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DL5050, a Selective Agonist for the Human Constitutive Androstane Receptor

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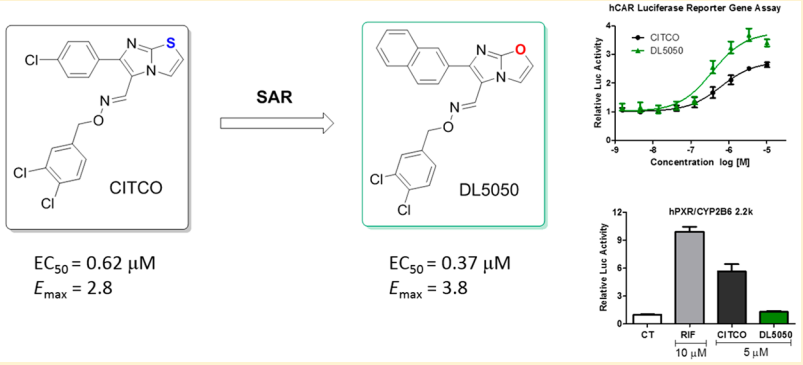
Supporting Information



ABSTRACT: The constitutive androstane receptor (CAR) is a xenobiotic sensor governing the transcription of genes involved in drug disposition, energy homeostasis, and cell proliferation. However, currently available human CAR (hCAR) agonists are nonselective, which commonly activate hCAR along with other nuclear receptors, especially the closely related human pregnane X receptor (hPXR). Using a well-known hCAR agonist CITCO as a template, we report our efforts in the discovery of a potent and highly selective hCAR agonist. Two of the new compounds of the series, **18** and **19** (DL5050), demonstrated excellent potency and selectivity for hCAR over hPXR. DL5050 preferentially induced the expression of CYP2B6 (target of hCAR) over CYP3A4 (target of hPXR) on both the mRNA and protein levels. The selective hCAR agonist DL5050 represents a valuable tool molecule to further define the biological functions of hCAR, and may also be used as a new lead in the discovery of hCAR agonists for various therapeutic applications.

KEYWORDS: *Nuclear receptor, constitutive androstane receptor, structure−activity relationship, pregnane X receptor*

The constitutive androstane receptor (CAR, NR1I3) belongs to the nuclear receptor (NR) subfamily 1I that also includes the pregnane X receptor (PXR, NR1I2) and vitamin D receptor (VDR, NR1I1).[1,2] CAR plays an important role orchestrating the metabolism and detoxification of potentially toxic chemicals,[3,4] by upregulating genes that are involved in the clearance of both xenobiotics and endobiotics.[5,6] Although CAR-mediated metabolism of drugs (e.g., acetaminophen) can lead to the production of toxic metabolites,[7] induction of CAR regulated genes has emerged as a therapeutic strategy for the treatment of various diseases. For instance, phenobarbital, a prototypical CAR activator, has been used to treat neonatal jaundice through its induction of UDP glucuronosyltransferase 1A1 (UGT1A1), the rate-limiting enzyme responsible for the metabolism of bilirubin.[8] Maglich and co-workers have reported an important regulatory role of CAR in the metabolism of thyroid hormones during caloric restriction,[9] while other studies revealed that activation of CAR ameliorates high-fat diet-induced obesity and diabetes in mice, suggesting that CAR modulators might be potential therapeutics for metabolic disorders.[10,11] It is well-known that cyclophosphamide (CPA), a widely used chemotherapeutic prodrug, is converted to a therapeutically active metabolite 4-hydroxy-CPA primarily by CYP2B6 (target of hCAR) and to an inactive metabolite N-DCE-CPA predominantly by CYP3A4 (target of hPXR).[12,13] Recent studies from our laboratory have shown that activation of CAR sensitizes cyclophosphamide (CPA)-based cancer treatment by enhancing biotransformation of CPA to its activate metabolite 4-hydroxy CPA.[14,15]

The activation mechanism of CAR begins with a ligand-triggered dissociation of CAR from its cochaperone partners, cytoplasmic CAR retention protein (CCRP), and heat shock protein 90 (Hsp90), in the cytosol (Figure 1).[16] Dephosphor-

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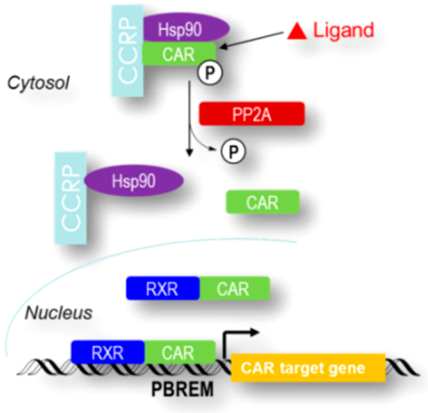


Figure 1. Activation mechanism of CAR.

ylation of CAR at Thr38 by protein phosphatase 2A (PP2A) generates free CAR,[17] which then translocates into the nucleus and forms a heterodimer with the retinoid X receptor (RXR). The complex of RXR-CAR regulates its target genes upon binding to cis-acting responsive enhancer modules upstream of target genes.[18] Different from most known NRs, CAR demonstrates constitutive and ligand-independent activity.[19]

Similar to most classical steroid receptors, CAR contains multiple functional domains including an *N*-terminal DNA-binding domain (DBD) that directly interacts with the promoters of its target genes, a hinge domain, and a multifunctional ligand-binding domain (LBD). The LBD contains a ligand binding pocket, a dimerization interface that binds to RXR, and a *C*-terminal ligand-dependent activation helix (AF2).[20] The LBD of human CAR (hCAR) can bind to a wide range of structurally diversified ligands.[21−23] Earning its name as an orphan nuclear receptor, physiologically relevant endogenous ligands of hCAR have not been identified as of yet.

Within the NR1I subfamily, hCAR shares a modestly conserved LBD and highly conserved DBD with human PXR (hPXR).[24] As a result, these two nuclear receptors bind to multiple common ligands[25] and regulate an overlapping set of genes (e.g., CYP2B6 and CYP3A4).[24,26,27] Compared to hPXR, hCAR has a much smaller LBD (675 Å^3 vs ∼1200 Å^3 for hPXR).[23] Unlike hPXR to which selective agonists (e.g., SR12813[28]) have been identified, no highly selective hCAR activator has been disclosed. The availability of potent and highly selective ligands for hCAR will not only benefit the confirmation of target genes for this important nuclear receptor but also verify whether hCAR is a viable therapeutic target.

In the past decade, efforts in both screens[29−32] and medicinal chemistry[33] have been devoted to the identification and development of agonists for hCAR. Among all known hCAR agonists, the GSK high-throughput screening hit CITCO is the most well-known for its good potency and selectivity.[5,20] CITCO potentiates constitutive activity of hCAR by stabilizing the constitutive AF2-coactivator interaction.[23] Although CITCO only indicates moderate selectivity for hCAR over hPXR, it is by far the most widely used chemical tool in elucidating the function of hCAR.[34−37]

Herein, we report the development of hCAR agonists based on the structure of CITCO (Figure 2). Specifically, the 4-Cl-phenyl, imidazothiazole, and 3,4-diCl-phenyl groups of CITCO (**1**) were modified using compounds **2**−**29**. Seven compounds were selected and further evaluated for their

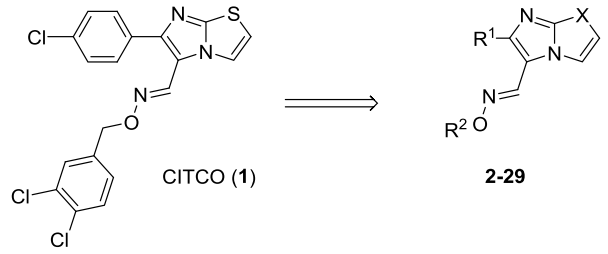
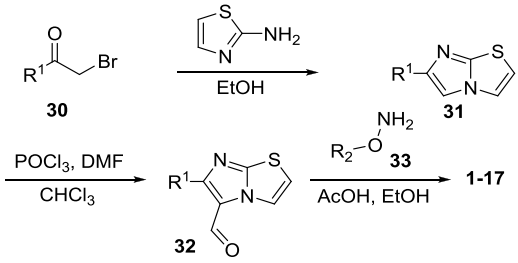


Figure 2. Design of compounds **2**−**29** from CITCO.

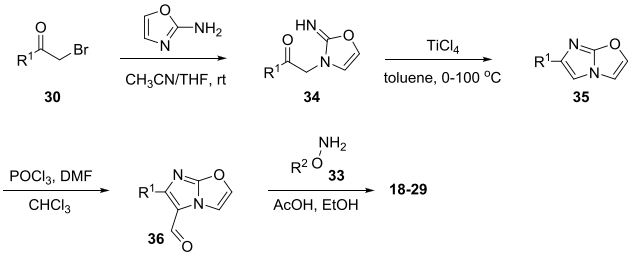
selectivity over hPXR. Two of the new compounds, **18** and **19** (DL5050), demonstrated excellent selectivity for hCAR over hPXR while maintaining similar hCAR agonistic potency to CITCO. In particular, compound DL5050 selectively induced the expression of cytochrome P450 CYP2B6 over CYP3A4 at both the mRNA and protein levels (Figure 4).

The synthetic strategies for compounds **1**−**29** were outlined in Schemes 1 and 2. Condensation of bromomethyl ketone

Scheme 1. Synthesis of Compounds 1−17



Scheme 2. Synthesis of Compounds 18−29



(**30**) with thiazol-2 amine provided imidazothiazole **31** (Scheme 1). Vilsmeier formylation of compound **31** gave aldehyde **32** in good yields. Treatment of aldehyde **32** with hydroxylamine **33** in EtOH led to the formation of final products **1**−**17**.

As shown in Scheme 2, a modified four-step route was used to synthesize compounds **18**−**29**. Condensation of bromomethyl ketone (**30**) with oxazol-2-amine in the mixture of THF and MeCN generated intermediate **34**. $TiCl_4$-mediated cyclization of compound **34** yielded imidazooxazole **35**. Vilsmeier reaction of compound **35** using $POCl_3$ in chloroform gave aldehyde **36** in excellent yields. Oximation of aldehyde **35** with substituted hydroxylamine **33** in the presence of AcOH led to the final compounds **18**−**29**.

The new compounds were first evaluated for their potential modulation of hCAR activity using a cell-based quantitative high-throughput screening (qHTS) luciferase reporter assay on a double stable HepG2-CYP2B6-hCAR cell line.[31] The potency was expressed as 50% effective concentration

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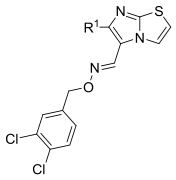
(EC_{50}), and the maximum luciferase signal fold increase of a new compound over vehicle control as E_{max}. In addition, cytotoxicity of new compounds was evaluated in a luciferase-coupled ATP quantitation cell viability assay (Table S1).

In order to optimize R^1, a collection of compounds (**1**−**17**) with a common (*E*)-imidazo[2,1-*b*]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime scaffold were tested (Table 1). The

Table 1. EC_{50} and E_{max} of Compounds 1−17[a]



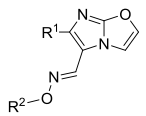
Cmpd	R^1	EC_{50} (μM)	E_{max}	E_{max}/EC_{50}
1	4-Cl-Ph	0.62 ± 0.08	2.8	4.5
2	Ph	0.58 ± 0.36	2.3	4.0
3	4-F-Ph	0.67 ± 0.10	2.6	3.9
4	4-Br-Ph	0.48 ± 0.22	2.4	5.0
5	4-Me-Ph	0.49 ± 0.23	2.7	5.5
6	4-CF_3-Ph	0.88 ± 0.09	2.3	2.6
7	4-MeO-Ph	0.49 ± 0.27	2.4	4.9
8	4-*i*Pr-Ph	0.59 ± 0.13	3.0	5.1
9	4-*t*Bu-Ph	2.4 ± 0.30	2.0	0.83
10	4-CN-Ph	3.4 ± 0.29	2.2	0.65
11	4-Cy-Ph	> 100	2.3	< 0.03
12	4-Ph-Ph	ND	1.1	-
13		ND	1.2	-
14	3-Cl-Ph	1.1 ± 0.08	2.3	2.1
15	2-Naph	0.38 ± 0.31	2.3	6.1
16	1-Naph	0.94 ± 0.73	2.2	2.3
17	Ethyl	2.6 ± 0.16	3.6	1.4

[a]EC_{50} and E_{max} values were calculated by nonlinear regression. Data are presented as mean ± SEM of at least three independent experiments in quadruplicate. ND (if the maximum tested concentration produced no effect).

E_{max} values were shown as relative values compared to the basal value (negative control). Under our assay conditions, CITCO (**1**) indicated an EC_{50} of 0.62 μM and E_{max} of 2.8, which led to an E_{max}/EC_{50} value of 4.5. Removing the Cl atom, the analogue **2** showed a slightly increased potency. However, the E_{max} of compound **2** was also decreased. While substitution of the Cl atom with an F led to a new compound (**3**) with similar EC_{50} and E_{max} values, a larger Br group (**4**) at this position brought increased agonistic activity (E_{max}/EC_{50} = 5.0). Similarly improved activities were also observed when substituting the Cl with an Me group (**5**). Addition of a more polar CF_3 group (**6**) yielded a weaker agonist. These results suggested that there was no demand for a hydrogen bond receptor at this position, instead, a small hydrophobic pocket might be available for new compound development. OMe (**7**) and *i*-Pr (**8**) groups at this position led to new compounds with slightly improved agonist activity. Large substituents, such as *t*-Bu (**9**), CN (**10**), Cy (**11**), and Ph (**12**), were also explored. Compounds **9** and **10** demonstrated weak agonistic activity for hCAR (E_{max}/EC_{50} < 1.0), and compounds **11** and **12** showed essentially no activity toward hCAR. Our results demonstrated that the hCAR agonist activity decreases while the size of substituents increase. Compound **13**, with a bulky substitution on the 3,4-position also indicated no activity for hCAR, which is in agreement with our previous discovery. Shifting the Cl atom to the 3-position from the 4-position yielded compound **14** with a 2.6-fold decreased EC_{50} and slightly lower E_{max} value, implying that 3-substitution can also be tolerated by the agonist. Replacement of the 4-Cl-phenyl group with naphthalen-2-yl group led to compound **15** with an improved potency although its E_{max} was also decreased. The E_{max}/EC_{50} value of compound **15** was 1.4-fold better than that of CITCO. The regioisomer naphthalen-1-yl **16**, however, indicated significantly lower EC_{50} and E_{max} values. Replacement of the aromatic group with an ethyl group led to compound **17** having a weak potency, highlighting the importance of an aromatic ring at this position. The cytotoxicity of new compounds was evaluated (Table S1). Similar to CITCO, our new compounds started to show toxicity at concentrations above 10 μM.

Next, in an attempt to increase the E_{max} value of the compounds, we synthesized compounds **18**−**29** (Table 2).

Table 2. EC_{50} and E_{max} of Compounds 18−29[a]



Cmpd	R^1	R^2	EC_{50} (μM)	E_{max}	E_{max}/EC_{50}
18	4-Cl-Ph	3,4-diCl-Bn	0.41 ± 0.09	4.2	10
19	2-Naph	3,4-diCl-Bn	0.37 ± 0.11	3.8	10
20	2-Naph	2,4-diCl-Bn	1.5 ± 0.12	3.5	2.3
21	2-Naph	2-Cl-Bn	1.1 ± 0.11	3.8	3.5
22	2-Naph	4-Cl-Bn	0.99 ± 0.15	4.2	4.2
23	2-Naph	Bn	0.84 ± 0.10	4.4	5.2
24	2-Naph		0.98 ± 0.10	3.7	3.8
25	2-Naph	perF-Bn	1.7 ± 0.13	3.1	1.8
26	2-Naph	4-CF_3-Bn	0.47 ± 0.18	2.8	6.0
27	2-Naph	4-MeO-Bn	4.4 ± 0.44	3.3	0.75
28	2-Naph	4-*t*Bu-Bn	7.9 ± 0.48	2.3	0.29
29	2-Naph	4-PhO-Bn	ND	1.0	-

[a]EC_{50} and E_{max} values were calculated by nonlinear regression. Data are presented as mean ± SEM of at least three independent experiments in quadruplicate. ND (if the maximum tested concentration produced no effect).

Replacing the imidazothiazole core with imidazoxazole, in CITCO and **15**, yielded two new compounds, **18** and **19**, with similar EC_{50} values but significantly larger E_{max} values (E_{max}/EC_{50} = 10 for both **18** and **19**). These results indicated that the imidazoxazole core is preferred over the imidazothiazole for generating larger E_{max} values. Using compound **19** as a template, we studied the substitution effects on R^2. When the 3-Cl atom was moved to the 2-position, the resulting 2,4-diCl-Bn analogue **20** indicated a 4.1-fold decreased EC_{50} value.

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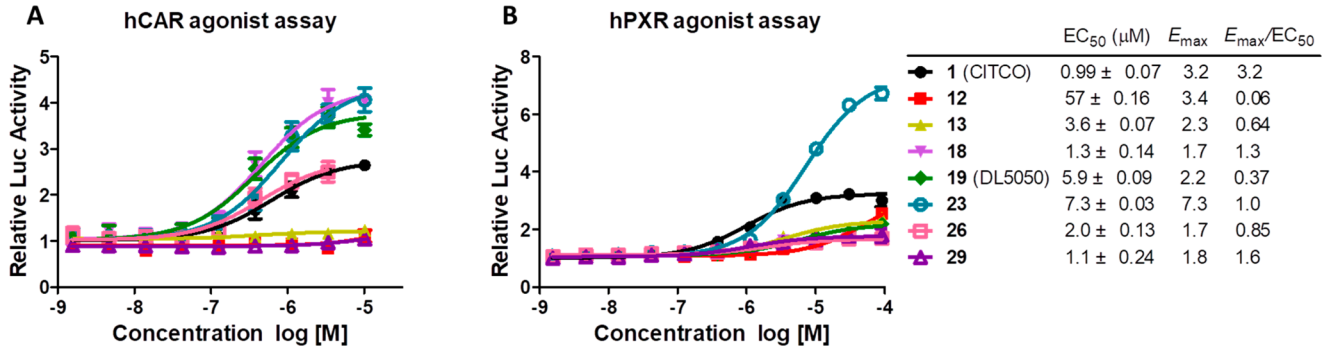

Figure 3. Selectivity of eight compounds **12**, **13**, **18**, **19** (DL5050), **23**, **26**, **29**, and CITCO (**1**) for hCAR over hPXR. (A) Dose-dependent activation of hCAR by CITCO, **12**, **13**, **18**, **19**, **23**, **26**, and **29** in high throughput hCAR agonist luciferase gene reporter assays. (B) Dose-dependent activation of hPXR by CITCO, **12**, **13**, **18**, **19**, **23**, **26**, and **29** in high throughput hPXR agonist luciferase gene reporter assays.

After removal of one of the two Cl atoms from **20**, the resulting compounds, **21** and **22**, indicated 3.0- and 2.7-fold decreased EC_{50} values, respectively. When both Cl atoms of compound **19** were removed, the resulting compound **23** was 2.3-fold weaker; however, this compound also indicated a large E_{max} value of 4.4. Replacement of the ethylene linker with a structurally rigid five-membered ring generated a new compound (**24**) with diminished values for both EC_{50} and E_{max}. This result indicated the necessity of a flexible linker. The analogue with a pentafluorophenyl group (**25**) was less potent and efficacious in comparison to the unsubstituted phenyl analogue **23**. In addition, different substitutions at the *para*-position of the Bn group, including CF_3 (**26**), OMe (**27**), *t*-Bu (**28**), and PhO (**29**) were introduced to probe the depth of the binding pocket for R^2. While compound **26** showed a comparable EC_{50} and E_{max} values to those of CITCO, compounds **27** and **28** indicated weak agonistic activities; in particular, the phenoxy analogue **29** demonstrated no agonistic activity toward hCAR. These results highlighted that large substitutions on the phenyl ring could not be tolerated in the pocket to which $R^2$was bound. It is worth noting that the imidazoxazole-based compounds displayed less cytotoxicity compared to the ones listed in Table 1. In particular, compounds **18**, **19**, **23**, and **26** showed no obvious cytotoxicity at the maximum tested concentration (92 μM) (Table S1).

Based on the predicted hCAR activity, we selected seven new compounds including four potent hCAR agonists (**18**, **19** [DL5050], **23**, and **26**) and three weak hCAR agonists (**12**, **13**, and **29**) and tested their capacity in hPXR activation using a luciferase reporter assay (Figure 3B). The three weak hCAR agonists (**12**, **13**, and **29**) also indicated minimum agonistic activity for hPXR (Figure 3B). These results showed that CITCO analogs with large hydrophobic substitutions on either aromatic tail could abolish the potency in both hCAR and hPXR activation. These results also provided valuable information to define the size limitation of the LBD for both hCAR and hPXR based on the scaffold of CITCO. Among the four potent hCAR agonists, **23** exhibited potent hPXR activation at high concentrations with an E_{max} of 7.3 and EC_{50} of 7.3 μM (Figure 3B). Notably, compounds **18**, **19**, and **26** indicated negligible agonistic activity of hPXR and thereby represent selective agonists for hCAR over hPXR.

Given that **26** demonstrated an hCAR activity that is markedly lower than that of **18** and **19** (DL5050), we next evaluated the effects of **18** and DL5050 on the induction of CYP2B6 and CYP3A4 genes in cultured human primary hepatocytes (HPH). As shown in Figure 4A, both **18** and

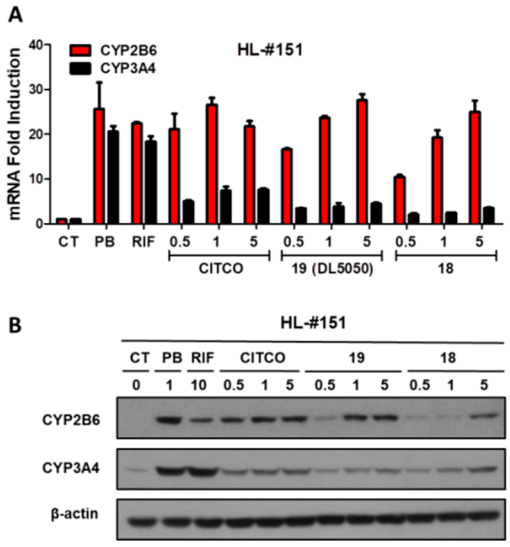

Figure 4. hCAR regulated induction of CYP2B6 and CYP3A4 in HPHs. (A) CYP2B6 and CYP3A4 mRNA levels were measured in HPHs prepared from human liver donors (HL-#151). The HPHs were treated with CITCO (0.5, 1, and 5 μM), PB (1 mM), RIF (10 μM), DL5050 (0.5, 1, and 5 μM), **18** (0.5, 1, and 5 μM), or vehicle control. (B) Representative immunoblots of CYP2B6 and CYP3A4 proteins in HPHs from liver donors #151. RT-PCR data obtained from three independent experiments were expressed as mean ± SD normalized against vehicle control.

DL5050 induced CYP2B6 mRNA expression in a concentration-dependent manner while only moderately altered the expression of CYP3A4. This was significantly different to the hCAR and hPXR dual activator (PB), the selective hPXR agonist (RIF), and even the moderately selective hCAR agonist (CITCO). At the protein level, the naphthyl analog DL5050 also demonstrated a similar concentration-dependent selective induction of CYP2B6 over CYP3A4 (Figure 4B). Conversely, compound **18** only showed marginal induction of CYP2B6 protein.

The binding mode of compound DL5050 in hCAR was also studied (Figure 5). The dichloro-phenyl ring of DL5050, which is the same as in CITCO, rests in the hydrophobic pocket flanked by Phe132, Tyr224, and Tyr 217. The imidazo[2,1-*b*]oxazole core of DL5050 fit deeper at the junction of helixes H5 and H3 than the imidazothiazole core of CITCO. Finally, the naphthyl ring of DL5050 establishes a $\pi-\pi$ stacking interaction with Phe234, which is not present in

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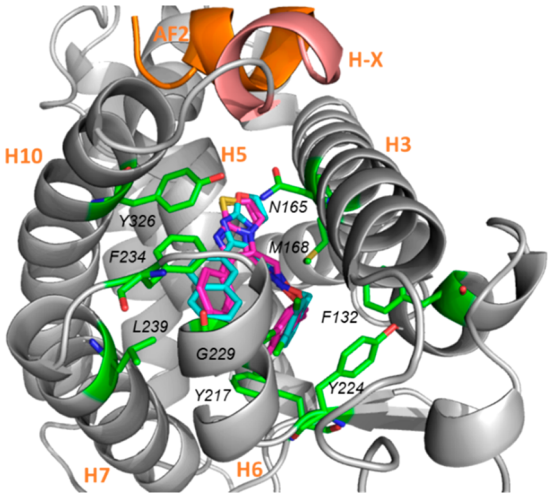

Figure 5. Docking result of compound DL5050 (orange) in the ligand-binding domain of hCAR in comparison with CITCO (magenta) using AutoDock Vina.[38] Key hCAR residues that are expected to mediate interactions with ligands are highlighted in green. Helix X is showed in light pink. The activation function 2 helix (AF2) is shown in orange.

CITCO, potentially providing a basis for the heightened activity of DL5050.

In summary, structure optimization of CITCO has yielded new hCAR agonists **18** and **19** (DL5050). While maintaining a similar hCAR activation potency as that of CITCO, these new compounds indicated a high specificity for hCAR over its closely related nuclear receptor, hPXR. One of the two new compounds, DL5050, exhibited potent induction of CYP2B6 gene at both the mRNA and protein levels while only resulted in minimal induction of CYP3A4. DL5050 displayed a strong induction preference in CYP2B6 over CYP3A4, which exceeds that of the known hCAR activator CITCO. The unique profile of compound DL5050 indicates this compound can be a tool for elucidating biological functions of hCAR with minimal hPXR interference. Additionally, the selectiveness of DL5050 for hCAR suggests it may be used as a therapeutic agent for various human diseases.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.9b00079.

General experimental procedures, supplementary data, and copies of ^{1}H and ^{13}C NMR spectra and HPLC of compounds (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

CAR, constitutive androstane receptor; NR, nuclear receptors; PXR, pregnane X receptor; VDR, vitamin D receptor;; CCRP, cytoplasmic CAR retention protein; RXR, retinoid X receptor; ER, estrogen receptor; GR, glucocorticoid receptor; DBD, DNA-binding domain; LBD, ligand binding domain; qHTS, quantitative high-throughput screening

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