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Discovery of substituted sulfonamides and thiazolidin-4-one derivatives as agonists of human constitutive androstane receptor

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ABSTRACT

The constitutive androstane receptor (CAR; NR1I3) is a nuclear receptor responsible for the recognition of potentially toxic endo- and exogenous compounds whose elimination from the body is accelerated by the CAR-mediated inducible expression of metabolizing enzymes and transporters. Despite the importance of CAR, few human agonists are known so far. Following a sequential virtual screening procedure using a 3D pharmacophore and molecular docking approach, we identified 17 novel agonists that could activate human CAR *in vitro* and enhance its association with the nuclear receptor co-activator SRC1. Selected agonists also increased the expression of the human CAR target CYP2B6 mRNA in primary hepatocytes. Composed of substituted sulfonamides and thiazolidin-4-one derivatives, these agonists represent two novel chemotypes capable of human CAR activation, thus broadening the agonist spectrum of CAR.

1. Introduction

The constitutive androstane receptor (CAR, NR1I3) is a member of the nuclear receptor (NR) superfamily, a group of ligand-regulated transcription factors which are involved in many physiological processes including cellular development, homeostasis and metabolism [1]. Together with the pregnane X receptor (PXR, NR1I2), CAR governs the drug-inducible expression of cytochrome P450s (CYPs), conjugating enzymes and transporter proteins [2]. As xenobiotic sensors, CAR and PXR recognize and are activated by many chemically diverse compounds including synthetic drugs, natural products and xenobiotics [3], and both receptors display wide species differences in their spectrum of activators [2,3]. For example,

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Abbreviations: CAR, constitutive androstane receptor; CITCO, 6-(4-chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)-oxime; CYP, cytochrome P450; EE2, 17α-ethynyl-3,17β-estradiol; H12, helix 12; LBD, ligand-binding domain; LBP, ligand-binding pocket; NR, nuclear receptor; MIF, molecular interaction field; PB, phenobarbital; PDB, Protein Data Bank; PXR, pregnane X receptor; S.D., standard deviation; SRC1, steroid receptor coactivator-1; TCPOBOP, 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene; TMPP, tri-*p*-methylphenyl phosphate; vdW, van der Waals; VS, virtual screening.

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endogenous substances including bilirubin, bile acids and testosterone metabolites have been identified as CAR ligands and/or activators [4]. The pesticide contaminant 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) and the synthetic drugs chlorpromazine, meclizine and 17α-ethynyl-3,17β-estradiol (EE2) are efficient activators of mouse CAR [5–7]. However, these agonists do not activate human CAR at all; in contrast, meclizine and EE2 even inhibit it [8–10].

In unexposed liver, CAR is located in the cytoplasm where it forms a complex with the cytoplasmic CAR retention protein and heat shock protein 90 which help retain CAR in the cytoplasm [11,12]. CYP inducers dissociate the cytoplasmic complex in a yet unknown manner, trigger translocation of CAR into the nucleus and lead to the transcriptional activation of CAR target genes. Recently, several protein kinases [13,14], protein phosphatases [15,16] and other factors [17] have been implicated in modulation of the CAR translocation process.

The common activation mechanism for NRs involves the direct binding of the ligand to a pocket that is harbored by the ligand-binding domain (LBD). Agonist binding modulates the orientation of the activation helix 12 (H12) so as to allow the LBD to interact with NR co-activators such as the steroid receptor coactivator-1 (SRC1) [1,18]. However, only a few agonists which activate human CAR via direct binding to the ligand-binding pocket (LBP) are known. Among these, the steroid 5β-pregnane-3,20-dione and the synthetic compound 6-(4-chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime (CITCO) have been co-crystallized with the human CAR LBD [18]. Tri-*p*-methylphenyl phosphate (TMPP) and clotrimazole have been shown to bind to the LBD and to activate human CAR [8,19,20]. In addition to the direct CAR agonism, an indirect activation mechanism has been postulated for CAR. In this case, certain chemicals including bilirubin, phenobarbital (PB), phenytoin and acetaminophen are able to translocate and activate CAR and exert their CYP-inducing effects despite the fact that no direct binding to the CAR LBP could be demonstrated for these compounds [3,21,22]. The details of this indirect action are not understood, and some interpretations may be based on problems in detecting CAR agonism in assays with high basal activity of CAR [20,23,24].

In order to identify novel bioactive molecules, virtual screening (VS) techniques are a well-established technology. Despite its limitations, *in silico* screening has emerged as increasingly important in the drug discovery process. While diverse G protein-coupled receptors, enzymes and ion channels have been targeted by different VS approaches, only a few success stories for NRs have been reported although NRs represent an equally important class of pharmaceutically relevant proteins [25]. So far, VS efforts have led to the identification of retinoic acid receptor and thyroid hormone receptor antagonists as well as estrogen receptor modulators [26–31].

We aimed to discover novel human CAR agonists by virtually screening a drug-like compound library. The selection of CAR-interacting candidates was first guided by a ligand-based screening approach followed by a molecular docking procedure. Among 30 molecules tested *in vitro*, 17 emerged as agonists in a cell-based human CAR activation assay, and their agonism was confirmed by assays measuring co-activator recruitment. Selected agonists also activated the CAR target *CYP2B6* gene expression. The sulfonamides and thiazolidin-4-one derivatives identified here represent two novel chemotypes as efficacious human CAR agonists.

2. Materials and methods

2.1. Molecular modeling

All calculations were performed on Intel-based Linux workstations. The compounds of the LeadQuest® database (Tripos Inc., St. Louis, USA) were transformed into 3D molecular structures using the Concord module within SYBYL 7.0 (Tripos Inc., St. Louis, USA). All compounds were generated in the protonation state assumed to exist under physiological conditions.

Crystal structures of the CAR LBD were obtained from the Protein Data Bank (PDB) [32]. The volume of the CAR LBP was ascertained using the MOLCAD module (SYBYL 7.0) by calculating the Connolly surface with a probe radius of 1.4 Å [33,34]. For the calculations all hydrogen atoms were assigned to the structures using the Biopolymer module (SYBYL 7.0).

2.2. Pharmacophore-based screening

A detailed description of the homology model generation and docking procedure of clotrimazole and TMPP as well as a comparison with the human CAR X-ray structures has been previously described [35,36]. The Tripos LeadQuest® database containing approximately 85,000 compounds was used as starting point for the 3D database search. Since the experimental testing was carried out in a cell-based assay, compounds violating Lipinski's "rule-of-five" [37] were excluded from the search. A pharmacophore query within the UNITY module (SYBYL 7.0) was defined consisting of the Connolly surface of the LBP and two hydrophobic features. Potentially active compounds were expected to fit into the spatial environment of the search query whereas inactive molecules would not. The tolerance of the surface was set to 0.5 Å. The two hydrophobic features (tolerance 1.0 Å) were assigned based on the human CAR/ligand interactions.

2.3. Molecular docking

The selected LeadQuest® compounds were docked into the CAR X-ray structure (PDB entry 1XVP, chain D) using GOLD version 2.2 (CCDC, Cambridge, UK) [38]. The program has been recently shown to reproduce the binding modes of agonists in human CAR X-ray structures [36].

All molecules were docked within a sphere (radius 20 Å) defined around atom CD1 of amino acid Leu206. For each compound a maximum number of 10 poses was allowed. Pyramidal nitrogens were allowed to flip. From the top-ranked molecules, 66 compounds were selected according to their GoldScores as well as visual inspection. Altogether 30 molecules could be obtained from the supplier and were tested for human CAR activation in a cell-based assay described in Section 2.6.

2.4. Molecular interaction fields calculations

Interaction possibilities between ligands and CAR were analyzed using version 22 of the GRID program (Molecular Discovery Inc., Pinner, UK) [39]. GRID is an approach to predict non-covalent interactions between a molecule of known three-dimensional structure (*i.e.* human CAR LBD) and a small group as a probe (representing chemical features of a ligand). The following probes were used for the calculations: methyl probe (C3), aromatic probe (C1=) and hydrophobic probe (DRY).

2.5. Chemicals

The test chemicals were purchased from Tripos Inc. PB was obtained from Kuopio University Apothecary (Kuopio, Finland), CITCO from Biomol (Plymouth Meeting, PA), clotrimazole and EE2 from Sigma (St. Louis, MO). TMPP was synthesized in-house as described [8].

2.6. Activation assay for human CAR

C3A cells (ATCC CRL-10741) were cultured overnight in 48-well plates to 50% confluence in phenol red-free DMEM (Gibco 11880-028, Invitrogen, Gaithersburg, MD) complemented with 10% fetal bovine serum (BioWhittaker, Cambrex, Belgium), 1% L-glutamine (Euroclone, Pero (Milano), Italy) and 100 U/ml penicillin-100 μg/ml streptomycin (Euroclone). Then the cells were transfected for 5 h using calcium phosphate method with previously described plasmids CMX-GAL4-CAR LBD (450 ng/well), UAS4-tk-luciferase (300 ng/well) and pCMVβ (600 ng/well) [10,20]. After transfection, the medium was replaced with fresh DMEM including either the vehicle control dimethylsulfoxide (DMSO), reference compounds or test chemicals (10 μM), 10 μM EE2 and 5% delipidated serum (HyClone, Logan, UT) instead of 10% regular serum. After 24 h chemical treatment, the cells were lysed and the luciferase and β-galactosidase activities [40] were measured from 20 μl of the cell lysate using the Victor^{2TM} multiplate reader (PerkinElmer Wallac, Turku, Finland). All luciferase activities were normalized to β-galactosidase activities and the results are expressed as mean ± standard deviation (S.D.) of at least three independent experiment.

2.7. Mammalian two-hybrid assay for human CAR/SRC1 interaction

The plasmids in the MatchMaker kit (Clontech, Mountain View, CA) were used. The SRC1 co-activator domain for NR interaction (amino acids 549–789) [41] was cloned in-frame downstream of the GAL4 DNA-binding domain present in the pM plasmid. The human CAR LBD (residues 108–348) was fused with the VP16 transactivation domain present in the pVP16 plasmid. These plasmids (250 ng/well) were co-transfected together with the control plasmid pCMVβ (600 ng) and the luciferase reporter pG5-luc (Promega, Madison, WI) (300 ng) into C3A cells. The transfected cells were treated with vehicle DMSO, reference compounds or test chemicals (10 μM) for 24 h, and assayed for reporter activities as described above.

2.8. Induction of CYP2B6 mRNA in human primary hepatocytes

Freshly seeded BD Gentest^{TM} human hepatocytes (lot 169) were procured from BD Discovery Labware (Woburn, MA). The liver donor was a 48-year-old non-smoking Caucasian male with no known exposure to known CYP inducers or inhibitors. The cells were negative for hepatitis B, hepatitis C, HIV1-2 viruses, HTLV I-II and syphilis (RPR), and they were seeded on collagen I coated six-well plates (BD^{TM} Biocoat Multiwell, 12×10^6 cells per well) in BD^{TM} Hepato-STIM hepatocyte culture medium. Upon receipt, the medium was replaced immediately (about 48 h after seeding) with serum-free Hepatocyte Culture Medium (BD Gentest) supplemented with 2 mM L-glutamine and 1% penicillin–streptomycin and allowed to stabilize at +37 °C/5% CO_2 prior to the chemical exposure. The hepatocytes were exposed to DMSO (0.1%, v/v), CYP inducer PB (1.5 mM), reference compounds CITCO (1 μM) and TMPP (10 μM) or the test chemicals (10 μM) for 24 h.

Total RNA was isolated with TRI Reagent (Sigma) and contaminating DNA was removed by treatment with DNase (Applied Biosystems/Ambion, Austin, TX). RNA was quantified with RiboGreen dye (Molecular Probes, Netherlands) and reverse transcribed to cDNA as described before [42]. Equal amounts of cDNAs were used for amplification and detection of the CYP2B6 (NM_000767) and the reference β-actin (NM_001101) mRNAs using TaqMan chemistry on an ABI Prism 7500 instrument (Applied Biosystems). Forward and reverse primers and the probe for the CYP2B6 were: 5′-gacttcgggatgggaaagc-3′; 5′-tggcggtaatggactggaa-3′ and 5′-FAM-cttcggaaatccaagggggc-3′ while β-actin was analyzed with: 5′-cggcaccaccatgtaccc-3′, 5′-tggcggtaatggactggaa-3′, and 5′-VIC-atcaagatcattgctcctc-3′, respectively. The fluorescence data were processed with Eq. (2) in the QGene program [43], and CYP2B6 mRNA levels were normalized to β-actin mRNA expression. Comparisons between the groups were analyzed by Student's *t*-test with Bonferroni's correction.

3. Results

3.1. Virtual screening and validation of CAR activation

Initially, we used a homology model of the human CAR LBD for virtual screening as no crystal structure was available at that time [20]. Structural comparison of the model and the recently published crystal structures revealed a good agreement of side chain conformations within the LBP [36]. Moreover, binding modes of the co-crystallized ligands could be reproduced quite well in the model. Based on docked agonists TMPP and clotrimazole, a 3D pharmacophore model was generated within the UNITY module of SYBYL which was employed to screen the LeadQuest® database containing about 85,000 compounds. The search query was defined based on the volume and shape of the LBP and its interactions with known agonists (Fig. 1). Approximately 9700 compounds were found to fit into the spatial environment of the search query. In a second step, hit molecules retrieved from the pharmacophore search were docked into the now available CAR X-ray structure (PDB entry 1XVP) using the molecular docking

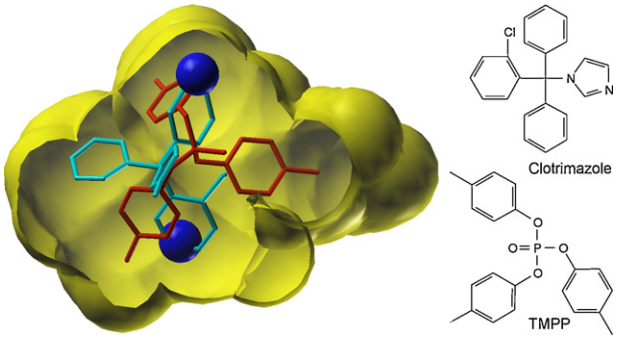

Fig. 1 – The search query for the 3D pharmacophore search. The query was based on the human CAR LBP volume and shape (surface in yellow) as well as two sites of vdW contacts (blue spheres) found between agonists clotrimazole (cyan) as well as TMPP (red) and the LBP. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

program GOLD and ranked according to the calculated GoldScore.

On the basis of the ascertained fitness scores as well as careful visual inspection, 66 compounds among the top-ranked poses were finally selected as potential CAR-interacting ligands. For experimental validation, altogether 30 compounds were available from the supplier and were tested in a cell-based CAR activation assay. A compound was considered as human CAR agonist when the basal activity was increased by 100% or more. According to this criterion, 17 compounds were identified as agonists, corresponding to a hit rate of 56.6%. Most agonists showed between twofold and fourfold activation, whereas three compounds were found to activate human CAR fivefold or eightfold, respectively (Table 1). For comparison, the known human CAR agonists CITCO and TMPP displayed a 1.44-fold and 2.45-fold activation, whereas clotrimazole activated CAR by 4.34-fold.

3.2. *Novel chemotypes as human CAR agonists*

All agonists discovered in this study can be grouped into two different chemotypes: substituted sulfonamides (**1–7**) as well as thiazolidin-4-one derivatives (**8–17**) (Fig. 2). Among sulfonamides, four *N*-substituted (**1–4**) and three *N*,*N*-disubstituted (**5–7**) compounds were found. The increase in reporter gene activation varied from about twofold to almost eightfold (Table 1). The sulfonyl group of all sulfonamides is either connected to a naphthyl or a substituted phenyl ring, whereas the amide nitrogen is connected to an (hetero)aromatic system (Fig. 2A). The second substitution of *N*,*N*-disubstituted sulfonamides consists either of a cyanoethyl (**5**) or an ethylacetate (**6**,**7**) group. In addition, 10 thiazolidin-4-one derivatives were found to activate CAR (Fig. 2B). Compounds **9** and **14** emerged as the strongest agonists of this chemotype, both showing about 5-fold CAR activation (Table 1). All thiazolidin-4-ones contain a phenyl or thiophene-based substituent at position 2 of the thiazolidinone ring whereas the substituent at the nitrogen atom shows greater variability with either dimethoxylated phenyl rings, benzodioxols or

Table 1 – Virtual screening hits from the LeadQuest database

Chemical class	Compound (10 μM)	LeadQuest code	CAR activity[a]
Substituted sulfonamides	**1**	1525-00805	2.24 ± 0.03
	2	1554-07963	2.03 ± 0.09
	3	1554-08382	7.76 ± 0.08
	4	1554-09455	3.18 ± 0.22
	5	1528-09425	2.35 ± 0.17
	6	1557-02157	4.18 ± 0.06
	7	1557-02174	2.10 ± 0.09
Thiazolidin-4-ones	**8**	1539-02527	2.22 ± 0.24
	9	1539-03130	5.04 ± 0.48
	10	1539-23375	2.83 ± 0.19
	11	1539-33770	3.95 ± 0.34
	12	1539-37655	2.80 ± 0.14
	13	1539-04498	3.01 ± 0.33
	14	1539-30112	5.34 ± 0.32
	15	1539-32407	2.44 ± 0.03
	16	1539-32332	2.88 ± 0.26
	17	1539-33682	2.28 ± 0.22
Reference compounds	CITCO (1 μM)		1.44 ± 0.04
	Clotrimazole (4 μM)		4.34 ± 0.28
	TMPP (10 μM)		2.45 ± 0.19

Cut-off values for the agonist = twofold activation over vehicle DMSO.
[a] CAR activity ($N = 3$, mean ± S.D.) relative to DMSO control (=1.00 ± 0.08).

furylmethylsulfanylethyl moieties. Although the compounds possess moieties capable of forming hydrogen bonds, no such interaction with the CAR LBP was observed in the docking results. Instead, ligand binding was found to occur exclusively through van der Waals (vdW) interactions.

3.3. *Analysis of the human CAR agonist binding mode*

Depending on the crystal structure, the human CAR LBP encompasses a volume of about 750 to 800 Å^3 (as calculated using MOLCAD). Thus the hydrophobic cavity provides sufficient space to allow agonist binding in different orientations. On the basis of the docking results two different binding modes emerged: (1) either the ligands were found to bind deep in the LBP, mostly adopting a compact conformation (Fig. 3A) or (2) exhibit a more stretched arrangement that allows binding to the small channel (C1) connecting the LBP with the activation helix H12 (Fig. 3B).

Of the 17 discovered CAR agonists, 10 (**3**, **5–13**) exhibit the first binding mode. The aromatic or aliphatic moieties of these ligands were found to interact with small hydrophobic subsites (S1 and S2) within the LBP (Fig. 3A). The subsite S1, framed by amino acids located on H2′ (Leu141), H3 (Leu157, His160, Phe161, Ile164), H6 (Asp228, Val232) and the β3 strand (Tyr224) is also occupied by the A-ring of 5β-pregnane-3,20-dione in the human CAR crystal structure (PDB entry 1XV9). In order to analyze the interaction possibilities at these cavities, molecular interaction fields (MIFs) were calculated for the empty LBP using GRID. Several probes (methyl, aromatic and the hydrophobic DRY probe) were used to determine favorable hydrophobic and vdW

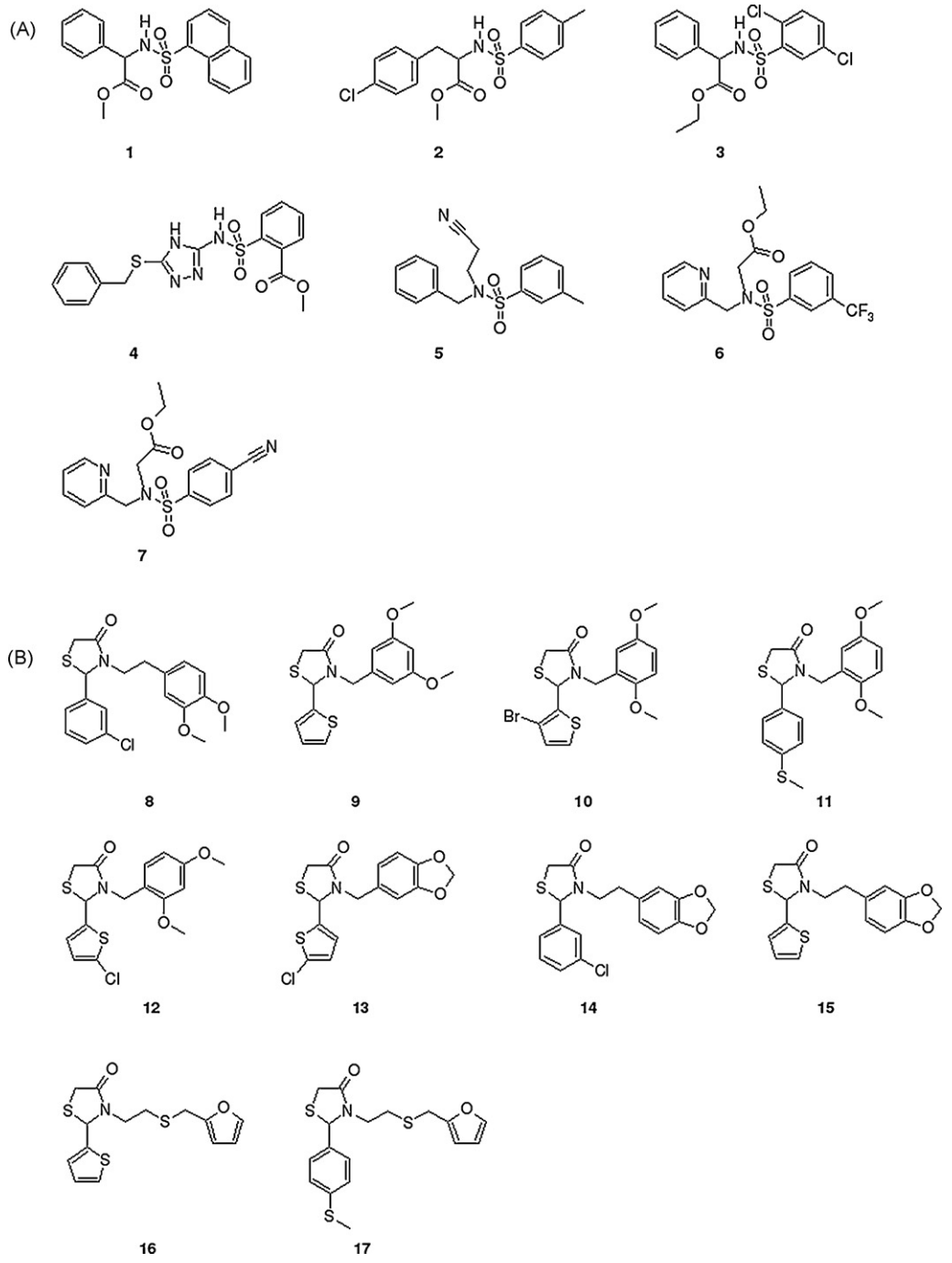

Fig. 2 – Structures of novel human CAR agonists as identified by the virtual screening procedure. Compounds activating human CAR at least twofold belong to the two chemotypes: (A) *N*-substituted and *N*,*N*-disubstituted sulfonamides as well as (B) thiazolidin-4-one derivatives.

interaction regions. A good agreement between hydrophobic moieties of the docked agonists and the calculated GRID fields was observed. Interaction fields obtained with the aromatic probe are shown as an example (Fig. 3A).

For the remaining seven agonists (**1**, **2**, **4**, **14**–**17**), the second binding mode was observed. In contrast to the previous ten compounds, these molecules were found to adopt an extended, L-shaped conformation (Fig. 3B). Aromatic moieties of the ligands may thereby bind into the C1 channel (consisting of amino acids Asn165, Met168, Val169, Ala198, Val199, Cys202, Tyr326) that connects the LBP and the activation helix H12. MIF calculations using the aromatic probe revealed a large favorable interaction region within C1 (Fig. 3B).

3.4. *Confirmation of human CAR agonism*

To confirm that the identified compounds were true human CAR agonists, we employed the mammalian two-hybrid

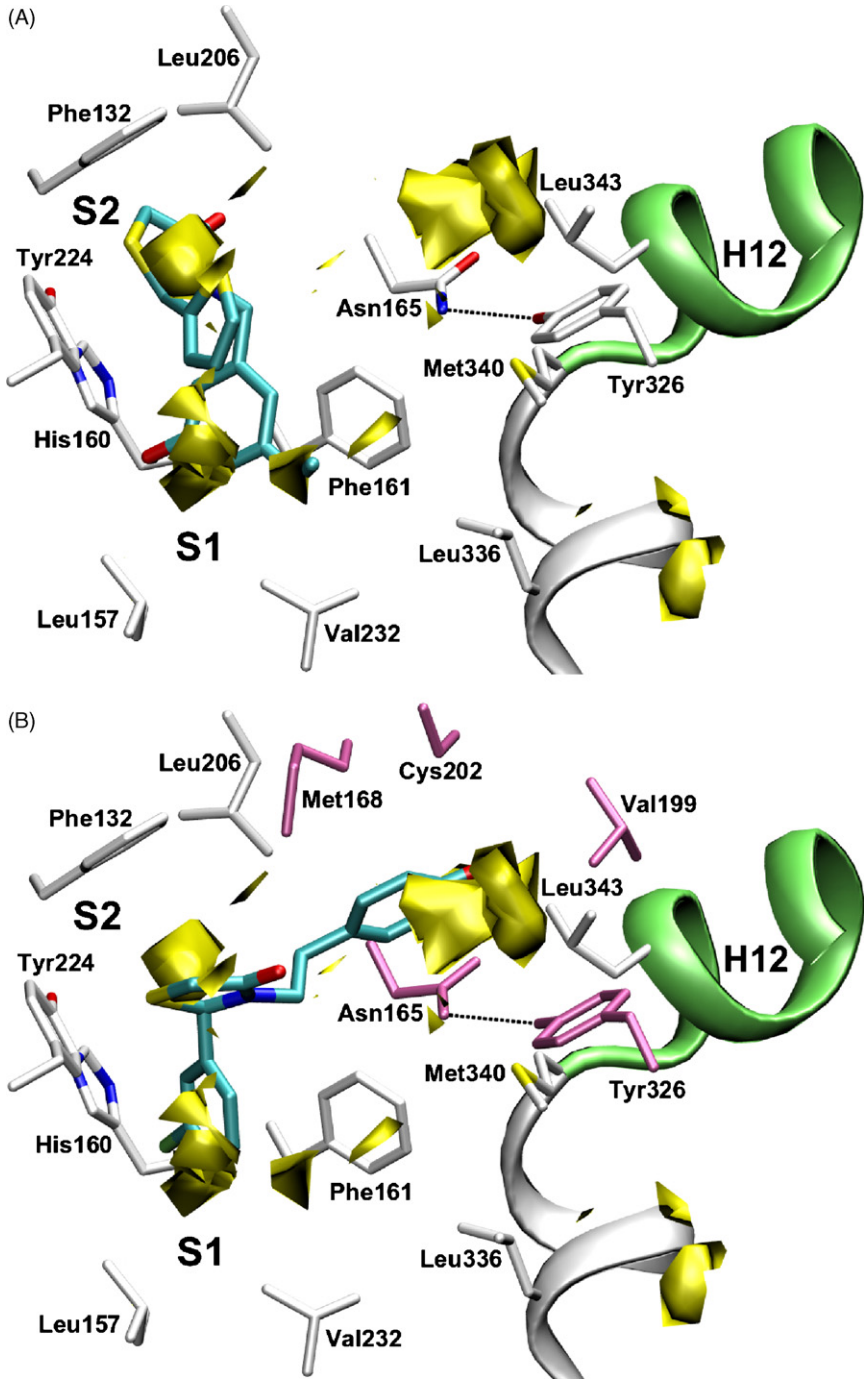

Fig. 3 – Agonist binding within the human CAR LBP. (A) Most ligands were found to enter the S1 and/or S2 subsite. The agonists (here compound 9) occupy the space between Phe161 and Tyr224. Favorable interactions between the protein and the aromatic GRID probe (yellow fields) coincide with the position of hydrophobic moieties of the bound ligands. The GRID map is contoured at an energy level of −2.7 kcal mol^{-1}. (B) Compound 14 shows additional vdW interactions with Asn165 and Tyr326 of the LBD/H12 interface and also contacts Leu343 of H12 by placing an aromatic group in the C1 channel (Asn165, Met168, Val169, Ala198, Val199, Cys202, Tyr326, colored in mauve). The energy level of the GRID contour map is −2.7 kcal mol^{-1}. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

system frequently used to ascertain agonistic actions of NR ligands [9,41,44]. In this assay, agonist binding induces a conformational shift in the human CAR LBD, resulting in enhanced recruitment of a NR-interacting peptide from co-activator SRC1 [45] and subsequent activation of the reporter gene expression (Fig. 4). All 17 identified CAR agonists enhanced the human CAR/SRC1 interaction to a variable degree, from 1.7-fold activation of the reporter by compound **10** to more than 150-fold with compound **3**. The reference agonists enhanced SRC1 recruitment by 3.5-fold to 70-fold

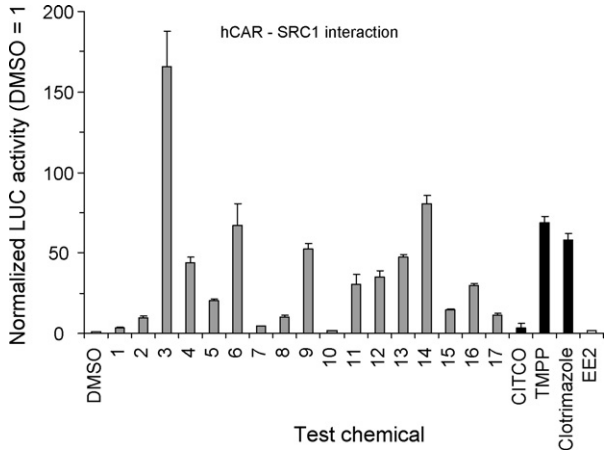

Fig. 4 – Novel human CAR agonists enhance SRC1 recruitment. Ligand-dependent association of human CAR LBD with the human co-activator SRC1 was analyzed by the mammalian two-hybrid assay after exposure to vehicle DMSO (0.1%, v/v), test chemicals 1–17 (10 μM), reference compounds CITCO (1 μM) TMPP (10 μM) and clotrimazole (4 μM) and the inverse agonist EE2 (10 μM).

while the negative control EE2 was without effect, as expected from a human CAR inverse agonist [20]. Most significantly, the human CAR activation results (Table 1) and the mammalian two-hybrid data were highly correlated ($r^2 = 0.942$, $n = 17$).

3.5. *Induction of CYP2B6 mRNA expression by selected human CAR agonists*

Finally, we wanted to learn whether the novel human CAR agonists could activate the transcription of *CYP2B6* gene in human liver. We selected two highly active *N*- and *N,N*-substituted sulfonamides (**3**, **6**), two thiazolidin-4-ones (**9**, **14**), and reference compounds CITCO and TMPP. We also included PB, a classical inducer of CYP2B6 which has been reported to activate human CAR indirectly [3]. Treatment of human hepatocytes for 24 h (Fig. 5) and analysis of CYP2B6 mRNA, normalized for β-actin expression, showed that the reference compounds TMPP and CITCO modestly induced the CYP2B6 mRNA by 1.5-fold to 1.9-fold, while PB gave a robust 7.8-fold induction. The thiazolidin-4-ones (**9**, **14**) displayed stronger 2.6-fold and 6.2-fold induction of CYP2B6 mRNA, while compounds **3** and **6** gave weaker responses of 1.6-fold and 1.1-fold, respectively.

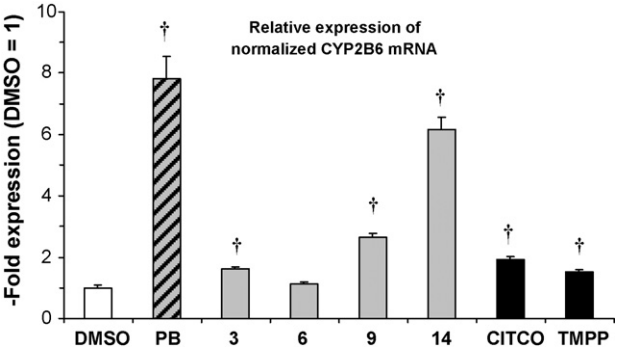

Fig. 5 – Novel CAR agonists increase CYP2B6 expression. Primary human hepatocytes were exposed to selected test chemicals (3, 6, 9, 14) and human CAR activators phenobarbital (PB, 1.5 mM), TMPP (10 μM) and CITCO (1 μM). CYP2B6 mRNA levels were normalized to β-actin mRNA expression and all normalized values were compared with DMSO control (set at 1.0). The results are mean ± S.E.M. of three separate wells per group, each analyzed in triplicate. The dagger indicates a statistically significant difference (with Bonferroni's correction) to DMSO control values.

4. Discussion

Here, we report the discovery of 17 novel human CAR agonists based on a two-step virtual screening approach. With the combination of a pharmacophore-based database screening and a molecular docking approach, we identified 30 potential CAR agonists. We obtained a high hit rate of 56.6% among these compounds as judged by our cell-based CAR activation assay. Of these 17 confirmed agonists, three ligands (**3**, **9** and **14**) were highly active (more than fivefold CAR induction). With nearly eightfold activation, compound **3** is one of the most efficacious human CAR agonists known so far. The mammalian two-hybrid assay indicated that these agonists act via direct binding to the human CAR LBD and that the most efficient agonists also had the highest abilities to recruit the co-activator SRC1 to the LBD, with an excellent correlation ($r^2 = 0.942$) between these two parameters.

In human hepatocytes, three out of four tested agonists were also able to induce the CYP2B6 mRNA, an established human CAR target. The induction response elicited by compounds **3**, **9** and **14** was at the same level or exceeded that of the reference compounds CITCO and TMPP, and with **14** even approaching the response of PB. It should be noted here that the rank correlation of CYP2B6 mRNA induction for the test and reference compounds did not match the human CAR activation or the two-hybrid results. As an example, compounds **3** and **6** displayed a high CAR activation but a low CYP2B6 mRNA induction while compounds **9** and **14** activated CAR by 5-fold and induced CYP2B6 mRNA by more than 2.5-fold. This poor correlation may result from several factors that relate, (i) to the intracellular levels of the compounds, and (ii) to the specificity for co-regulators by the compounds. First, the low CYP2B6 mRNA induction might appear because compounds **3** and **6** contain ester groups that might be readily hydrolyzed in metabolically active hepatocytes in contrast to the non-ester compounds **9** and **14**. Permeability differences due to *e.g.*, efflux pump expression between hepatoma cells and primary hepatocytes cannot be ruled out either. Second, it is well known that NR agonists can recruit different co-regulators in a ligand-selective manner [46]. In addition, several estrogenic and xenobiotic agonists for mouse CAR are able to recruit both co-activators and co-repressors, resulting in a submaximal induction of CYP2B10 mRNA [7]. If operative for human CAR as well, both these processes will result in deviations from correlation between CYP2B6 mRNA induction

and in vitro CAR activation assays that employ only one or limited number of co-activators. Further studies with larger number of chemicals are needed to address conclusively the correlation between CYP2B6 mRNA expression and CAR activation.

With respect to ligand spectrum, human CAR is more promiscuous as compared to the classical steroid hormone receptors [2,3,11,12]. Several structurally diverse compounds comprising both synthetic compounds and natural products are known to modulate CAR activity [2,3]. With substituted sulfonamides and thiazolidin-4-one derivatives, our study adds two novel chemotypes to the list of human CAR agonists that chemically differ significantly from currently known activators. Despite the chemical diversity of the database used for screening, the structural variety of the agonists described here is limited as only two different chemotypes were detected. In fact, of the 66 compounds selected for experimental testing virtually all molecules can be grouped into substituted sulfonamides and thiazolidin-4-ones with only two exceptions. All 30 compounds available to us could be allocated to either of the two novel scaffolds. The low structural variability is most likely a consequence of the initial pharmacophore search for which a restrictive search query was defined. In particular, the chosen size of the hydrophobic features appears to limit the structural variability of the resulting database subset. Using a larger tolerance for the hydrophobic features would probably have resulted in a more diverse set of chemicals for the subsequent docking approach and thus might have led to the identification of additional scaffolds as potential CAR agonists.

Several of the human CAR agonists identified in this study contain a chiral centre. Because all compounds were available only as racemic mixtures, the effects of individual stereoisomers remain unknown. Therefore, both enantiomers of the chiral compound were considered in the docking study. In the most cases, differences in binding mode for *R*- and *S*-enantiomers are marginal, and interactions of the hydrophobic groups with the subpockets and/or the C1 channel were observed for both enantiomers. However, it cannot be completely excluded that the activating effect is based on one particular enantiomer, especially when the chiral substituent is of considerable size. The role of the ligand configuration for NR activation has been shown in several studies [45,47,48]. For instance, an ureidofibrate-like derivative *R*-enantiomer emerged as a full PPARγ agonist, while the *S*-enantiomer was a partial agonist with 50% efficacy [47], and a human PXR ligand showed about fourfold difference in receptor activation between the enantiomers [48]. Diarylketones with a chiral *O*-methoxy group could both activate mouse CAR with at most twofold differences in the extent of response (unpublished results).

Using MIF calculations, specific areas within the LBP important for ligand binding were identified. The favorable interaction regions correspond to several subsites (S1, S2, C1) within the binding crevice that are targeted by the novel agonists. As the CAR LBP is mainly composed of hydrophobic amino acid residues, ligand binding occurs predominantly via vdW interactions. Thus the identification of specific favorable interaction areas for aromatic moieties gives a first insight into ligand recognition and may be further used to more precisely identify the molecular properties of compounds that activate the receptor.

The compounds presented here not only provide information about human CAR activation, but might also be applied for the development of CAR antagonists. Usually, NR antagonists exert their inhibiting effect by preventing H12 from adopting the active conformation as shown for raloxifene and tamoxifen in the estrogen receptor [49,50]. In case of the current CAR agonists, further enlargement of the molecule part located within the C1 channel is expected to result in displacement of H12 from its active conformation. Modifications of the current agonists will finally show whether this approach will be successful. In a recent study, a similar effort failed for the PXR [51], most likely because of its spacious and flexible LBP [52]. In contrast to PXR, the human CAR LBP is significantly smaller and less flexible and the above approach may succeed in this case.

In conclusion, our study demonstrates the potential of the chosen VS approach for identifying novel efficacious NR ligands. The two-step pharmacophore and receptor-based VS protocol [53] guided the selection of efficient activators with minimal experimental effort. As a result, we were able to identify two novel chemotypes that exhibit strong agonism in the cell-based CAR activation assay. Moreover, binding modes of the newly discovered agonists allow to propose specific protein/ligand interactions as important for ligand-dependent human CAR activation.

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