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New *in Vitro* Tools to Study Human Constitutive Androstane Receptor (CAR) Biology: Discovery and Comparison of Human CAR Inverse Agonists

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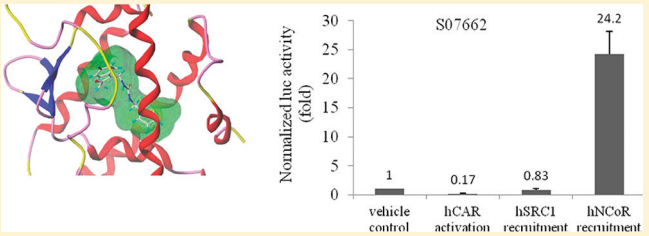
Supporting Information

ABSTRACT: The human constitutive androstane receptor (CAR, NR1I3) is one of the key regulators of xenobiotic and endobiotic metabolism. The unique properties of human CAR, such as the high constitutive activity and the complexity of signaling, as well as the lack of functional and predictive cell-based assays to study the properties of the receptor, have hindered the discovery of selective human CAR ligands. Here we report a novel human CAR inverse agonist, 1-[(2-methylbenzofuran-3-yl)methyl]-3-(thiophen-2-ylmethyl) urea (S07662), which suppresses human CAR activity, recruits the corepressor NCoR in cell-based assays, and attenuates the phenytoin- and 6-(4-chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime (CITCO)-induced expression of CYP2B6 mRNA in human primary hepatocytes. The properties of S07662 are also compared with those of known human CAR inverse agonists by using an array of different *in vitro* and *in silico* assays. The identified compound S07662 can be used as a chemical tool to study the biological functions of human CAR and also as a starting point for the development of new drugs for various conditions involving the receptor.



KEYWORDS: *nuclear receptor, constitutive androstane receptor, inverse agonist, virtual screening, cell-based assays*

INTRODUCTION

Among the 48 members of the nuclear receptor (NR) superfamily, the constitutive androstane receptor (CAR, NR1I3) and pregnane X receptor (PXR, NR1I2) have been established as the master regulators of hepatic drug and xenobiotic metabolism and transport.[1−4] In addition, there are several recent reports[5−9] which link CAR and PXR to the regulation of endogenous lipids, bile acid, bilirubin, and glucose metabolism in animal models.[10,11] It is well-documented that dramatic species differences in CAR- and PXR-mediated induction of cytochrome P450 (CYP) expression are caused by differential recognition of ligands by human and rodent NRs due to evolutionary changes of critical residues within the NR ligand-binding pockets (LBPs).[12,13] Because modulation of CAR and PXR activity by drugs and xenobiotics has considerable importance in drug development and in the evaluation of drug−drug interactions and compound toxicity, there is increasing interest in the development of both *in silico* and *in vitro* assays to assess the potential of drug candidates to interact with CAR and PXR.[14−16] In addition, receptor-specific agonists and antagonists would be valuable in deciphering the roles and interplay of CAR and PXR in human metabolism of endogenous compounds. Currently, based on such assays, potent human PXR agonists[2,17] and antagonists[18,19] have been identified and proved useful for *in vitro* and *in vivo* investigations.

In contrast, the ligand specificity of human CAR is still obscure, and this has been attributed mostly to lack of robust assays and complexity of CAR signaling.[14,20] The first problem is the high constitutive activity of CAR which may impede detection of human CAR modulators in cell-based reporter assays.[14,17] Another complicating factor is the so-called "indirect activation" of CAR where a compound is able to elicit translocation of CAR from the cytosolic compartment into the nucleus in primary hepatocytes but *trans*-activation of CAR-regulated reporter gene cannot be demonstrated.[21] Next, cell- and assay-based differences may cause confusion in assignment of a CAR-interacting compound. For instance,

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clotrimazole has been reported both as a modest agonist[22,23] or an inverse agonist.[12,24,25] Also, many CAR activators appear to be partial agonists[26,27] that are capable of recruiting both NR coactivators and corepressors that are essential in conveying activating and inhibiting signals to the general transcription machinery.[28] Because the expression levels of these coregulators often vary between cell lines, and thus, influence ligand effects,[29] it is important to carefully select a suitable cell line for CAR reporter assays. In addition, it is prudent to verify any activating and/or inhibitory effects with, for example, two-hybrid systems that interrogate CAR interactions with specific NR coactivators and corepressors, respectively.[30,31] Despite the above problems, the current list of CAR-activating compounds has expanded to contain several drugs, environmental chemicals, herbal medicines, and flavonoids.[2,26,32,33]

In contrast, the range of human CAR inverse agonists is currently very narrow. The high constitutive activity can be repressed by androstenol, an inverse agonist of mouse CAR with an IC_{50} value below 1 μM.[34,35] However, androstenol is much weaker in inhibiting human CAR activity, with the maximal effect at 10 μM (E_{max}) being about 30%. Other reported inverse agonists for human CAR include the above-mentioned clotrimazole (E_{max} ~30−60%),[12,24,25] 17α-ethinylestradiol (EE2) (E_{max} ~50%),[36] the antiemetic meclizine (E_{max} ~50%),[37] and the peripheral benzodiazepine receptor ligand PK11195 (E_{max} ~80%).[38] The latter three compounds appear to be partial agonists for mouse CAR.[27,37] In ligand- and structure-based virtual screening campaigns for additional human CAR ligands, we have identified several novel human CAR agonists.[30,31] Because these screening projects were based on the occupancy and good fit of chemicals within the human CAR LBP with no requirements on receptor activity, both agonists and inverse agonists could be identified during screening. Here, we report a novel compound S07662 which is able to strongly inhibit human CAR activity via recruitment of NR corepressors and to attenuate inducer-elicited CYP2B6 expression in human primary hepatocytes. Moreover, we compare and contrast the properties of other reported human CAR inverse agonists with the novel compound S07662.

MATERIALS AND METHODS

Chemicals. Meclizine was a kind gift from H. Wang (University of Maryland, MD) and T. Sueyoshi (NIEHS, NC). Tri-*p*-methyl phenyl phosphate (TMPP) was synthesized as described previously.[39] Phenobarbital was obtained from Kuopio University Apothecary (Kuopio, FI), 6-(4-chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime (CITCO) from Biomol (Plymouth Meeting, PA), efavirenz from Toronto Research Chemicals, Inc. (North York, ON, CA), and simvastatin from Synfine Research, Inc. (Richmond Hill, ON, CA). The steroids were purchased from Steraloids, Inc. (Newport, RI) or Sigma Aldrich (St. Louis, MO). Other chemicals were of at least analytical grade from Sigma Aldrich, Calbiochem, or Riedel de-Haën. Apart from phenobarbital, all chemicals, including those ordered from Maybridge, Inc. (Trevillet, UK), were diluted in dimethyl sulfoxide (DMSO).

Discovery of S07662. The novel compound 1-[(2-methylbenzofuran-3-yl)methyl]-3-(thiophen-2-ylmethyl) urea (S07662) was found in a search for novel human CAR ligands by creating a set of 500 molecules from the Maybridge database. First, all compounds with molecular weights between 250 and 500 were chosen. Then, so-called random numbers were calculated for these compounds in Molecular Operating Environment (MOE) (Chemical Computing Group Inc., Quebec, Canada), the list of compounds arranged according to the random numbering, and the first 500 compounds in the list were picked. For pharmacophore searches, multiple conformations of these 500 compounds were created using program OMEGA 2.0 (OpenEye Scientific Software Inc., Santa Fe, USA) (with default settings except maxconfs = 500, rms = 0.4, buildff = mmff94s_Trunc). The pharmacophore for the searches was created based on aromatic GRID fields (calculated with GRID 22a, Molecular Discovery Ltd., Pinner, UK) and static hydrophilic and hydrophobic contact fields (calculated with MOE) for the human CAR crystal structure 1XVP, chain D. The excluded volumes were created with MOE based on the pharmacophores of 18 previously identified ligands.[30] The 500 compounds were docked into 1XVP using the GOLD docking suite (Cambridge Crystallographic Database, Cambridge, UK). The docking site was defined in GOLD by using the ligand molecule extracted from the crystal structure, and the dockings were performed with default settings, 7−8 times speed-up, and with two hydrophobic constraints which were chosen based on the most favorable positions in the static hydrophobic contact fields. The radius of the constraints was 3 Å. GoldScore was used as a scoring function, and hydrophobic atoms that fit inside the constraint spheres in docking solutions were given extra points in GoldScores. The pharmacophore used in the search was utilized as an additional scoring method and combined with the docking results; GoldScore of 0 was given to the molecules that did not fit into the pharmacophore. Pharmacophores created based on 18 previously identified ligands[30] were also tested in the searches, but they did not prove to be as selective. The 35 top-ranking compounds including S07662 were purchased from Maybridge and screened for human CAR activity with the mammalian 1-hybrid assay as described below. The normalized reporter activity data for each compound was standardized to the reference compound clotrimazole and vehicle control DMSO reporter activities on each plate (see the Supporting Information, SI). After the initial activity screening, compound S07662 was synthesized to high purity in-house (see the SI).

Mammalian 1-Hybrid and Cytotoxicity Assays. C3A hepatoma cells (ATCC CRL-10741, a clonal derivative of the HepG2 cell line) were seeded onto 48-well plates (183 000 cells/cm^2) in phenol red free Dulbecco's modified Eagle's medium (DMEM; Gibco 11880, Invitrogen, Gaithersburg, MD) complemented with 10% fetal bovine serum (FBS; BioWhittaker, Cambrex, Belgium), 1% L-glutamine (Euroclone, Pero (Milano), Italy), and 100 U/mL penicillin−100 μg/mL streptomycin (Euroclone) and grown overnight at 37 °C in a 5% CO_2 atmosphere. The cells used for transfections were from passages 7−25 and were transfected with CMX-GAL4-hCAR or hPXR LBD (450 ng/well), UAS4-tk-luciferase (300 ng/well), and pCMVβ (600 ng/well) by using the calcium phosphate method, as described previously.[31] After transfection, the medium was replaced with fresh DMEM complemented with 5% delipidated serum (HyClone, Logan, UT) instead of 10% FBS and including either the vehicle control (0.1%), reference compounds, or tested chemicals in triplicates at indicated concentrations. After treatment for 24 h, the cells were lysed and assayed for reporter gene activities as described.[40] The cytotoxicity of the tested chemicals was determined by the MTT assay.[41]

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Mammalian and Yeast 2-Hybrid Assays. The NR interaction domains of human corepressor NCoR (residues 1972−2290) and coactivator SRC1 (residues 549−789) were cloned between the *Eco*RI and *Bam*HI sites of the pM vector (Matchmaker kit, BD Clontech, USA) and the NdeI and *Bam*HI sites of the CMX-GAL4-vector, respectively. CMX-GAL4-SRC1 or pM-NCoR coactivator domains (250 ng/well) and VP16-human CAR LBD (residues 108−348) (250 ng/well) were cotransfected together with the control plasmid pCMVβ (600 ng) as well as the luciferase reporter pG5-luc (300 ng/well) to C3A cells grown on 48-well plates, treated with test chemicals, and assayed for reporter gene activities as described above. For the yeast 2-hybrid assay, pGBKT7 and pGADT7 plasmids (BD Clontech, USA) encoding the human CAR LBD and the human NCoR interaction domain have been described.[36] Yeast colonies expressing both CAR LBD and the interacting partner (NCoR) were grown, treated with DMSO vehicle (0.1%) or selected test chemicals, and assayed as before for β-galactosidase activity and cell density.[27]

Production of Guman CAR LBD and Limited Protease Digestion Assay. *Escherichia coli* BL21 (DE3) cells were transformed with the His_6-hCAR LBD (residues 103−348) construct in pET-15b expression vector (Novagen). Protein production was induced overnight at 20 °C with 0.75 mM isopropyl thio-β-D-galactoside in Luria−Bertani medium. The fusion protein was purified on a metal-affinity resin (Clontech), washed, and eluted with stepwise addition of imidazole (10−250 mM) in elution buffer (50 mM Tris-HCl at pH 8.0, 100 mM NaCl, 10% glycerol). The fractions were analyzed using sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE), and the 50 mM imidazole fraction contained highly purified His_6-human CAR LBD.

In predetermined conditions, His_6-human CAR LBD (~100 pmol in 9 μL) in elution buffer was preincubated with DMSO or selected human CAR inverse agonists (300 μM) for 25 min at 25 °C. Subtilisin A from *Bacillus licheniformis* (Sigma-Aldrich) was added to 1 ng/μL, and the digestion was continued for 30 min at 25 °C. The reaction was terminated by addition of 3 μL of 5× SDS protein loading buffer. The samples were denatured, separated by electrophoresis through 16% SDS-PAGE gels, and stained with Coomassie Blue, and gel images were captured (ImageQuant System, GE Healthcare Life Sciences).

Docking of the Ligands and Molecular Dynamics (MD) Simulations. Briefly, the dockings were performed with GOLD docking suite (version 4.0, Cambridge Crystallographic Database: Cambridge, UK, 2008). Some 10 ns MD simulations were carried out to study the structural behavior of selected ligand/LBP complexes at a longer time scale. The cutoff for Lennard−Jones interactions was 8 Å, and the particle-mesh Ewald (PME) method was used for the treatment of electrostatic interactions.[42] The trajectories were analyzed for root-mean-square deviations (rmsd), atomic positional fluctuation (APF), and protein secondary structure with the PTRAJ program of Amber Tools 1.4.[43] The contours of LBPs were visualized with the MOLCAD module of Sybyl-X (version 1.1.2) (Tripos International, St. Loius, MO, USA) using a 1.6 Å probe radius. Structures were visually examined with the assistance of Sybyl-X and the VMD program.[44] A detailed description of ligand docking and subsequent MD simulation is available upon request.

Induction of CYP mRNA in Human Primary Hepatocytes. Freshly seeded primary hepatocytes from a 73-year-old male were obtained from Biopredic International (Rennes, France). The cells were seeded on collagen I-coated 96-well plates (0.16−0.18 × 10^6 per cm^2) in Williams E medium. After a medium change, the cells were exposed to DMSO (0.1%, v/v) and selected chemicals for 24 h in triplicate wells like previously described.[31] Total RNA was isolated and reverse-transcribed to cDNA using the TaqMan Gene Expression Cells-to-CT kit (Applied Biosystems/Ambion Inc., Austin, TX) according to the manufacturer's instructions. Real-time quantitative reverse transcription polymerase chain reactions (RT-PCRs) for each sample were performed in triplicate reactions by using TaqMan chemistry on an ABI Prism 7500 Instrument and the following TaqMan Gene Expression assays: CYP3A4 (Hs00430021_m1, NM_017460), CYP2B6 (Hs03044634_m1, NM_000767), β-actin (4326315E, NM_001101), and GAPDH (4326317E, NM_002046). The fluorescence data were processed with eq 2 in the QGene program,[45] and the measured CYP mRNA levels were normalized to β-actin and GAPDH mRNA expression.

Data Analysis. All experiments were performed in triplicate. Data are presented as mean ± SEM (standard error of the mean). Differences between treatments were compared with the paired Student's *t*-test with the Šidák correction and considered significant when $p < 0.05$.

RESULTS

Discovery and Comparison of Human CAR Inverse Agonists. Virtual screening was used to find molecules which fit into the ligand binding pocket of human CAR. The 35 chemicals selected in virtual screening were assayed at 10 μM final concentration for human CAR activity with the mammalian 1-hybrid assay (see SI, Table S1.). Our definition of an agonist was a compound with a standardized response of 50% or more, as compared to the reference compound clotrimazole. Altogether 14 out of 35 compounds (40%) fulfilled this criterion. Three compounds that inhibited the basal human CAR activity by more than 50% were assigned as inverse agonists: HAN00020 (51%), HTS09666 (60%), and S07662 (89%). Upon closer examination, HTS09666 proved to be slightly cytotoxic and was not carried further in the analysis. To compare a larger set of inverse agonists in further studies, we selected the novel compounds HAN00020 and S07662, the reported inverse agonists androstenol, EE2, meclizine, and PK11195[34−38] and reanalyzed 1,9-dideoxyforskolin and etiocholanolone which had displayed at least a 30% decrease in human CAR activity in our prior, unpublished screening studies. Apart from meclizine and HAN00020, all of these chemicals (10 μM) showed a decrease in human CAR activity (10−77% of DMSO control) in the M1H assay (Table 1), while the agonistic reference compounds CITCO, clotrimazole, and TMPP displayed, as expected, 21.6-, 4.5-, and 2.9-fold activation, respectively. Rather surprisingly, meclizine increased human CAR activity by 2.1-fold.

To study the mechanism of inhibition, these compounds were tested for the ligand-dependent interaction of human CAR LBD with NR coregulators SRC1 and NCoR (Table 1). Recruitment of the coactivator SRC1 to human CAR LBD in the mammalian 2-hybrid assay was robust with the agonists clotrimazole, TMPP, and CITCO (34- to 136-fold activation) and quite low (<5-fold) for all inverse agonists except for HAN00020 (13-fold) and PK11195 (18-fold), suggesting that the latter two compounds may possess characteristics of a partial agonist. In the mammalian NCoR assay, only the inverse agonists S07662 and PK11195 responded strongly (24- and 20-fold, respectively), while other inverse agonists and reference agonists yielded modest 1.5- to 4.1-fold increases in NCoR recruitment. However, in the yeast cells, the human CAR/NCoR interaction was significantly enhanced

Table 1. Human CAR (hCAR) Activity and Cofactor Recruitment in Mammalian and Yeast Assays with Selected Inverse Agonists and Reference Compounds[a]

	M1H	M2H		Y2H
chemical (μM)	hCAR	hCAR + SRC1	hCAR + NCoR	hCAR + NCoR
DMSO (0.1% v/v)	100 ± 6	100 ± 10	100 ± 5	100 ± 9
androstenol (10)	76.8 ± 0.3	467 ± 49	167 ± 27	1450 ± 27.9
1,9-dideoxyforskolin (10)	47.9 ± 6.1	117 ± 0.04	158 ± 4.7	315.3 ± 15.9
EE2 (10)	29.7 ± 3.9	239 ± 42	152 ± 1.0	1083 ± 52.4
etiocholanolone (10)	62.4 ± 3.8	199 ± 0.06	198 ± 12	2538 ± 79.0
HAN00020 (10)	110.4 ± 20.1	1311 ± 38.1	405 ± 4.8	ND
meclizine (10)	212 ± 4.0	296 ± 20.8	169 ± 11	175.8 ± 1.94
PK11195 (10)	49.8 ± 2.3	2038 ± 419	1961 ± 102	2197 ± 41.4
S07662 (10)	21.4 ± 1.8	186 ± 15	2422 ± 250	3358 ± 255
CITCO (1)	2164 ± 216	13609 ± 720	239 ± 8.4	112.0 ± 3.71
clotrimazole (4)	453.8 ± 35.2	3435 ± 254	363 ± 17	468.9 ± 24.3
TMPP (10)	294.4 ± 21.6	2708 ± 205	196 ± 6.8	99.12 ± 6.27

[a]The results are presented as fold ± SEM over DMSO set as 100. Control reactions with empty expression vectors or yeast expressing only NCoR with reference compounds (agonists and inverse agonists) were performed. No reporter activity was seen in these experiments.

not only by S07662 (34-fold) and PK11195 (22-fold) but also by all inverse agonists with a steroidal structure: androstenol (14-fold), EE2 (11-fold), and etiocholanolone (25-fold). Other compounds, including meclizine, had more modest responses (<5-fold). Competition experiments indicated that the strong human CAR/NCoR interaction elicited by inverse agonists was attenuated by reference agonists CITCO, clotrimazole, and TMPP (for PK11195 by 20−70% and for S07662 by 60−85%), presumably by their competition for the same or overlapping binding site (Figure 1).

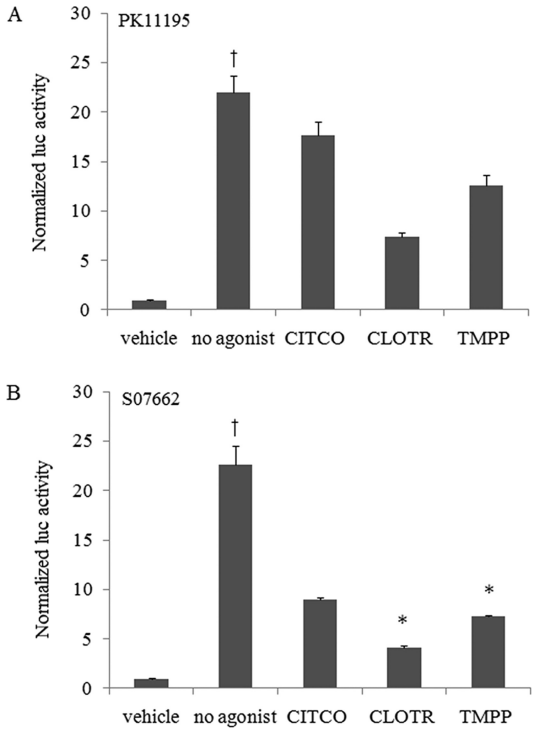

Figure 1. Competition experiments (hCAR/NCoR interaction, mammalian 2-hybrid) of (A) PK11195 (10 μM) and (B) S07662 (10 μM) with reference agonists CITCO (0.1 μM), clotrimazole (CLOTR, 4 μM), and TMPP (10 μM). The results are presented as mean ± SEM. $^{\dagger}p < 0.05$ (inverse agonist vs vehicle), $^{*}p < 0.05$ (inverse agonist alone vs inverse agonist + reference agonist).

The potency of the most efficacious compounds was sought after in dose−response studies. In the mammalian 1-hybrid assay, EE2 and androstenol showed a dose-dependent decrease in human CAR activity with IC_{50} values of about 1.5 μM and 4 μM, respectively (Figure 2). Meclizine did not show a progressive change in human CAR activity, while clotrimazole displayed a modest, dose-dependent increase reaching about 2-fold activation. At concentrations higher than 10 μM, clotrimazole appeared to be toxic to the cells. Both PK11195 and S07662 inhibited the human CAR activity with IC_{50} values of about 0.8 μM and 0.7 μM, respectively. In the mammalian 2-hybrid assay for human CAR/NCoR interaction (Figure 3), androstenol, PK11195, and S07662 showed a clear concentration-dependent increase in reporter activity up to 11-, 21-, and 46-fold, respectively. Meclizine showed no response, and clotrimazole yielded about a 3-fold activation. The approximate half-maximal concentrations in this assay seemed higher than in the mammalian 1-hybrid system, which may be related to the fact that the former assay involves coregulator *peptides*, whereas the mammalian 1-hybrid system relies on intact, cell-derived coregulators.

The recruitment of corepressors suggested that inverse agonists are able to evoke conformational changes in the human CAR LBD. Using the purified human CAR LBD, we conducted limited protease digestion *in vitro* with subtilisin A (Figure 4). In the absence of any added ligand, human CAR LBD was efficiently digested by the protease, whereas agonists clotrimazole and CITCO protected the LBD from degradation. Similarly, inverse agonists also stabilized the human CAR, and PK11195 and S07662 afforded better protection than the weaker inverse agonist EE2. Even though we cannot distinguish agonists from inverse agonists by specific proteolysis patterns, these results indicate that both types of ligands can stabilize the human CAR LBD to a conformation that is permissive of appropriate coregulator binding.

Molecular Dynamics (MD) Simulations. Because S07662 inhibited human CAR activity by attracting NCoR and appeared to stabilize the LBD, we concentrated in defining its mechanism of action by MD simulations. As judged from the root-mean-square deviation data (see SI, Figure S2), human CAR LBD remained stable during the simulations. The most noticeable change in the ligand-free (*apo*) structure was the

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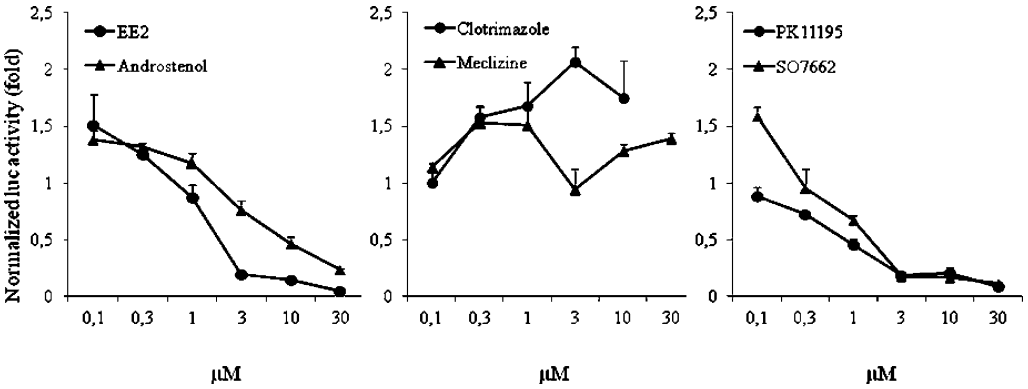

Figure 2. Reporter gene activities in the human CAR mammalian-1-hybrid assay. The results are presented as mean ± SEM. The vehicle control DMSO was set as 1, and the 1 μM CITCO used as a control on all plates showed an average fold-activation of 13 ± 0.6 in the experiments. Clotrimazole was toxic to the cells at a 30 μM concentration.

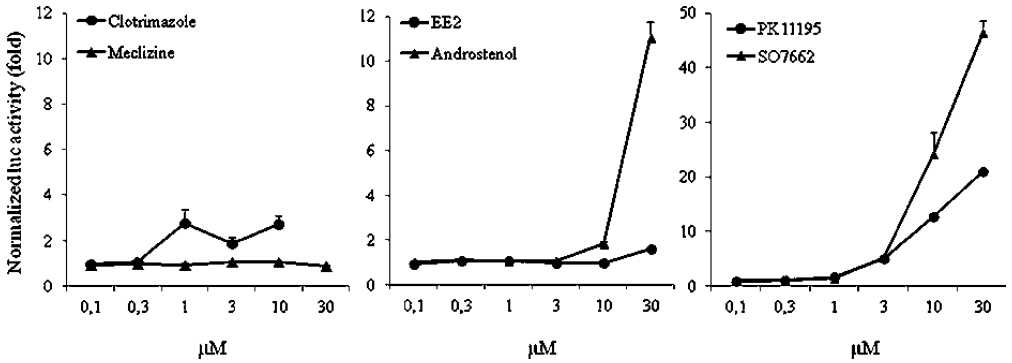

Figure 3. Reporter gene activities from corepressor (NCoR) recruitment in a mammalian-2-hybrid assay. The results are presented as mean ± SEM. The vehicle control DMSO was set as 1, and the 10 μM EE2 used as a control on all plates showed an average fold-activation of 0.7 ± 0.1 in the experiments. Clotrimazole was toxic to the cells at a 30 μM concentration.

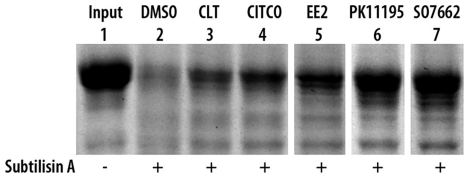

Figure 4. Limited protease digestion by selected human CAR ligands. About 100 pmol of purified His6-hCAR LBD (lane 1, input) was preincubated for 25 min with solvent DMSO (lane 2), human CAR agonists clotrimazole (CLT, lane 3), or CITCO (lane 4) or inverse agonists EE2 (lane 5), PK11195 (lane 6), or S07662 (lane 7) (300 μM each) before a 30 min digestion reaction in the presence of subtilisin A (lanes 2–7).

increase in rmsd at 8.5–10 ns which is caused by the opening of the loop between helices 2 and 3 (see SI, Figure S3), thought to be one of the possible ligand entries into the LBP.[46,47] The agonist CITCO and the inverse agonists PK11195 and S06772 appear to stabilize the overall LBD structure as judged by the decrease in the APF values around the loop region between helices 2 and 3 and also at the β sheet and helix 6 (see SI, Figure S3). This finding coincides very well with our limited protease digestions suggesting that both types of ligands can stabilize the human CAR LBD. Next, we focused on the C-terminal helices 10, X, and 12 which are important for human CAR basal activity and coactivator recruitment.[22,48] When the final structures from the simulations were inspected, the LBP was opened to the direction of helix 12 more by inverse agonists PK11195 and S07662, as compared to the ligand-free *apo* structure or by CITCO (Figure 5). Especially, S07662 projected further toward the helix 12 than other compounds and, thus, likely interfered with helix 12 in occupying the so-called "active position" critical for SRC1 recruitment. On the other hand, PK11195 (which can recruit SRC1 to some extent) pointed toward the loop between helices 6 and 7 which is located underneath the loop connecting the helices 10 and X. Thus, it is conceivable that PK11195 might influence the conformation of helix X.

When the C-terminal helices were analyzed in detail in the absence or presence of these ligands (Figure 6), we found that the agonist CITCO further stabilized both helices X and 12 because they assumed more highly helical conformations as compared to the *apo* form during the MD simulations (Figure 6A). This suggested that helix 12 was retained by CITCO in the active position for coactivator recruitment, which is supported by the final structure (Figure 6B) and by the APF monitored during the MD run (Figure 6C). In contrast, the inverse agonists S07662 and PK11195 do not increase the helical content of helix X, and the latter compound seems to distort this region of the LBD. Although both inverse agonists appear to stabilize helix 12 (Figure 6A,B), its position deviated significantly during the MD runs, especially for S07662, as judged by the APF data at the end of helix 12 (Figure 6C). In the final structures of the MDs (Figure 5), the distance between the helix 12 residue Leu342 α-carbon atoms between the *apo* and S07662-bound forms was 2.6 Å. This distance is enough to disrupt hydrophobic and hydrogen-bonding interactions.

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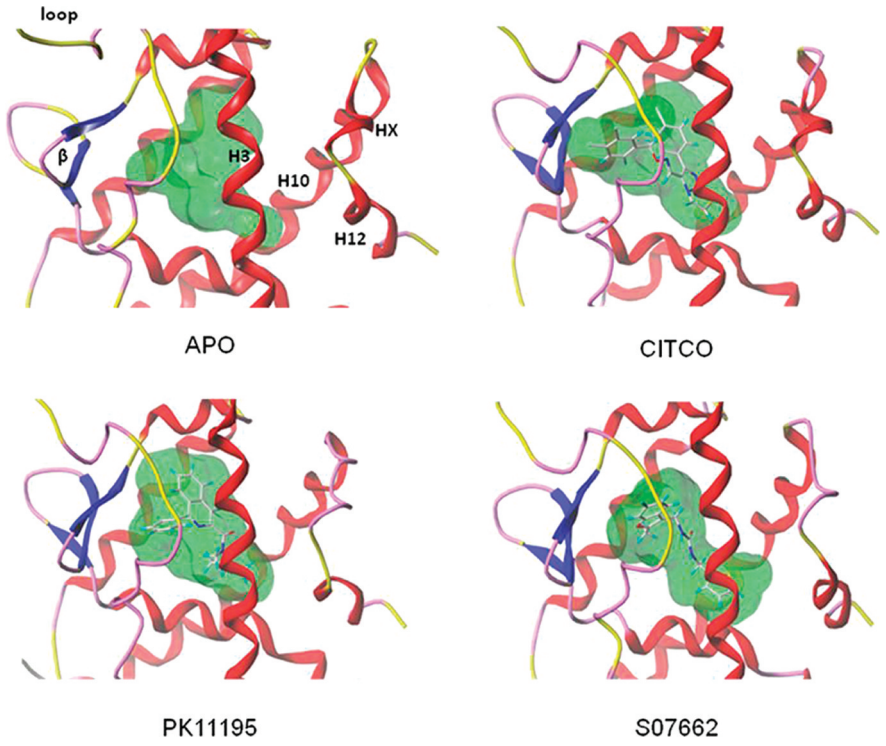

Figure 5. Ligand-induced changes in the cavities of the human CAR LBP and subsequent effects in the coregulator recruitment. The structural figures (final conformations from MD runs) show how inverse agonists are able to open the cavity toward H12. The Connolly surfaces of the LBP cavities are shown in green.

Suppression of CYP2B6 Expression in Human Primary Hepatocytes. To test the ability of the inverse agonists to suppress the expression of human CAR target genes, we measured their ability to attenuate induction of CYP2B6 mRNA by phenytoin and CITCO. These CYP inducers are rather specific for human CAR[49,50] but are thought to mediate their effects either by an indirect mechanism or by direct agonist binding, respectively. However, there is some recent evidence that phenytoin might bind weakly to the CAR LBD.[51] In human primary hepatocytes (Figure 7), phenytoin treatment (15 μM) resulted in about 24-fold increase in CYP2B6 mRNA level, which was inhibited about 66% and 84% by the steroidal inverse agonists EE2 and androstenol at 10 μM, respectively. PK11195 and S07662 attenuated CYP2B6 mRNA expression by 71% and 88% at 10 μM, and progressed to basal levels or even below at the highest 40 μM concentration (PK11195 99%, p = 0.07 and S07662 96%, p = 0.03). A similar but slightly weaker overall trend of suppression was observed with the CITCO-induced (0.1 μM) CYP2B6 expression (PK11195 91% and S07662 70% at 40 μM; $p > 0.05$). In the absence of added human CAR agonist, CYP2B6 mRNA level was moderately increased by androstenol (4.4-fold), EE2 (6.4-fold), and PK11195 (4.3-fold), while S07662 affected it only slightly (1.8-fold). Because the *CYP2B6* gene can be activated by both CAR and PXR,[36,52] it was possible that some inverse agonists activated the human PXR in addition to inhibiting human CAR. We therefore measured the effect of inverse agonists on human PXR activity in mammalian 1-hybrid assay and on CYP3A4 mRNA expression in primary hepatocytes (Figure 8). Androstenol and EE2 showed no effect, S07662 moderate (<8-fold, p = 0.03), and PK11195 strong (13-fold, p = 0.02) activation of human PXR as compared to the positive control rifampicin (9.7-fold) (Figure 8A). For CYP3A4 expression in primary hepatocytes (Figure 8B), low induction was observed with androstenol and EE2, while PK11195 and S07662 elevated CYP3A4 mRNA by 4.9- (p = 0.02) and 6.8-fold (p = 0.08), respectively.

DISCUSSION

The present study aimed first at discovering novel compounds that would expand the repertoire of ligands as tools to study human CAR biology. Second, we performed a systematic comparison of all reported inverse agonists, because such studies have not yet been made. In the search process, we utilized a virtual screening protocol that combined both ligand- and protein-based search criteria. The high success rate of the virtual screening (17 actives/35 tested) reflects its efficiency in finding novel agonists and inverse agonists for human CAR. The fact that agonists (14) were found more often than inverse agonists (3) is understandable in the light of previous 3D-QSAR studies on human CAR.[30] The occupancy of three regions within the CAR LBP was connected with increased agonism, whereas only one region was associated with decreased activity.

Our virtual and *in vitro* screening experiments utilizing several different assays provide an efficient means for systematic comparisons between ligands. We can now verify that EE2 and androstenol inhibit human CAR by the recruitment of corepressors. Presently, we cannot explain the dissimilar efficacy of NCoR recruitment between mammalian and yeast cells for these steroids, but plausible reasons include differential metabolism and/or transport of ligands, presence of interfering coregulators or other proteins in mammalian cells, or differences in post-translational modification of coregulator peptides between yeast and mammalian cells that might affect the sensitivity of the assays. As a further proof of their inverse agonism, EE2 and androstenol suppressed phenytoin- and CITCO-induced CYP2B6

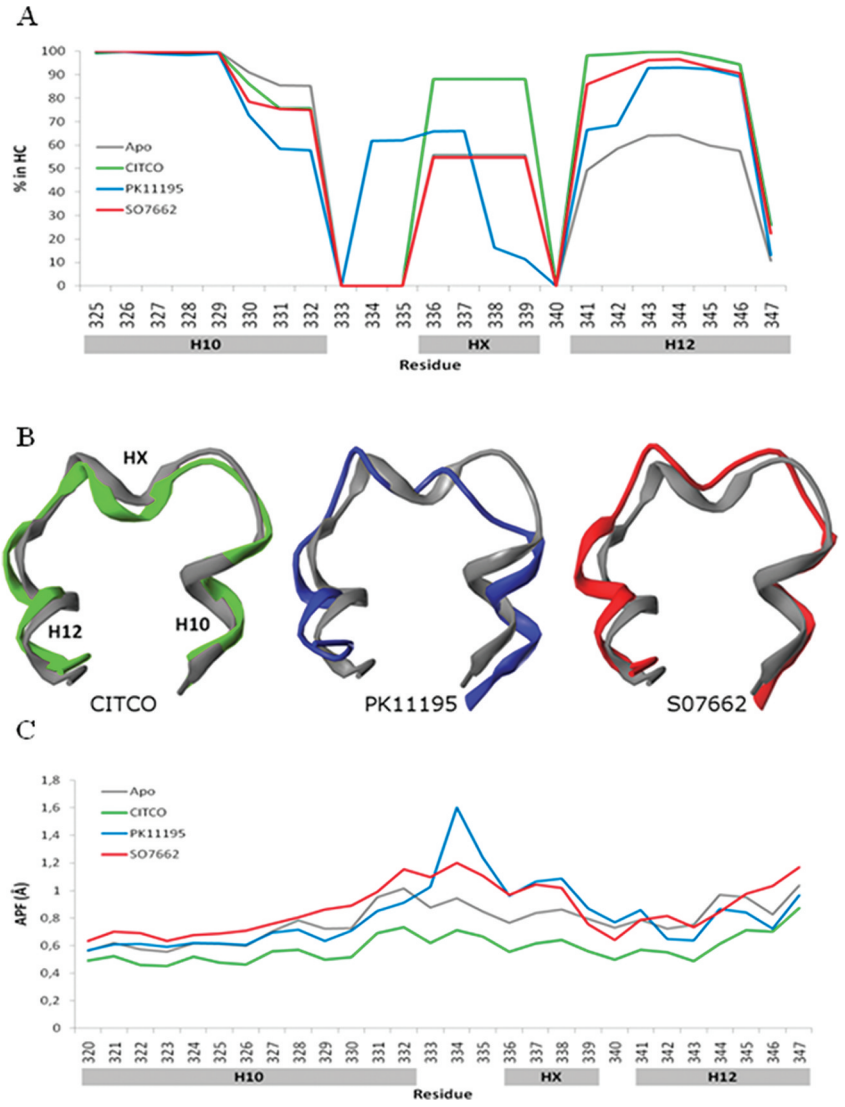

Figure 6. Conformational changes in helices 10, X, and H12 of human CAR induced by ligands during 10 ns MD simulations. (A) The relative time (HC %) residues assume a helical conformation in the MD runs. (B) The structural figures of the final conformations from MD runs show movements in the presence of ligands (in color) relative to the apo structure (gray). (C) Atomic positional fluctuation (APF) for the backbone atoms of the human CAR C-terminus is illustrated.

mRNA expression in human primary hepatocytes. With these assays, we could confirm the report that PK11195 inhibits human CAR activity but is a human PXR agonist.[38] However, we could not reproduce the finding that meclizine is a human CAR inverse agonist.[37] First, human CAR activity was not decreased by meclizine, and second, meclizine could not recruit NCoR to human CAR in mammalian or yeast cells. These findings support the very recent report that meclizine could not repress constitutive or CITCO-enhanced human CAR reporter activity and CYP2B6 expression in primary hepatocytes.[53] Together, these two studies strongly suggest that meclizine is not an inverse agonist for human CAR. In contrast, we identified an unrelated chemical S07662 that is, at equimolar concentrations, more efficacious than the previously reported inverse agonists. Collectively, S07662 inhibited human CAR reporter activity to the greatest extent, elicited the strongest response in NCoR recruitment, and minimally attracted the coactivator SRC1 as compared to PK11195 and other compounds. In addition, S07662 suppressed phenytoin- and CITCO-induced CAR target gene *CYP2B6* expression by more than 90 and 70%, respectively.

On a minor note, despite having a better inverse agonist profile, S07662 repressed CYP2B6 to a lesser degree than PK11195 in the presence of CITCO. Although direct comparisons of results from reporter assays in hepatoma cells and CYP2B6 expression in hepatocytes are problematic due to dissimilar contents of NR coregulators and metabolic capacities of the cells, it is possible that PK11195 occupies and interacts with the ligand-binding pocket more effectively due to its larger size and Y-shaped structure in comparison to the smaller, L-shaped S06772. This explanation seems to match with our competition results showing that human CAR agonists interfered more efficiently with NCoR interaction elicited by S07662 than by that of PK11195. It must also be acknowledged that both PK11195[38] and to a lesser extent S07662 suffer from the fact that they activate human PXR and, thus, induce CYP3A4 in primary hepatocytes. Despite the above-mentioned drawbacks, S07662 seems to be as potent as PK11195 and in some cases even outperforms it, in most of the assays performed. Given its better coregulator profile and weaker propensity for human PXR activation, we feel that S07662 will be a useful tool for the interrogation of human CAR biology.

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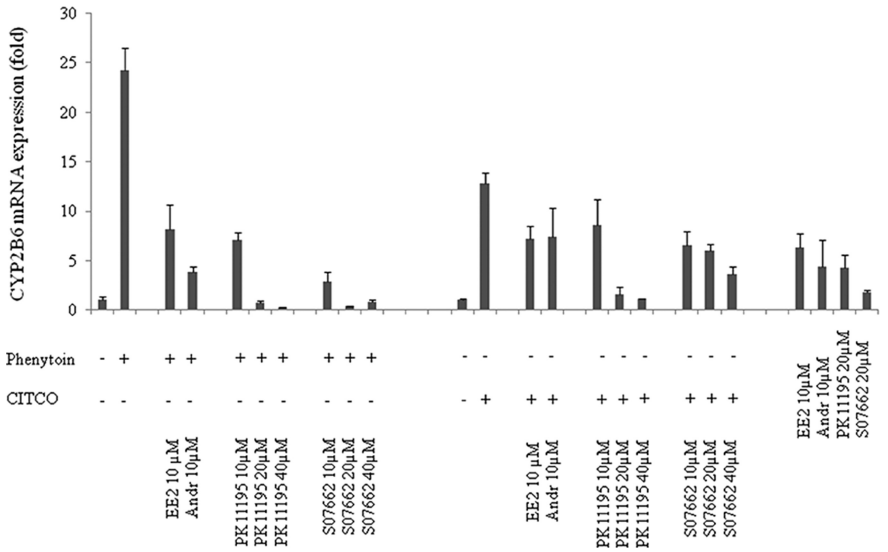

Figure 7. Effects of the inverse agonists on basal and induced (CITCO 0.1 μM and phenytoin 15 μM) CYP2B6 mRNA expression. The results are presented as fold-activation/expression ± SEM over vehicle control (DMSO) set as 1.

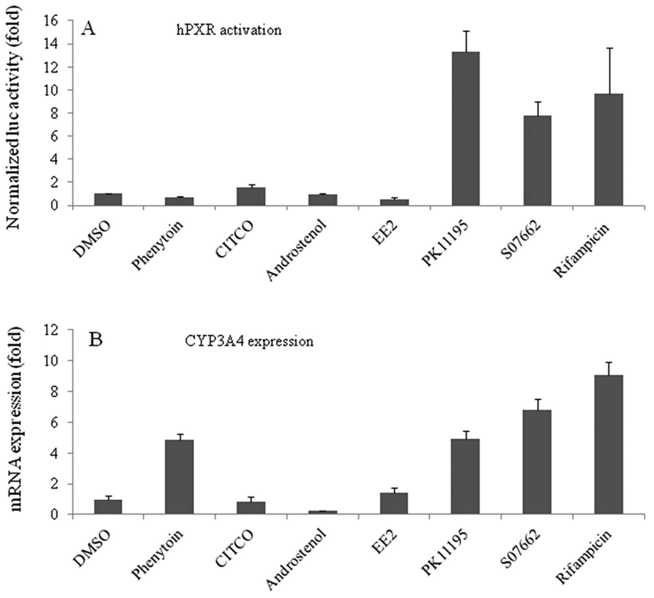

Figure 8. Effects of phenytoin (15 μM), CITCO (0.1 μM) ,and inverse agonists (androstenol and EE2 10 μM, PK11195 and S07662 20 μM) on hPXR activity (A) and CYP3A4 mRNA expression (B). The results are presented as fold-activation/expression ± SEM over vehicle control (DMSO) set as 1.

Our limited protease digestion and NCoR/SRC1 recruitment studies suggested that all human CAR ligands stabilize the human CAR LBD in such a way that the binding of an appropriate coregulator is enhanced. The MD simulation studies emphasize this idea because movement of the entire CAR LBD backbone was attenuated upon binding of CITCO, S07662, or PK11195. However, these ligands show intriguing differences at the C-terminus of the LBD encompassing the functionally important helices X and 12. In contrast to agonist CITCO, inverse agonists did not stabilize helix X. Although all ligands increased the helical content of helix 12, its orientation was shifted by inverse agonists. These two changes result in opening the LBP toward helix 12, allowing inverse agonists to occupy some of this space. This finding matches well with our previous study that the presence of a ligand near helix 12 is associated with decreased human CAR activity.[30] In MD runs, no major rearrangements of helix 12 due to sterical reasons were seen, as described for raloxifene and estrogen receptor and for LG100754 and RXR.[54,55] There are several reasons for such a modest displacement of helix 12. The potential contacts between the ligand and helix 12 are limited by a narrow channel from the LBP to helix 12, so the inverse agonists do not create a major steric clash with helix 12. Because helix 12 is rather short, it may accommodate the corepressor by yielding toward helix 10. A similar movement has been observed with progesterone receptor and asoprisnil.[56]

In conclusion, the power of virtual screening combined with comprehensive *in vitro* assays resulted in identification of a novel human CAR inverse agonist that surpasses the previously reported compounds in efficacy and other characteristics. In addition, MD runs complemented with *in vitro* binding and recruitment assays give insight as to molecular mechanisms of inverse agonism. The compound S07662 will be useful addition as a chemical tool to address various biological functions or CAR, and by utilizing these comprehensive methods, a valuable starting point for novel drug discovery for dyslipidemias and liver diseases that involve CAR.[57,58]

■ ASSOCIATED CONTENT

Supporting Information

Synthesis protocol of S07662, the M1H screening data of the Maybridge compounds, and the calculated root-mean-square deviations (rmsd) of the α carbon atoms of CAR as well as the atomic positional fluctuation (APF) analysis of the CAR backbone. This material is available free of charge via the Internet at http://pubs.acs.org.

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