenic agents against this mutant. In our results not shown here, R1881 is able to exert significant agonism activity against mutant hART877A, however, compounds **1b** and **1h** did not

Table 1. Fluorescence polarization competitive binding assay with recombinant AR protein



Compound	*n*	*R*	Relative biding affinity
1a	0	2′,3′-CH_3	6.8
1b	0	3′,5′-CH_3	6.4
1c	0	3′,5′-F	3.9
1d	0	3′-F,2′-CH_3	2.9
1e	0	3′-F,4′-CH_3	5.1
1f	0	3′-OH,4′-CH_3	2.3
1g	0	3′,4′-OH	6.8
1h	0	4′-CH_2–$(CF_3)_2$(OH)	12.6
2a	1	3′,5′-CF_3	6.4
2b	1	3′-F,4′-OH	13.4
Flutamide			1
CPA			150

AR binding: The polarization values versus test molecule concentration curves were analyzed by nonlinear least-squares curve fitting in the graphfit software and generated IC_{50} value. The IC_{50} value was further converted to relative binding affinity (RBA) by using flutamide's IC_{50} as a standard. The RBA value of each test molecule was quantified as RBA = IC_{50} of flutamide/IC_{50} of test molecule.

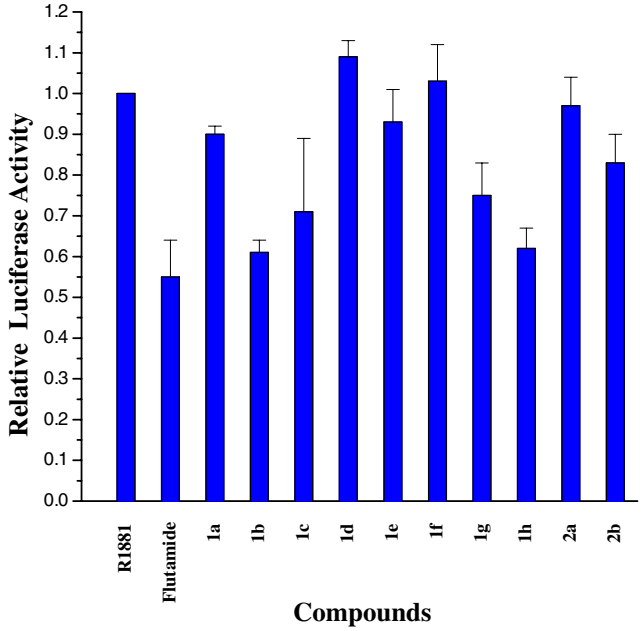


Figure 2. Transient transfection reporter assay in HepG2 cells. AR antagonistic effect was determined by the ARE-driven transactivation luciferase activity in the presence of 0.5 nM R1881. The relative luciferase activity was quantified as RLA of 5 μM tested compound/RLA of 0.5 nM R1881. The RLA data represent means ± SD for three determinations.

show any obvious agonistic effects compared with the vehicle, DMSO.

Since compounds **1b** and **1h** possessed optimal antiandrogen activity, further modeling with InsightII program was undertaken to elucidate their potential binding modes. The lack of a cyano or nitro group on the A ring of compound **1b** precludes it from interacting with Arg 752 like other novel antiandrogens. On the other hand, the hydroxymethyl group on the 3 position of compound **1b** can mimic the hydroxyl group of hydroxyflutamide making hydrogen-bond interaction with the backbone of Leu 704 or the side chain of Asn 705, and as mentioned previously, this hydrogen-bond interaction could compensate for the lack of hydrogen-bond interaction with Arg 752. In addition, the dimethyl-substituted D ring of compound **1b** may insert into a small hydrophobic pocket formed by the residues, Leu 873, Phe 876, Ile 899, and Thr 877. As previously pointed out, the hydroxyl group on the A ring of compound **1h** is less favored to bind to AR compared with the nitro and cyano groups on the A ring of hydroxyflutamide. Alternatively, it should be favored to interact with Thr 877. Apart from the terminal hydroxyl group, compound **1h** also contains fluoro groups that may enhance binding by interacting with Thr 877. The importance of the hydroxymethyl group on the 3 position of compound **1h** was further demonstrated by synthesizing a compound with similar structure but lacking this moiety. This modified compound did not show any significant antiandrogenic effects (RLA = 98%, data not shown here) as well as exerted reduced binding affinity to AR when compared with compound **1h**.

FXR is another member of nuclear receptor superfamily and its native ligands are bile acids, such as CDCA (chenodeoxycholic acid) with a steroidal skeleton and a carboxylate moiety at the 24 position. Our compound **1h** possesses a structural similarity to TO91317, a novel liver x receptor (LXR) agonist, which is known to act as a FXR agonist.[16] We therefore tested them against FXR. For agonist and antagonist activities against FXR, a transient transfection reporter assay in HepG2 cells was employed. The HepG2 cells were transfected with three plasmids including hFXR LBD expression

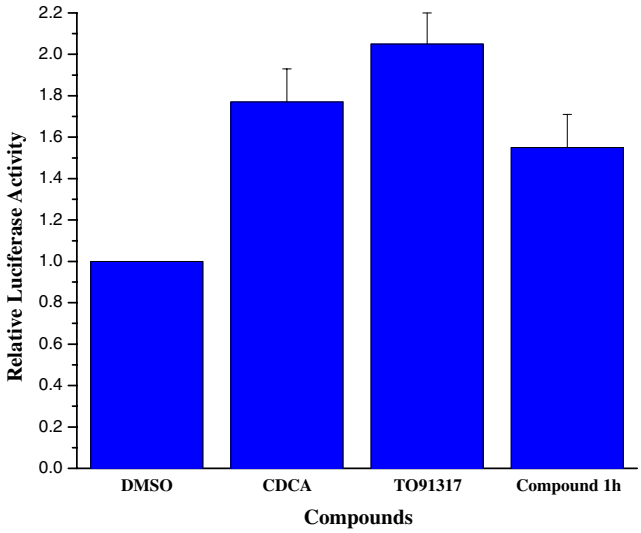


Figure 3. Mammalian one-hybrid assay of ligand-mediated transactivation of FXR in HepG2 cells. FXR agonistic effect was determined by GAL4/FXR LBD-driven transactivation luciferase activity. The relative luciferase activity was quantified as RLA of 10 μM tested compound/RLA of DMSO. The RLA data represent means ± SD for three determinations.

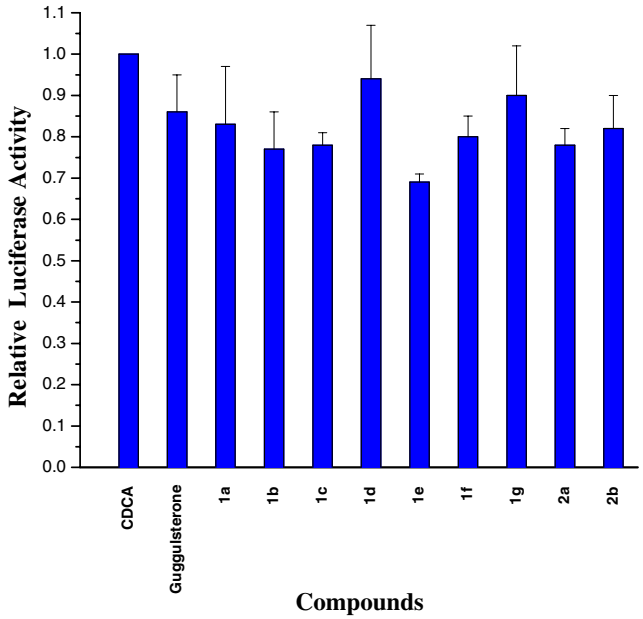


Figure 4. Mammalian one-hybrid assay of ligand-mediated transactivation of FXR in HepG2 cells. FXR antagonistic effect was determined by GAL4/FXR LBD-driven transactivation luciferase activity in the presence of 10 μM CDCA. The relative luciferase activity was quantified as RLA of 10 μM tested compound/RLA of 10 μM CDCA. The RLA data represent means ± SD for three determinations.

plasmid pGal-hFXR LBD that is generated by subcloning the cDNA of hFXR LBD, produced by PCR method, into pBIND vector (Promega, WI) and fusing the hFXR LBD to the C-terminus of Gal4 DNA binding domain, a luciferase reporter plasmid containing repeated Gal4 response element pG5-Luc (Promega, WI), and a normalization control, β-galactosidase reporter plasmid pCMVβ. The compounds, CDCA and T091317, both agonists, and guggulsterone, a novel FXR antagonist, were used as the standard reference for comparison.[17] Only compound **1h** (RLA = 1.55 of vehicle, DMSO effects) showed moderate agonist activity as shown in Fig. 3, with activity less than CDCA (RLA = 1.77 of DMSO effects) and TO91317 (RLA = 2.05 of DMSO effects) at 10 μM. The other tested compounds did not exert any significant agonistic effect against FXR (data not shown).

Fig. 4 shows the antagonist activity of these compounds at 10 μM, with similar activity as guggulsterone. Compound **1e** is the most potent antagonist among them. The crystal structure of rat FXR LBD complexed with CDCA reveals three main hydrogen-bond interactions: (1) the 3α-hydroxyl group of CDCA orients toward helix 11 of FXR LBD to make hydrogen-bond interaction with His 444 and Trp 466; (2) the 7α-hydroxyl group interacts with Y366; and (3) the 24-carboxyl group serves as a hydrogen-bond acceptor for Arg 328 (Arg 335 of human FXR) of helix 5.[18] In addition to these residues, there are other residues in the ligand pocket of FXR with potential hydrophobic interactions. Structure–activity relationship studies of CDCA derivative agonists suggest that among these hydrogen-bond interactions, the one between the 24-carboxyl group of CDCA and Arg 328 of helix 5 plays the most essential role in the agonistic effects of these compounds. On the other hand, it was proposed that the 3β, 7β as well as 12β-hydroxyl substituents on CDCA are not critical for the FXR activation.[19] For the putative binding mode of our compounds, we suggest that the compounds shown here might have two possible orientations, with the D ring either mimicking the A ring or D ring of CDCA. Since compounds **1a**–**2b** (except for **1h**) did not show any agonist but only antagonistic effects, we hypothesize that they might bind to FXR LBD by orienting their D ring to interact with helix 11 in the same manner as the A ring of CDCA, allowing the D ring substituents to make contact with FXR LBD through hydrophobic or hydrogen-bond interactions. In this orientation, the 3-hydroxymethyl group on the core scaffold mimics the 12β-hydroxyl group of CDCA which has been shown not only to diminish the effects of FXR activation, but to enhance the compound's binding affinity to FXR, allowing them to act as competitive inhibitor of FXR.

On the contrary, compound **1h**, which contains a similar moiety found in T0901317 (a mixed FXR and liver x receptor LXR agonist), might adopt different binding mode due to its unique agonist effects among this type of derivative. T0901317 only contains hydrophilic substituents on one of its phenyl moieties but can still activate FXR. Compound **1h** might bind to FXR in the same manner as T091317 due to the fact that it shares similar structural moieties, as well as exerts similar actions as T091317. T0901317 is also a liver x receptor (LXR) agonist. Unlike a natural LXR ligand, such as 22*R*-hydroxyl cholesterol, the A ring of T091317 cannot make hydrogen-bond interactions with Glu 267 and Arg 305.[20] T0901317 binds to LXRβ LBD by orienting its

hydrophilic group toward helix 11 so that its terminal hydroxyl group makes contact with His 421. However, in FXR LBD, compound **1h** and T0901317 may have different orientation with their hydrophilic phenyl substituents, preferably leaning toward helix 5 of FXR LBD to make contact with Arg 328. Hydrogen-bond interaction with Arg 328 of helix 5 could be important for the activation of FXR with CDCA derivatives. Interestingly, fexarene, a novel non-steroidal FXR agonist does not interact with Arg 335 of human FXR, but still activates FXR transcription action.[21] This result indicates that the mode of binding and receptor activation may be more complicated and co-crystal structure will be required to understand the mode of action of compound **1h** in FXR LBD.

The above studies indicate that the tetrahydroisoquinoline-*N*-phenylamide derivatives can exert antagonist effects against AR, and the 3-hydroxymethyl moiety might play an essential role in that action. Additionally, these compounds are also capable of regulating FXR action by either competing with CDCA to bind to FXR LBD or by activating FXR in the same way as T0901317. Since the structural requirements to develop AR antagonists and FXR modulators are quite different, we believe that we may be able to generate selective AR and FXR modulators based on further structure–activity relationship and crystal structure analyses.

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