CHARLES UNIVERSITY

Faculty of Pharmacy in Hradec Králové

Department of Pharmacology and Toxicology


# Nuclear receptors – study of new ligands and influence of gene variability

**Doctoral thesis**

Mgr. Alejandro Carazo Fernández

Hradec Králové, 2017

Hereby I declare that this thesis is my original work of authorship that I developed under the supervision of Professor PharmDr. Petr Pávek, PhD. All literature and other sources, which I used in the development of this work, are listed and properly cited. The work was not used for acquisition of another or the same degree.

Prohlašuji, že tato práce je mým původním autorským dílem, které jsem vypracoval samostatně. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpal, jsou uvedeny v seznamu použité literatury a v práci řádně citovány. Práce nebyla využita k získání jiného nebo stejného titulu.

Alejandro Carazo Fernández

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# ABSTRACT IN ENGLISH LANGUAGE

**Candidate:** Mgr. Alejandro Carazo Fernández

**Supervisor:** Prof. PharmDr. Petr Pávek, PhD.

**Title of the doctoral thesis:** Nuclear receptors - new ligands study and importance of the genetic variability

Nuclear receptors (NRs) constitute a superfamily of transcription factors, which regulate the expression of target genes upon the binding of a ligand. These receptors can be classified in hormonal receptors, “adopted orphan receptors” and “orphan receptors” depending on the affinity to an endogenous ligand. Nuclear receptors play important roles in physiological processes and are widely distributed in the human body. Thus, adipogenesis, lipolysis, insulin sensitivity, oxidative metabolism, fatty acid homeostasis, cholesterol homeostasis, gluconeogenesis, glycogen homeostasis, triglyceride metabolism, among other processes, are regulated by nuclear receptors.

During my study, we have tested several sets of drugs, endogenous, natural and synthetic, in several nuclear receptors, focusing mainly on constitutive androstane receptor (CAR) and to a lesser extent on pregnane X receptor (PXR). My main aim was to find a new and reliable ligand or activator for human CAR. In addition, I aimed to study the mechanism of action by which these compounds interact with the receptor and how they trigger downstream pathways and target genes. For this purpose I used *ex vivo*, *in vitro* and *in silico* models.

In the first research project, I described the human CAR activation by several flavonoid compounds via inhibition of epidermal growth factor receptor (EGFR). This way of indirect CAR activation was described for antiepileptic drug phenobarbital (PB), so we proposed that these flavonoids would interact with the receptor similarly. For this purpose, we employed several methods including CAR assembly assay in cultured cells and LanthaScreen® Time Resolved – Fluorescence Energy Transfer (TR-FRET) cell-free assay.

In the second project, I optimized the TR-FRET method that we used in the previous research work. This method is based on the fluorescence resonance energy transfer (FRET) between two fluorophores that change the emission spectrum upon presence of a ligand interacting with CAR ligand binding domain. In this work, I characterized well-known agonists, antagonists and inverse agonists of CAR employing the method.

In the third research project, I studied interactions of a set of rationally developed acetylated and oxidized derivates of parent bile acids with several nuclear receptors in HepG2 hepatic cells. In this work, I established that derivate 3,12-diacetate DCA is able to strongly activate PXR and its target genes. However, we were not able to find traces of this derivate in human or mice bile samples with HPLC method suggesting that the compound is not an endogenous ligand of PXR.

In the last research project, I am working on the mechanism, which is involved in interaction of leflunomide, a drug for the treatment of rheumatoid arthritis, with human CAR. To date, no drug used in human therapy has been reported to directly activate this receptor with high affinity and only CITCO (a toxic oxime) is able to stably activate human CAR. The importance of finding a CAR ligand among current medication is critical for the study of CAR activation in human beings.

I believe our results will help us understand how CAR, PXR and other nuclear receptors work. In addition, our research sheds some light on the understanding of the molecular mechanisms by which nuclear receptors exert their activity and how they are involved in metabolic and physiological processes. Moreover, our work aimed to apply this knowledge in the development of new ligands for the potential applications in therapy of metabolic diseases.

# ABSTRAKT V ČESKÉM JAZYCE

**Kandidát:** Mgr. Alejandro Carazo Fernández

**Školitel:** Prof. Petr Pávek, PhD.

**Název dizertační práce:** Nukleární receptory – studium nových ligandů a význam genové variability

Nukleární receptory (NR) náleží do superrodiny transkripčních faktorů, které regulují expresi cílových genů. Nukleární receptory se dělí na hormonální receptory, tzv. "osvojené sirotčí receptory" (z angl. Adopted orphan receptors) a „sirotčí receptory". Receptory, které nemají žádný identifikovaný endogenní ligand, jsou nazývány jako „sirotčí receptory". Nukleární receptory hrají důležitou roli ve fyziologických procesech a jsou široce distribuovány po celém lidském těle. Jejich role je důležitá například při regulaci adipogeneze, glukoneogeneze, lipolýze, odpovědi na inzulín, oxidativním metabolismu, homeostáze mastných kyselin, homeostáze cholesterolu, homeostáze glykogenu a triglyceridů.

Během své doktorské práce jsem testoval několik sad látek endogenní, přírodní a syntetické povahy na interakce s několika jadernými receptory se zaměřením zejména na konstitutivní androstanový receptor (CAR) a v menší míře na pregnanový X receptor (PXR). Mým hlavním cílem bylo najít nové a spolehlivé ligandy nebo aktivátory lidského CAR. Kromě toho, cílem bylo studovat mechanismus účinku, kterým tyto sloučeniny interagují s CAR receptorem a jakým způsobem regulují jeho cílové geny. Pro tento účel jsem použil *ex vivo*, *in vitro* a *in silico* modely.

V první výzkumném projektu, který jsem prvoautorky publikoval, jsem popsal aktivaci lidského CAR několika flavonoidními látkami prostřednictvím inhibice receptoru epidermálního růstového faktoru (EGFR). Tento způsob nepřímé aktivace CAR receptoru byl popsán pro antiepileptikum fenobarbital (PB). Pro tento účel jsme použili několik metod, včetně tzv. CAR assembly assay a LanthaScreen® TR-FRET CAR coactivator metody.

Ve druhém projektu jsem dále optimalizoval metodou TR-FRET, kterou jsem použil v předchozí výzkumné práci. Tato metoda je založena na přenosu fluorescenční rezonanční energie (FRET) mezi dvěma fluorofory, který mění emisního spektra v přítomnosti ligandu interagujícího s vazebnou doménou CAR receptoru. Touto nebuněčnou metodou jsem detailně popsal interakce známých agonistů, antagonistů a inverzních agonistů s lidským CARem.

Ve třetím výzkumném projektu jsem studoval interakce několika nově vyvinutých acetylovaných a oxidovaných derivátů žlučových kyselin s několika jadernými receptory v HepG2 buňkách. Ukázal jsem, že 3,12-diacetát deoxycholové kyseliny (DCA) je schopen silně aktivovat PXR a regulovat jeho cílové geny. Nicméně jsme nebyli schopni identifikovat tuto látku v lidské nebo myší žluči metodou HPLC, což naznačuje, že sloučenina není endogenní ligand PXR.

V posledním výzkumného projektu jsem pracoval na mechanismu, který se podílí na interakci leflunomidu s lidským CAR. K dnešnímu dni žádné léčivo používané v humánní terapii nebylo identifikováno jako přímý aktivátor CAR receptoru s vysokou afinitou a jen látka CITCO vycházející strukturálně z oximu, je schopna stabilně aktivovat lidský CAR. Význam nalezení ligandů CAR receptoru je rozhodující pro studium aktivace CAR u lidí, jelikož zvířecí ortholog se značně od lidského liší.

Věřím, že naše výsledky nám pomůžou pochopit, jak CAR, PXR a jiné jaderné receptory vykonávají své fyziologické a detoxifikační funkce v lidském organizmu. Kromě toho, náš výzkum vrhá trochu světla na pochopení molekulárních mechanismů, kterými jaderné receptory vykonávají svůj účinek a jak se podílejí na metabolismu a fyziologických procesech. Konečně, naše práce se pokusila aplikovat tyto znalosti při vývoji nových ligandů CAR receptoru i jiných nukleárních receptorů pro potenciální aplikaci v léčbě metabolických poruch.

INDEX

# 1. INTRODUCTION

## 1.1. Genetic regulation

The concept of gene regulation includes all biological processes and mechanisms by which cells regulate the transcription of genetic material from DNA to mRNA and finally to proteins. The importance of gene regulation rely on that cells are able to, upon requirement or necessity, synthesize the needed proteins. Almost all cells in the body contain the same genetic information, and due to genetic regulation, each cell expresses a set of genes depending on the localization. This specific genetic expression is how cells specialize in a specific function in a given tissue or organ. The differential expression of genes is established by extracellular and intracellular signals (growth factors, ligand binding, phosphorylation, chemical messengers, etc.).

Regulation of gene expression takes place in two different locations in the cell, the nucleus and the cytoplasm. In the nucleus, the transcription machinery accesses the DNA and transcribes the information of a gene to an mRNA molecule. Later, the newly synthesized mRNA leaves the nucleus and translocates to the cytoplasm were, after several steps, a new protein is synthesized.

In the basal state, DNA is found as a compact chromatin structure which needs to be uncondensed to allow the transcriptional machinery to reach the DNA. This compacted structure is possible thanks to proteins called histones. Histones can be post-transcriptionally modified by different enzymes, which allow the compaction of the chromatin but also the opening of the structure so the transcriptional complex can reach to the DNA helix. This compact structure is itself a regulatory point. In this same step, regulation is also exerted by transcription factors and enhancers. Transcription factors are proteins that selectively bind to DNA and regulate the expression of a given target gene. As the topic of this doctoral work is the family of nuclear receptors belonging to transcription factors, they are described in detail in the following sections.

In this process, several steps control and regulate the genetic expression. These steps involve transcriptional control (the process by which DNA is transcribed to mRNA, the most common regulation point), RNA processing control, RNA transport

control, translational control (the process of translation from mRNA to protein) and protein stability or degradation control (Fig. 1).

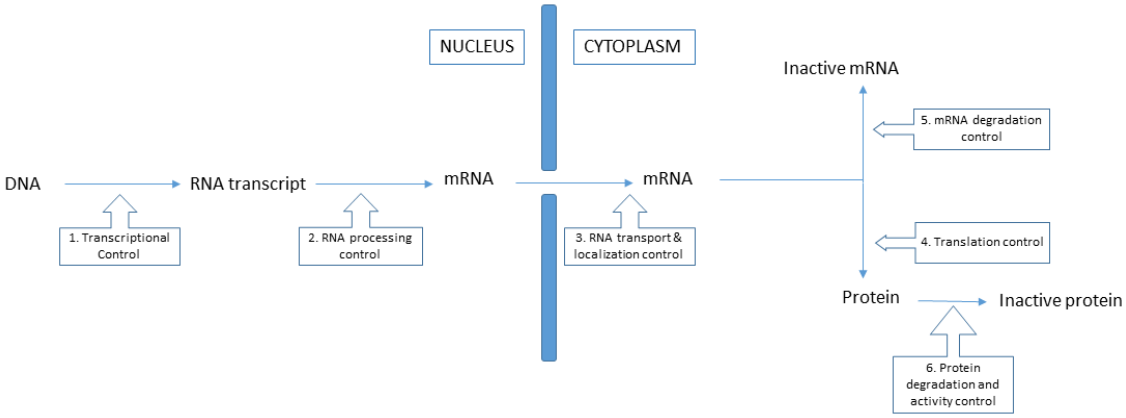


Figure 1. Gene expression regulation in eukaryotic cells.

The main regulatory step is the transcriptional control point, before the DNA gets transcribed to RNA. This regulatory process is exerted by several factors and molecules, which enable the access to the genetic material. From the biological point of view, it is logic to have the main regulatory step as soon as possible in the transcription chain, to avoid synthesis of useless genetic material which would not be used later on by the cell.

The RNA processing control step includes all the modifications that take part in the newly synthesized mRNA. This includes the splicing and editing of the molecule to the mature mRNA which will be transported to cytoplasm and its interaction with regulatory RNAs.

Another key regulatory steps are the translational control from mature mRNA to protein, which takes place in the cytoplasm, and the protein activity control, which determines if the synthesized protein will be active or not. Finally, in the mRNA degradation control point, the mRNA not transcribed into protein is inactivated due to interaction with regulatory nucleic acids such as miRNAs.

## 1.2. Transcription factors

Transcription factors (TFs) are proteins that control the expression of genetic information that is transcribed from DNA to mRNA. Mechanistically, transcription factors exert their function via forming dimers with other transcription factors, forming homo or heterodimers via forming transcription complexes, or without association to other regulatory factors.

A characteristic of these transcription factors is that they possess structures that specifically recognize the promoter regions of genomic DNA. These structures are called DNA-binding domain (DBD). Transcription factors can bind to DNA in the promoter sequence or to distal regulatory sequences, which may be upstream or downstream from the target gene. In addition, these factors can associate to other proteins that enhance or repress the genetic expression, called activators and repressors, respectively. Besides genetic transcription, TFs self-regulate themselves through other TFs. This self-regulation can take place upon activation, through feedback regulation or upon access to the DNA chain.

TFs are involved in different functions and processes such as cell cycle control, response to environmental and intracellular signals. There is growing interest on focusing transcription factors as therapeutic targets, since they can actively regulate gene expression and influence many physiologic pathways.

It is not clear how many transcription factors exist in the human being, however, a study reported about 2 600 transcription factors for humans (Babu et al 2004).

## 1.3. Nuclear receptors

Nuclear receptors are ligand activated transcription factors which play key roles in the organism such as metabolism, cell differentiation and proliferation. Importantly, these receptors are linked with health conditions and unbalanced physiological processes, among others, cardiovascular diseases, energy metabolism, inflammation and cancer.

Initially, the human genome was reported to encode 48 nuclear receptors (Mangelsdorf et al 1995). At present, different sources report a higher number of NRs. Thus, an online nuclear receptor database collects 58 nuclear receptors (Nuclear Receptor Signaling Atlas - www.nursa.org), whereas the International Human Genome Sequencing Consortium reports 60 nuclear receptors contained in the human genome (Lander et al 2001).

Interestingly, in the fly *Drosophila melanogaster* only 21 nuclear receptors were reported (Adams et al 2000), and more than 270 genes have been identified in the nematode *Caenorhabditis elegans* (Sluder & Maina 2001).

The first nuclear receptors described were GR and ERα in the 1980's (Green et al 1986, Hollenberg et al 1985, Weinberger et al 1985). Interestingly, a same nuclear receptor is able to regulate a different set of genes depending on where it is found, or develop different functions in organs depending on its localization. For example, estrogen receptor (ER) is widely present in the human being (breast, brain, uterus, etc.) and is known to code different genes depending on the organ were is present (Enmark et al 1997). This is due to a differential binding of the nuclear receptors to DNA, which is determined by the characteristics of each cell, which allows specific chromatin portions to be transcribed.

Nuclear receptors are key transcription factors which control many physiological processes and are involved in the good functioning of the organism. Dysfunction in the processes regulated by the NRs can lead to highly relevant diseases and pathological conditions such as obesity, diabetes mellitus type II, atherosclerosis, cancer or hyperlipidemia (di Masi et al 2009, Ma et al 2008).

### 1.3.1. Structure of nuclear receptors

Since the first nuclear receptors were described in the late decades of last century, many other receptors have been identified, and all of them proved to have a significant structural similarity. These transcription factors form a superfamily, which is composed of many different receptors, sharing a highly conserved structure, since they all evolved from a common ancestor (Escriva et al 1997). The phylogenetic study (the study of the evolutionary relationships between, organisms, genes or other biological entities), demonstrates a common origin for all NRs, shown in Figure 2.

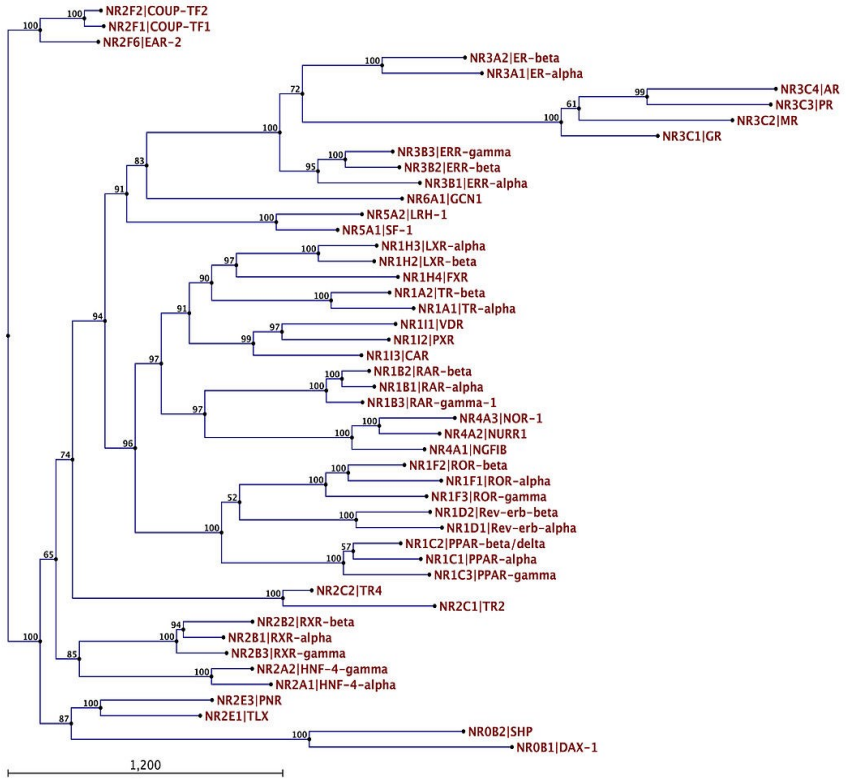


Figure 2. Phylogenetic scheme of human nuclear receptors (The numbers represent the length of units/amino acids of the NR).

The common structure of the nuclear receptors can be divided into 5 parts (Figure 3.):

- N-terminal domain: contains activation function-1 (AF-1) which is a transactivation domain able to be induced even in absence of a ligand, present in most of the NRs.
- DNA binding domain (DBD): is a well conserved structure that comprehends two zinc fingers which specifically interact with DNA response elements.
- Hinge region: a flexible domain connecting DBD and LBD.
- Ligand binding domain (LBD): is the responsible of the species specificity, and for the dimerization with other transcription factors. It is formed by several alpha helixes arranged forming a hydrophobic pocket, determined by the amino acid residues from the alpha helixes. This LBD varies in size, being the largest more than 1500 $Å^3$ (Li et al 2003). It also contains the activation function-2 (AF-2) which, unlike AF-1, requires the presence of a ligand. AF-2 consists of an open conformation in absence of a ligand, but in its presence, this conformation changes and allows interaction with coactivators (Nolte et al 1998).
- C-terminal domain or Hinge region: the most variable domain between nuclear receptors, as it is not present in all receptors.

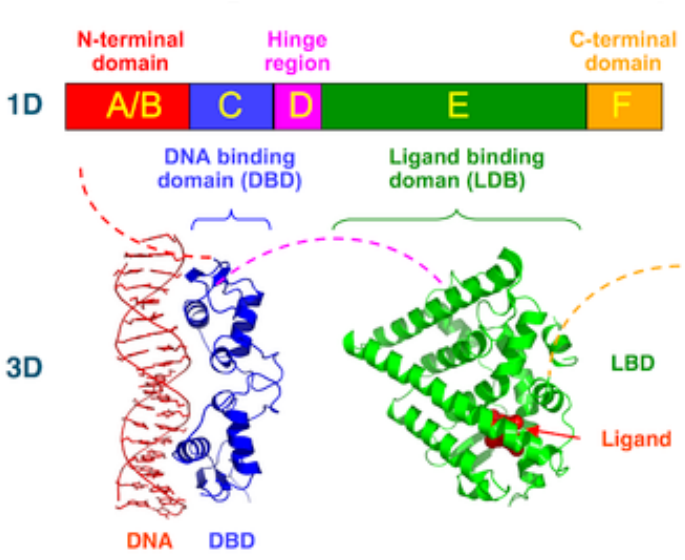


Figure 3. Structure of nuclear receptors.

Ligands are able to control the interactions of the NRs with coactivators or corepressors through the modification of the conformation of AF-2, located in the LBD (Glass & Rosenfeld 2000). Although all nuclear receptors share a similar structure, the mechanisms of action and functions of these receptors are very diverse. To exert their function, these receptors need to bind to DNA and the binding can happen as homodimers, heterodimers or monomers (unassociated with another NR). Interestingly, several studies reported that the transcription of the target genes starts in positions that can be quite far from the binding place were the transcriptional machinery binds into the promoter DNA (Carroll et al 2006).

### 1.3.2. Classification of nuclear receptors

Nuclear receptors can be classified according to different criteria: mechanism of action, sequence composition or regarding their ligand affinity. Here I present a classification into subfamilies according to its sequence homology (Nuclear Receptors Nomenclature 1999):

- Thyroid hormone receptor-like (NR1)
- Retinoid X receptor like (NR2)
- Estrogen receptor like (NR3)
- Nerve growth factor IB like (NR4)
- Steroidogenic factor like (NR5)
- Germ cell nuclear factor like (NR6)
- Miscellaneous (NR0): DAX1 and SHP (The receptors from this group lack of DBD and LBD)

Nuclear receptors are also typically classified according to the existence of known endogenous ligands. Thus, nuclear receptors can be classified into: hormonal/ endocrine NRs (high-affinity endogenous ligands), adopted orphan NRs (low-affinity endogenous ligands) and orphan NRs (with evidence of some ligands or without evidence of affinity for some ligand) (Table 1.). It should be kept in mind that some receptors can be activated even in absence of a ligand (CAR, HNF4α, etc.).

Table 1. List of human nuclear receptors (NRs) and its ligands.

| NRNC* | Classification | NR name | Abbreviation | Endogenous ligand |
|---|---|---|---|---|
| **NR0B1** | Orphan NR | Dosage-sensitive sex reversal | DAX1 | - |
| **NR0B2** | Orphan NR | Small heterodimer partner | SHP | - |
| **NR1A1** | Hormone NR | Thyroid hormone receptor α | TR α | Thyroid hormone |
| **NR1A2** | Hormone NR | Thyroid hormone receptor β | TR β | Thyroid hormone |
| **NR1B1** | Hormone NR | Retinoic acid receptor α | RAR α | Retinoic acid |
| **NR1B2** | Hormone NR | Retinoic acid receptor β | RAR β | Retinoic acid |
| **NR1B3** | Hormone NR | Retinoic acid receptor γ | RAR γ | Retinoic acid |
| **NR1C1** | Adopted orphan NR | Peroxisome proliferator-activated receptor α | PPAR α | Fatty acids |
| **NR1C2** | Adopted orphan NR | Peroxisome proliferator-activated receptor β/δ | PPAR β/δ | Fatty acids |
| **NR1C3** | Adopted orphan NR | Peroxisome proliferator-activated receptor γ | PPAR γ | Fatty acids metabolites |
| **NR1D1** | Orphan NR | Rev-ErbAα | Rev-ErbA α | Heme |
| **NR1D2** | Orphan NR | Rev-ErbAβ | Rev-ErbA β | Heme |
| **NR1F1** | Orphan NR | RAR-related orphan receptor α | ROR α | Melatonin |
| **NR1F2** | Orphan NR | RAR-related orphan receptor β | ROR β | Melatonin |
| **NR1F3** | Orphan NR | RAR-related orphan receptor γ | ROR γ | Melatonin |
| **NR1H2** | Adopted orphan NR | Liver X receptor β | LXR β | Oxysterols |
| **NR1H3** | Adopted orphan NR | Liver X receptor α | LXR α | Oxysterols |
| **NR1H4** | Adopted orphan NR | Farnesoid X receptor | FXR | Find |
| **NR1I1** | Hormone NR | Vitamin D receptor | VDR | Vitamin $D_3$ |
| **NR1I2** | Adopted orphan NR | Pregnane X receptor | PXR | Pregnane |
| **NR1I3** | Adopted orphan NR | Constitutive androstane receptor | CAR | Androstane |
| **NR2A1** | Adopted orphan NR | Hepatocyte Nuclear Factor 4α | HNF4 α | Fatty acids |
| **NR2A2** | Adopted orphan NR | Hepatocyte Nuclear Factor 4β | HNF4 β | Fatty acids |

| | | | | |
|---|---|---|---|---|
| **NR2B1** | Adopted orphan NR | Retinoid X receptor α | RXR α | Rexinoids |
| **NR2B2** | Adopted orphan NR | Retinoid X receptor β | RXR β | Rexinoids |
| **NR2B3** | Adopted orphan NR | Retinoid X receptor γ | RXR γ | Rexinoids |
| **NR2C1** | Orphan NR | Testicular receptor 2 | TR2 | - |
| **NR2C2** | Orphan NR | Testicular receptor 4 | TR4 | - |
| **NR2E1** | Orphan NR | Homologue Drosophila tailless gene | TLX | - |
| **NR2E3** | Orphan NR | Photoreceptor cell-specific NR | PNR | - |
| **NR2F1** | Orphan NR | Chicken ovalbumin upstream promoter – transcription factor I | COUP-TF I | - |
| **NR2F2** | Orphan NR | Chicken ovalbumin upstream promoter – transcription factor II | COUP-TF II | - |
| **NR2F6** | Orphan NR | V – erbA - related | EAR-2 | - |
| **NR3A1** | Hormone NR | Estrogen receptor α | ER α | Estrogen |
| **NR3A2** | Hormone NR | Estrogen receptor β | ER β | Estrogen |
| **NR3B1** | Orphan NR | Estrogen-related receptor-α | ERR α | - |
| **NR3B2** | Orphan NR | Estrogen-related receptor-β | ERR β | - |
| **NR3B3** | Orphan NR | Estrogen-related receptor-γ | ERR γ | - |
| **NR3C1** | Hormone NR | Glucocorticoid receptor | GR | Cortisol |
| **NR3C2** | Hormone NR | Mineralcorticoid receptor | MR | Aldosterone |
| **NR3C3** | Hormone NR | Progesterone receptor | PR | Progesterone |
| **NR3C4** | Hormone NR | Androgen receptor | AR | Testosterone |
| **NR4A1** | Orphan NR | Neuron-derived clone 77 | NUR77 | - |
| **NR4A2** | Orphan NR | Nuclear receptor related 1 | NURR1 | - |
| **NR4A3** | Orphan NR | Neuron-derived orphan receptor 1 | NOR1 | - |
| **NR5A1** | Adopted Orphan NR | Steroidogenic factor 1 | SF1 | 25-OH cholestrol |
| **NR5A2** | Adopted Orphan NR | Liver receptor homolog-1 | LRH-1 | - |
| **NR6A1** | Orphan NR | Germ cell nuclear factor | GCF | - |

*Nuclear Receptor Nomenclature Committee names

### 1.3.3. Mechanism of action of nuclear receptors

The triggering effect for a nuclear receptor to be activated is the binding of a ligand, however, not every nuclear receptor requires a ligand to be activated. Upon binding of a ligand, the nuclear receptors experience a conformational change, release corepressors, if they are bind to them, and recruit coactivators through their AF-2 sites. After the recruitment of these, the cascade of activation is started, leading to the transcription of a given gene. Alternatively, a ligand can lead to a repressing effect, which would promote the binding of corepressors instead of coactivators and restrain the genetic transcription. The effect depends on the nature of the ligand, which enhances or inhibits the nuclear receptor and starts the expression of target genes (Gronemeyer et al 2004).

When NRs bind corepressors in the gene promoter regions, gene expression is prevented by stabilization of the chromatin, the transcription complex cannot reach the DNA helix and transcription does not start (Moehren et al 2004). The effect of coactivators is just the opposite, destabilizing the chromatin and allowing the transcription complex to reach the DNA and start the transcription process (Harmsen et al 2007).

According to the mechanism of action, nuclear receptors can be classified into two groups:

- Type I: in this group we can find ER (estrogen receptor), PR (progesterone receptor), GR (glucocorticoid receptor) and AR (androgen receptor) among others. The receptors are initially found in the cytoplasm, associated to other proteins such as Hsp90 (Heat Shock Protein 90), which are chaperones (Echeverria et al 2010). After the binding of a ligand, the nuclear receptor unbinds the Hsp90, translocates to the nucleus and homodimerizes. Once in the nucleus, the complex recruits coactivators or corepressors (depending on the nature of the ligand) (Bulynko & O'Malley 2011, Glass & Rosenfeld 2000) and this transcription complex binds to the DNA through its HRE (Hormone Response Elements) (Figure 4. a.).

- Type II: the main representants of this group are TR (thyroid hormone receptor), PPAR (peroxisome proliferator-activated receptor) and RAR (retinoic acid receptor). In basal state, these receptors form a complex with another nuclear receptor, typically RXR and corepressors (NCoR and/or SMRT) or histone deacetylases (HDACs). After the binding of a ligand, which can be endogenous (oxysterols for LXR) or exogenous (drugs), corepressors dissociate and the complex binds coactivators, triggering the start of gene transcription. However, a ligand can also repress the genetic transcription (Figure 4. b.).

  Other orphan nuclear receptors, such as SHP (small heterodimer partner), TR2/4 (testicular receptor 2 and 4) and NURR1 (NUR-related protein 1), are included in this type of activation. For these nuclear receptors, no endogenous ligand is known and thus, activation or repression of their activity does not depend upon binding of a ligand. These receptors bind to the DNA as monodimers or as homodimers, however, some orphan NRs can also heterodimerize with RXR (NURR1) (Fig 4. c.).

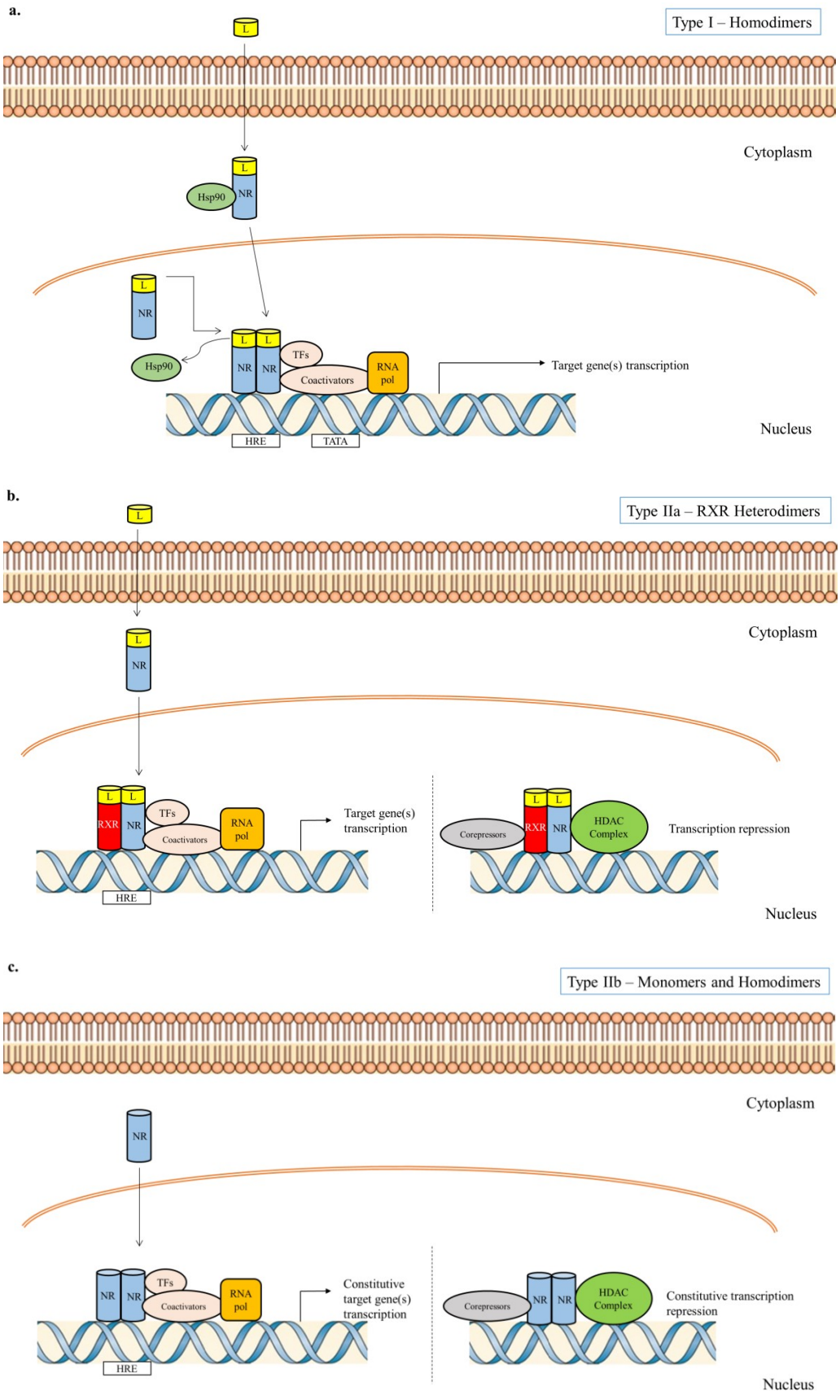


Figure 4. (a.) (b.) (c.) Types of NRs activation.

### 1.3.4. Species specificity

Nuclear receptors are only present in animals. Among animals, a same nuclear receptor presents different structural composition. These different variants of the receptors are called orthologs. For instance, nuclear receptor CAR is found, among other animals, in mouse and human being. Due to the species specificity, a potent ligand in the mouse receptor (Car) such as compound TCPOBOP (1,4-Bis-[2-(3,5-dichloropyridyloxy)]benzene, 3,3′,5,5′-Tetrachloro-1,4-bis(pyridyloxy)benzene), is inactive in human CAR. At the same time, the human CAR ligand CITCO (6-(4-chlorophenyl)imidazo(2,1-b)(1,3)thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl) oxime), is inactive in mice Car. Pregnane X receptor (PXR) is also differently activated regarding the species where is found. Thus, in human being, the reference activator rifampicin remains inactive in the mouse ortholog (Pxr). Consequently, the mouse Pxr reference activator PCN (Pregnenolone 16α-carbonitrile) lacks of activator effect in human PXR. This phenomenon is common for many NRs.

It should be kept in mind that some of the ligands that bind to these receptors can also bind to other NRs LBD, representing a dual activity. Such a function has been described for several CAR and PXR ligands, such as CITCO (Maglich et al 2003), clotrimazole (Moore et al 2000), TCPOBOP (Moore et al 2000), (5β)-Pregnane-3,20-dione (Lehmann et al 1998, Moore et al 2000), 17β-estradiol (Kawamoto et al 2000) or phenobarbital (Moore et al 2000). Nonetheless, also bile acids have been reported to have a dual activation of NRs, such as PXR, CAR and FXR (Fiorucci et al 2010). This promiscuity is restricted due to the specific characteristics and properties of the LBDs of each receptor. In addition, the sharing of a common ligand does not imply that the molecule will have the same effects in the receptors to which binds, but can act as an agonist in one and as an antagonist or partial agonist in another.

This specificity is present in all nuclear receptors among different species but also, among the same species. For instance, a given nuclear receptor in an individual will modulate the expression of different genes according to its localization. Thus, estrogen receptor (ER) will code a different set of genes depending if it is localized in breast, testis, uterus or central nervous system (Enmark et al 1997, Riggs & Hartmann 2003).

### 1.3.5. Functions of the nuclear receptors

Nuclear receptors are ligand-activated transcription factors. Upon binding of a ligand, it is reasonable to suppose that not only activation of the receptor can happen, but also inhibition. Thus, ligands that bind to the receptors can be agonists, antagonists, partial agonists or inverse agonists. The nature of ligands determines the expression of target genes, their transcription to mRNA and later on, to proteins. In addition, as commented above, a same nuclear receptor can specifically express a gene or set of genes depending on its localization, as has been previously found for ER (uterus, breast, central nervous system, bone or cardiovascular system) (Enmark et al 1997, Riggs & Hartmann 2003).

The superfamily of nuclear receptors involves many different receptors, each with its own characteristics and localization, and they can determine many physiological processes and mediate different functions. There is solid evidence showing that these receptors are involved in the following functions: homeostasis (glucose, glycogen, cholesterol, fatty acids, triglycerides and hormones), oxidative metabolism, xenobiotic detoxification, enhance insulin sensitivity, inflammation and many others.

Overlapping functions can happen among nuclear receptors. For instance, CAR and PXR are receptors involved in drug metabolism among other functions. Both receptors can regulate several common genes (CYP1A2, CYP2C19, CYP3A4, and MDR1) (Table 2.) and influence metabolism of glucose and lipids (Banerjee et al 2015, Pavek 2016). Many other examples of overlapping functions have been described such as the homeostasis of bile acids, which is determined by VDR, CAR, FXR and PXR (Fiorucci et al 2010, Lim & Huang 2008).

### 1.3.6. Potential therapeutic applications of the nuclear receptors

Many ligands of hormonal receptors are used in therapy of immune diseases and as hormonal substitution agents (ethinyl estradiol, glucocorticoids) (Coursin & Wood

2002, Mallappa & Debono 2016, Norjavaara et al 2016). In addition, nuclear receptors are important therapeutic targets in several metabolic diseases, where they influence endogenous metabolic pathways and, obviously, the expression of target genes such as in obesity, DM II (PPARγ agonists), dyslipidemias (PPARα ligands) or liver cholestatic diseases (FXR ligands). However, the signaling cascades and the level of influence of these NRs are not fully understood yet, and this presents a limiting factor in the potential application of nuclear factors in the treatment of human conditions.

An important aspect for the discovery of new ligands has been the development of *in silico* methods, in which the structure of the LBD is represented and allows to predict the affinity of interaction of a given ligand with the nuclear receptor cavity. Through study of the chemical nature of a ligand and the amino acidic composition of the ligand pocket, the information obtained is useful to predict if a given molecule would interact. This tool can be used in the development of synthetic drugs which would fit the pocket. Physical-chemical interactions should be also taken into account, as the LBD is often highly hydrophobic.

## 1.4. Genetic variability

Genetic variation is the naturally occurring genetic differences among organisms in the same species. These modifications happen through mutations of the genetic material and are the basis of the evolution. These genetic variations can be classified according to the size of genetic material modified or by their consequence on the organism. Small size variations comprehend changes of 1Kbp (1 000 base pairs) or less, and large variations refer to changes bigger than 1 Kbp. Small variations are single nucleotide polymorphisms (SNP), a mutation of a single base pair, or indels, insertions or deletions of few base pairs. Large size variations can be divided in: copy number variation and in chromosomal rearrangement. From the therapeutic point of view, SNP are most important mutations with significant clinical consequences that are present in each gene. A SNP in coding regions or in regulatory regions have significant impact on protein activity or expression. In case of receptors or biotransformation enzymes, SNP can cause an altered therapeutic outcome after the application of a drug. Thus, SNPs are the scope of study for pharmacogenetics or pharmacogenomics.

Each individual may present variations of the genetic material, which leads to a unique answer to the environment and obviously to pharmacological treatment. Understandably, genetic variation does not happen only among individuals but also between races. Thus, different human races will react differently to environmental situations or medication (Fujikura et al 2015, Zanger & Schwab 2013, Zimmer et al 2015).

Due to the advances in medicine and scientific research, genetic variability is revealing as an important factor to take into account in pharmacotherapy. This is leading to personalized therapy, in which each patient would be prescribed a drug or set of drugs according to their genetic characteristics (Fujikura et al 2015, Mancinelli et al 2000).

Nuclear receptors are also affected by genetic variations, the sequence of these proteins can vary from and individual to another. The most common variations regarding these receptors are SNPs, which have been also described for the majority of the nuclear receptors and also for PXR and CAR (Auerbach et al 2003, Lamba et al 2004a, Lamba et al 2004b). For PXR, rs3814055 SNP has been found as important in pharmacotherapy of tacrolimus, lamotrigin, cyclosporine and rifampin (www.pharmgkb.org/gene/PA378?previousQuery=pxr#tabview=tab1&subtab=31).

In the case of CAR, 11 SNPs have been reported and are listed in PharmGKB databases (www.pharmgkb.org). Nevertheless, CAR has more than 30 transcription variants, and transcription variant hCAR3 is widely studied as highly inducible variant in human representing about 20% of all NR1I3 transcripts. This splicing variant of human CAR (hCAR3) contains an insertion of five amino acids (APYLT) and exhibits low basal but xenobiotic-inducible activities (Chen et al 2010).

These facts add yet more complications to the study of these particular transcription factors.

# 2. NUCLEAR RECEPTORS: CHARACTERISTICS, LIGANDS, EXPERIMENTAL APPROACHES AND GENETIC VARIATION

Among all the nuclear receptors described to date, in my doctoral study I focused on two members belonging to the "adopted" orphan receptors subgroup: CAR and PXR. In addition to these receptors, I also worked on other receptors such as VDR (Vitamin D Receptor), FXR (Farnesoid X Receptor) and GR (Glucocorticoid Receptor). All these receptors belong to the thyroid-like nuclear receptors subfamily, except VDR, which belongs to the endocrine receptors subfamily (Section 1.3.2.).

Despite the great advances and information obtained in the last decades since nuclear receptors were first cloned, many processes and physiological mechanisms regarding their functioning remain to be elucidated. These receptors are involved in metabolic and endocrine systems and inflammation among other physiological processes. Hence, the attention attracted by these important transcription factors is growing exponentially.

As ligand activated receptors, these transcription factors show different affinities to ligands and are able to bind not only endogenous molecules but also synthetic compounds. As reported above, drugs used in therapy can modulate the effect of the receptors. Interestingly, a molecule can bind in different positions in a given LBD.

In this section, I describe in detail CAR and PXR, focusing on their ligand affinity and functions. I also outline the most relevant aspects of FXR, VDR and GR. I find necessary to remark that these receptors are found not only in humans but also in other animals. In this doctoral work I refer to the human variants of these receptors except when specified otherwise.

## 2.1. CAR and PXR: Current state of art

When first described, both CAR and PXR were included in the "orphan nuclear receptors" group due to the lack of known endogenous ligands. Eventually, both receptors were later reported to bind endogenous molecules but with low affinity (such as androstenol and androstanol in case of CAR and 17β-estradiol and 3,20-pregnanedione in PXR) (di Masi et al 2009). Therefore they were consequently relocated to the "adopted orphan receptors" group.

For several years, these receptors were considered merely xenobiotic sensors playing an important role in regulation of the metabolism of drugs and many other xenobiotics. Extensive research on these receptors showed that their roles and functions are wider and more complex than originally believed. Therefore, they are not only involved in xenobiotic metabolism but also in metabolism of glucose, lipids, amino acids, in tumor progression, etc. In addition, these two receptors showed to cross-talk and regulate common target genes and pathways (Gerbal-Chaloin et al 2001, Pavek 2016, Tien & Negishi 2006). Not only they regulate a common set of genes but also are able to regulate other nuclear receptors (Kodama & Negishi 2013). It is obvious that further functions remain to be characterized for these receptors.

Ligand binding is not the only factor that influences the activity of CAR and PXR and nuclear receptors in general. Environmental factors, cellular signaling cascades activated by cytokines and growth factors can also determine the activity of these receptors. Key steps for the determination of the function of these receptors are post-translational regulation and post-transcriptional modifications. Post-translational modifications represent are:

- Phosphorylation: the main regulatory mechanism for "orphan receptors" that can regulate the activation even in absence of a ligand (Berrabah et al 2011, Rochette-Egly 2003).
- SUMOylation: promotes repressive activity (Treuter & Venteclef 2011).
- Ubiquitylation: this process is not deeply studied but probably contributes to the termination of hormone signaling and nuclear receptor degradation (Lee & Lee 2012).

- Acetylation: common process for protein post-translational modification (Staudinger et al 2011).

  These processes are not completely understood yet and often provide confusing data regarding nuclear receptor regulation. For instance, phosphorylation is a process that not only happens in NRs, but also in the proteins that associate to these receptors and determine activities of coactivators and corepressors (Staudinger & Lichti 2008).

Regarding post-transcriptional modifications, different processes determine the expression and activity of a RNA molecule. Some of these processes are: RNA editing, alternative splicing and structural modifications in the sequence. Additionally, regulatory RNA molecules can control RNA expression. MicroRNA (miRNA) are small, non-coding RNA molecules, that are able to bind to newly synthesized mRNA molecules and promote their degradation or repress their translation (Nakajima & Yokoi 2011). In our group, colleagues recently comprehensively described miRNA regulation of both PXR and CAR (Smutny et al., 2015).

Table 2. Metabolizing enzymes and drug transporters regulated by CAR and PXR.

| Nuclear receptor | Metabolizing enzymes phase I | Metabolizing enzymes phase II | Drug transporters |
|---|---|---|---|
| CAR | CYP2B6<br>CYP3A4<br>CYP2A6<br>CYP2C8<br>CYP2C9<br>CYP2C19 | UGT1A1<br>UGT2A1 | MDR1<br>MRP2<br>MRP3 |
| PXR | CYP3A4<br>CYP3A5<br>CYP3A7<br>CYP2C8<br>CYP2C9<br>CYP2B6<br>CYP1A2<br>CYP2A6<br>CYP2C19<br>AKR1C1<br>AKR1C2 | SULT2A1<br>UGT1A1<br>UGT1A3<br>UGT1A4 | MDR1<br>MRP2<br>OATP1B1 |

Many studies have shown an overlap in the regulation of target genes between CAR and PXR. The duplicated functions can be found in different processes such as in xenobiotic metabolism, regulating a common set of enzymes and drug transporters (Gerbal-Chaloin et al 2001, Staudinger et al 2003, Zhou et al 2005) (Table 2.), and lipid or glucose metabolism (Guo et al 2003, Kast et al 2002).

### 2.1.1. Constitutive Androstane Receptor (CAR)

Human CAR (hCAR, NR1I3) was first described in 1994 (Baes et al 1994) and was included in the "orphan nuclear receptors" subgroup. Posterior work reported that endogenous steroids androstenol and androstanol bind to the receptor (Forman et al 1998) and inhibit its activation, shifting the receptor to the "adopted orphan nuclear receptors" category. Later, other endogenous ligands, such as estrone and estradiol (E2) and even primary bile acid cholic acid (CA) were reported to be low affinity CAR ligands and to activate the receptor (Fiorucci et al 2010, Swales & Negishi 2004).

CAR is mainly expressed in liver but it is also found in other tissues and organs, such as small intestine epithelium, brain, skeletal muscle, lungs, kidneys and heart. In basal state, CAR is found in the cellular cytoplasm forming a complex with proteins that keep the receptor inactivated and phosphorylated. The cytoplasmic complex is formed by CCRP (CAR Cytoplasmic Retention Protein), Hsp90 (Heat shock protein 90) and GRIP1 (Glutamate Receptor Interactiong Protein 1) (Kobayashi et al 2003, Min et al 2002b, Timsit & Negishi 2007). In presence of a ligand, CAR releases from the complex, dephosphorylates, and translocates to the nucleus, where heterodimerizes with RXRα and recruit coactivators (SRC-1, SMC-1, PGC-1α, GRIP1/TIF2, ASC-2 (Choi et al 2005, Inoue et al 2006, Muangmoonchai et al 2001, Shiraki et al 2003). All these proteins, in combination with RNA polymerase (RNApol) form the transcription machinery complex, which binds to the DNA in the responsive elements and triggers the transcription.

Activation of the receptor can happen through different mechanisms: ligand-dependent, ligand-independent or indirectly.

- Ligand-dependent: this activation happens when a ligand interacts directly with the receptors LBD and promotes the translocation and the formation of the transcription machinery.
- Ligand-independent or "constitutive activation": the receptor, without the binding of a ligand is able to dephosphorylate, release the associated proteins and translocate to the nucleus, triggering the genetic transcription (Swales & Negishi 2004).
- Indirectly: a ligand binds to a different receptor in the cell, this causes the activation of a signaling cascade and the receptor is activated. The first drug described to trigger this activation was the anti-epileptic agent phenobarbital (PB-like activation) (Mutoh et al 2013). Since then, other compounds (flavonoids) were reported to act similarly as we recently reported in our paper (Carazo Fernandez et al 2015, Mutoh et al 2013).

Interestingly, CAR expression can be regulated by other NRs such as HNFα, GR and PPARα (Tamasi et al 2009). This fact and the reported activation of several biological processes by two or more of these nuclear receptors is denominated cross-talk and is very relevant in their study.

To date, the most potent and high affinity CAR ligand reported is the synthetic compound 6-(4-chlorophenyl)imidazo(2,1-b)(1,3)thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime (CITCO) (Huang et al 2003, Maglich et al 2003). Despite its high affinity to CAR-LBD, this compound cannot be used in humans due to its toxicity and the search for new ligands with better characteristics continues. CAR activation is also enhanced upon presence of glucocorticoids and by some other hormones (Pascussi et al 2001, Pascussi et al 2003). In addition, CAR is suspected to regulate the levels of thyroid hormone in mice, but the hormone does not seem to regulate CAR activity (Maglich et al 2004, Qatanani et al 2005). Finally, CAR seems to reduce the activation of another nuclear receptor, ERα (Ganem et al 1999, Min et al 2002a, Min et al 2002b).

CAR possesses a quite small LBD and this determines its ability to bind ligands (Moore et al 2000). Nonetheless, CAR-LBD is flexible and is able to bind structurally different molecules: endogenous, synthetic and natural (Carazo et al 2017, Carazo Fernandez et al 2015, Chang 2009). However, after the binding of a ligand,

translocation to the nucleus takes place but this process not necessarily leads to an enhancement of gene expression (Swales & Negishi 2004).

Most of the information and findings regarding CAR physiological functions have been obtained using Car $^{-/-}$ knockout mice. As mice express their own ortholog of the receptor, humanized mice models have been also developed in which they express the human ortholog (Scheer et al 2008). These advantages have enabled a more comprehensive study of the effects of human CAR. In addition to the original function attributed to CAR, wider roles for the receptor have been described in the humanized model. Nevertheless, in CAR humanized mice, CAR still functions in mouse cells with mouse transcription machinery proteins and under mouse cellular signaling, which may modify true CAR function. Therefore clinical trials with human CAR ligands would help us to study real physiological roles of the receptor in humans. However, the lack of a CAR ligand used in human therapy is a limiting factor to closely characterize the receptor in clinical trials and study the consequences of its activity directly in the human organism. In my ongoing project, I am working with leflunomide, a drug used to treat rheumatoid arthritis patients which consistently showed to slightly activate the receptor and induce its major target gene CYP2B6, however, data points to an indirect activation, just as PB. Even though there are yet experiments to be performed and hypothesis to be confirmed, to our knowledge this would be the first CAR activator able to be used in humans in clinical investigation. This fact would open a new dimension in the study of the receptor. The consequences of its activation could be therefore closely studied and characterized.

As a metabolic sensor, CAR regulates the expression of several phase I and II enzymes and drug transporters. Among these enzymes and transporters are found CYP2C9, CYP3A4, UGT1A1, ABCC2 or MDR1 and especially CYP2B6, which are the most important target genes in the liver (di Masi et al 2009, Goodwin et al 2002, Kast et al 2002). As reported before, data shows an overlapping regulation between CAR and PXR in the regulation of metabolic enzymes and drug transporters (Banerjee et al 2015, Chai et al 2016). However, PXR seems to be a more important regulator than CAR in case of CYP3A4 and CYP2C9 enzymes and more pronounced upregulation of these genes can be seen in comparison with CAR-mediated induction of these genes.

Several works point to a beneficial effect of CAR activation in hyperglycemic situations after high-fat diet feeding of mice (a metabolic syndrome animal model). The regulation of glucose synthesis by CAR happens through repression of target enzymes of gluconeogenesis after CAR activation. CAR activation was reported to repress the enzyme G6Pase (glucose 6-phosphatase) and and PEPCK1 (phosphoenolpyruvate carboxykinase 1) that regulates initial step in gluconeogenesis (Kodama et al 2004, Ueda et al 2002, Yarushkin et al 2013). In addition, in mice models, enhanced sensitivity to insulin was observed in high-fat diet fed mice (Dong et al 2009b, Gao et al 2009, Gao & Xie 2012). These facts may be helpful for patients suffering DM type II in metabolic syndrome, since mice Car activation prevent weight gain and many of these patients suffer also from hyperlipidemia (Dong et al 2009b, Gao & Xie 2012, Roth et al 2008).

CAR activation has been reported to reduce the white adipose tissue increase in mice fed with high-fat diet (HFD) and to improve the lipid levels and dyslipidemia in obese mice (Dong et al 2009a, Gao et al 2009, Gao & Xie 2012, Sberna et al 2011). These effects are consequence of the repression of enzymes and proteins involved in the synthesis of lipids, such as lipogenic transcription factor SREBP-1 (sterol regulatory element-binding protein 1) (Roth et al 2008). In addition to lipid metabolism, CAR was reported to play a role also in the homeostasis of bile acids. Despite the important physiological functions of bile acids, an accumulation of them can lead to toxic situations. So far, studies performed in animal models reported that CAR was able to enhance enzymes involved in bile acid elimination and promote a protective effect of the receptor upon bilirubin accumulation (Guo et al 2003, Huang et al 2003). In addition, CAR was reported to enhance the metabolism of bilirubin to non-toxic derivates and to be involved in the metabolism of bile acids in mice (Huang et al 2003, Sugatani et al 2001).

Contradictory conclusions were reported regarding the role of CAR in cancer. No influence of CAR activation has been linked with development of cancer in humans and even, some reports point to an antineoplastic effect (Elcombe et al 2014, Chakraborty et al 2011, Wang et al 2013). However, in mice, several studies reported that the receptor activation by PB promoted liver cancer and hepatomegaly (Huang et al 2005, Yamamoto et al 2010). In additional experiments in mice, directly relationship with Car and tumorigenesis has been established as mice not expressing the receptor did

not develop liver cancer. In addition, when administered together an antineoplastic agent and a CAR agonist, reduction of efficacy against tumor was observed in human ovarian cell lines (Wang et al 2014).

Hormonal homeostasis is also regulated by CAR. Several works reported that the activation of CAR in mouse was linked to a reduction of the serum levels of thyroxine (T4), with the enhancement of steroid metabolism through activation of the target enzymes, and the metabolism of estrogens and androgens (Maglich et al 2004, Qatanani et al 2005, Swales & Negishi 2004).

CAR was also reported to be involved in other physiologic situations. Thus, an effect in bone metabolism was proposed for the receptor since CAR activators (PB) are involved in vitamin D homeostasis and have been linked with drug-induced osteomalacia (Hosseinpour et al 2007, Ma et al 2008). Lastly, a recent paper reported a CAR mediated healing role in intestinal damaged epithelium in mice and in human intestinal cultured cells (Hudson et al 2017). .

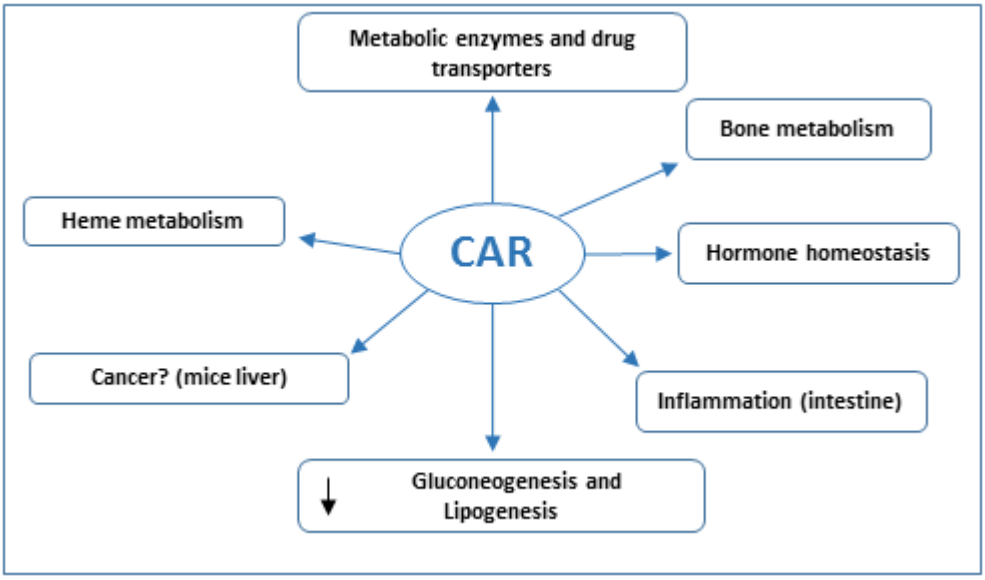


Figure 5. CAR functions in the organism. CAR activation has been reported to directly enhance xenobiotic metabolism, heme metabolism, decrease the synthesis of glucose and lipids, influence the hormonal homeostasis and probably, bone metabolism. The role of CAR in cancer remains to be clarified, however, in mice models, the activation of the receptor induces liver cancer.

### 2.1.2. Pregnane X Receptor (PXR)

PXR (hPXR, NR1I2) was first described after CAR in 1998 (Bertilsson et al 1998) and called steroid xenobiotic receptor (SXR). Similarly to CAR, it was reported to be a xenosensor but soon attracted bigger interest from the scientific community. This receptor is more widely present in the human body than CAR, and it is mainly found in liver and intestine but it is also expressed in kidneys, lung, heart, breast, uterus, brain, bone marrow, etc. (di Masi et al 2009).

PXR possesses a large PXR-LBD that makes possible the binding of very different ligands including endogenous steroids, synthetic ligands and many natural compounds (Moore et al 2003, Willson & Kliewer 2002). Rifampicin (an antibiotic used in the treatment of tuberculosis) is the reference activator of PXR (Bertilsson et al 1998). A variety of natural compounds are able to activate PXR. Importantly St. John's Wort extract, used in the treatment of depression and nervous conditions (bad sleep, anxiety) and artemisinin showed intense activation of the receptor (Burk et al 2005, Moore et al 2000). Due to species-specificity, human PXR activators do not activate rat or mice Pxr and vice versa as reported in section 1.3.4. Endogenous ligands that bind to PXR are some bile acids and hormones (Carazo et al 2017, di Masi et al 2009, Fiorucci et al 2010). In addition, PXR can also bind molecules which showed affinity to CAR. For instance, CAR, prototypical activator CITCO is a weak PXR activator (Maglich et al 2003).

No reliable PXR antagonists are known, however, the antineoplastic agent ET-743 (ecteinascidin-743 or trabectedin) showed inhibitory effect for the receptor (Synold et al 2001). However, the use of ET-743 is not possible due to high toxicity and thus its use is restricted. In addition, antifungal ketoconazole and antidiabetic agent metformin showed PXR inhibitory effects too (Huang et al 2007, Krausova et al 2011, Shan et al 2017). Interestingly, leflunomide was reported to also inhibit PXR *in vitro* (Ekins et al 2008).

PXR has a similar pattern of activation as CAR, however, there are contradictory data regarding the cellular localization of the receptor. Whereas some studies report that PXR is found mainly in the cytoplasm (Kawana et al 2003, Squires et al 2004), others indicate a nuclear localization of the receptor (Koyano et al 2004, Saradhi et al 2005). Independently of its localization, the receptor requires the presence of a ligand to trigger

its activation. After the ligand binding, the corepressors are released, the receptor heterodimerizes with RXRα (Frank et al 2005), recruits coactivators (SRC-1, PGC-1α) and binds to the DNA through its responsive elements (Bhalla et al 2004, Lehmann et al 1998). Unlike CAR, PXR is activated only by direct ligand binding. Through post-translational modifications, PXR activation can also be regulated through phosphorylation processes (Doricakova et al 2013, Elias et al 2014, Lichti-Kaiser et al 2009).

As a consequence of PXR activation, drug-drug interactions due to drug metabolism augmentation, especially in patients treated with multiple medications, can occur. This activation can lead to drug toxicity or sometimes to therapeutic failure. However, PXR activation does not necessarily induce its target genes. It is logic to think that the stimulation of transcription factors not only enhance the expression of target genes, but it can also repress them (Hyrsova et al 2016).

PXR was originally described as an important metabolic regulator able to modify the metabolism of xenobiotics and endogenous products. Target genes involved in metabolism involve phase I and phase II enzymes and drug transporters (Kliewer et al 2002, Maglich et al 2002). PXR is able to regulate a wider range of metabolic enzymes than CAR, although there is evidence of significant overlapped regulation of some enzymes (CYP1A2, CYP2C9, CYP2B6, CYP3A4 and MDR1) (Banerjee et al 2015, Chai et al 2016).

Bile acids are able to activate PXR, especially the secondary bile acid litocholic acid (LCA). Activation of PXR is able to prevent the hepatic damage caused by toxic LCA since is able to repress the main enzyme in bile acid production Cyp7a1 in mice (Staudinger et al 2001b). This effect is complemented with the enhancement of metabolizing pathways of bile acids after PXR activation and induction of intestinal and hepatic CYP3A4 (Li & Chiang 2005, Staudinger et al 2001a, Timsit & Negishi 2007). In addition, PXR activation induced the expression of key enzymes and transporters involved in the detoxification pathway of heme, whose accumulation leads to hyperbilirubinemia and toxic syndromes (Saini et al 2005, Wagner et al 2005).

After PXR activation, lipids metabolizing enzymes are repressed and lipogenesis is enhanced, which leads to an increased level of lipids in the liver (Bitter et al 2015,

Kodama & Negishi 2013). However, there are obvious species-specificities in these processes between mice models and humans. In mice, several works reported enhanced levels of plasma lipids after PXR activation, but contradictory data has been also obtained regarding the regulation of lipogenic genes (Moreau et al 2009, Zhou et al 2006b). Human studies showed a PXR-mediated effect on the expression of lipogenic genes (Bitter et al 2015). Obesity is another health condition believed to be modulated by PXR. Some works have linked prevention of weight gain in mice treated with Pxr agonists (Ma & Liu 2012), whereas other studies reported the same effect in knockout mice (He et al 2013). Doubt remains if the methods employed by these research groups decrease the appetite of the mice and thus the net effect is weight loss.

Interesting effects has been described regarding the effect of PXR activation on hepatic glucose homeostasis. Activation of the receptor led to repression of gluconeogenic enzymes in mice models and hepatic-derived human cultured cells (Bhalla et al 2004, Kodama et al 2004). In human hepatocytes, contradictory effects of the activation have been reported (Gotoh & Negishi 2014, Gotoh & Negishi 2015, Hakkola et al 2016). It is difficult to assert if this effect is consequence of specific PXR activation since many PXR activators are also CAR ligands (phenobarbital, phenytoin, etc.)

A significant amount of research papers report the protective effect of PXR activation in inflammatory processes. Among them, PXR was shown to have protective effect in inflammatory bowel disease (IBD) (Shah et al 2007). IBD is a chronic inflammation of the digestive tract of unknown origin/etiology. Reports showed that in induced IBD in mice, activation of PXR reverts the effects of IBD (Shah et al 2007). Similarly, in humanized mice models, hPXR activation lead to improvement in IBD (Cheng et al 2010). In addition, the expression of the key regulator of the inflammatory response NF-KB was repressed after PXR activation (Zhou et al 2006a).

Importantly, PXR has been reported to play a role in the course of cancer. Many genes regulated by PXR (MDR1, CYP3A, etc.) are classified as chemoresistance genes, as they enhance metabolism and transport of antineoplastic agents and PXR is thought to indirectly promote tumorigenesis (Harmsen et al 2007, Chen et al 2007).

Other physiological processes were reported to be conditioned by PXR, although the implication of the receptor is not fully clarified. In bone, PXR was reported to

mediate a protective effect, although also discrepant reports exist regarding drug induced osteomalacia (Igarashi et al 2007, Pascussi et al 2005) and to inhibit the development of fibrogenesis in hepatic tissue (Haughton et al 2006). PXR activation was connected with an imbalance of the steroid homeostasis. In mice, the activation of the receptor was reported to enhance the levels of corticosterone and aldosterone (Zhai et al 2007).

Much data on PXR have been obtained in mice or animal models, even though many of them were also obtained in PXR humanized mice (Scheer et al 2008). Species-specificity should be always kept in mind and the effects of rodent Pxr may not be extrapolated to the human. In humans, other pathways and interactions take place and thus, further research is required for the precise determination of the physiological effects of human PXR activation/ inhibition.

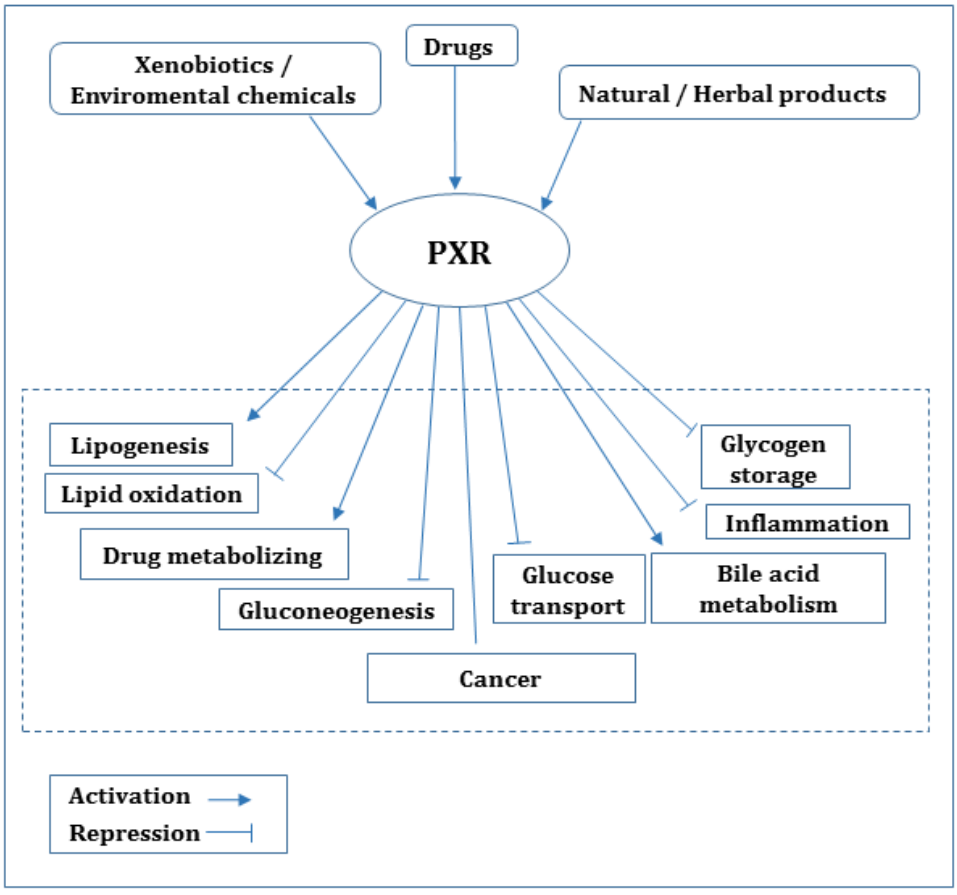


Figure 6. PXR functions in the organism. PXR can be activated by multitude of different molecules and has been linked with many different physiological processes. The activation of the receptor leads to the metabolism of drugs and xenobiotics, enhancement of lipid synthesis and the metabolism of bile acids. On the other hand,

PXR represses the lipid oxidation, gluconeogenesis and inflammatory processes. Importantly, evidence points to a cancer promoting effect for PXR.

### 2.1.3. Other nuclear receptors

During my doctoral study, I did not only focus on CAR and PXR, but I also worked with some other members of the nuclear receptor superfamily. Since there is growing evidence showing the interconnection between receptors in many physiologic processes, I briefly studied the potential relationship between CAR/PXR activity and other fellow receptors.

**Farnesoid X receptor** (FXR, NR1H4) was the first nuclear receptor described to be bile acid receptor (Forman et al 1995). Structurally, the receptor is closely related to PXR and CAR, and is mainly expressed in intestine, especially in ileum (Bookout et al 2006). Endogenous ligands of FXR are bile acids CDCA, CA and LCA (Carazo et al 2017). Obeticholic acid (OCA) is a high affinity synthetic FXR ligand that has been approved for the treatment of primary biliary cholangitis (primary biliary cirrhosis), in combination with ursodeoxycholic acid (UDCA) in 2016 (FDA Press release www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm503964.htm). In basal state, FXR binds to corepressors that are released after the binding of a ligand and recruits coactivators. FXR is involved in the bile acid homeostasis, metabolism, lipogenesis, gluconeogenesis and cancer (Claudel et al 2005, Deuschle et al 2012, Goodwin et al 2000, Pircher et al 2003). Importantly, FXR participates in the regeneration of the liver and the reversion of its inflammation (Hollman et al 2012, Chen et al 2011).

**Vitamin D receptor** (VDR, NR1I1) is expressed in bones, pancreas, intestine, kidneys, liver, adipocytes, monocytes and vascular muscles (Han & Chiang 2009, Norman 2006). The lack of vitamin D leads to bone diseases (osteomalacia, rickets, etc.). The functions of VDR include bone metabolism, cellular growth and mineral homeostasis (Norman 2006). The receptor also enhances several phase I metabolizing enzymes including CYP3A4 and CYP2C9 (Makishima et al 2002). Endogenous ligands calcitriol and bile acid LCA have high affinity for VDR (Adachi et al 2004). Activation mechanism is similar to that one of the previously described nuclear receptors, being ligand dependent.

**Glucocorticoid receptor** (GR, NR3C1) is another ligand-activated transcription factor. This receptor was first described in 1985 (Hollenberg et al 1985, Weinberger et al 1985), being the first nuclear receptor to be cloned. GR is mainly found in the cytoplasm in combination with proteins (Hsp90 among others) (Vandevyver et al 2012). Interestingly, GR was reported to be able to migrate from cytoplasm to nucleus and back again (Madan & DeFranco 1993). Upon ligand binding, GR releases the proteins that form the complex and translocates to the nucleus (Oakley & Cidlowski 2013). However, translocation of the receptor is also possible in absence of a ligand. Interestingly and unlike the other nuclear receptors, GR homodimerizes and binds to DNA through its GREs (Ratman et al 2013). This receptor, as indicated by his name, binds glucocorticoids, both natural and synthetic (cortisone, cortisol), but also non-steroidal molecules (De Bosscher et al 2010). GR receptor plays a role in inflammation, cancer, glucose metabolism and fatty acids metabolism (Oakley & Cidlowski 2011, Ratman et al 2013, Wu et al 2013).

## 2.2. Approach to ligand discovery

The discovery of a high affinity ligand for a given therapeutic target is a complicated, time and resources consuming task. In molecular biology, the majority of methods used for this purpose are *in vivo* and *in vitro* assays but with the advances in computational technology, *in silico* assays are gaining relevance.

Since nuclear receptors are found mainly in the liver, in our laboratory, we focused on *in vitro* hepatic models using cultured human hepatocyte-derived cells to study the activation patterns of PXR, CAR and other NRs. In these cell-based models, we used mainly traditional reporter gene assays (Carazo et al 2017), but also innovative CAR assembly assay (Carazo Fernandez et al 2015). Despite the information provided by these methods, complementary assays are usually required due to intricate mechanisms of activation that may not be adequately described through these methods. Ideally, the interactions of tested ligands with the target nuclear receptor need to be assessed with different methods, both cellular and non-cellular with recombinant proteins.

a. b.

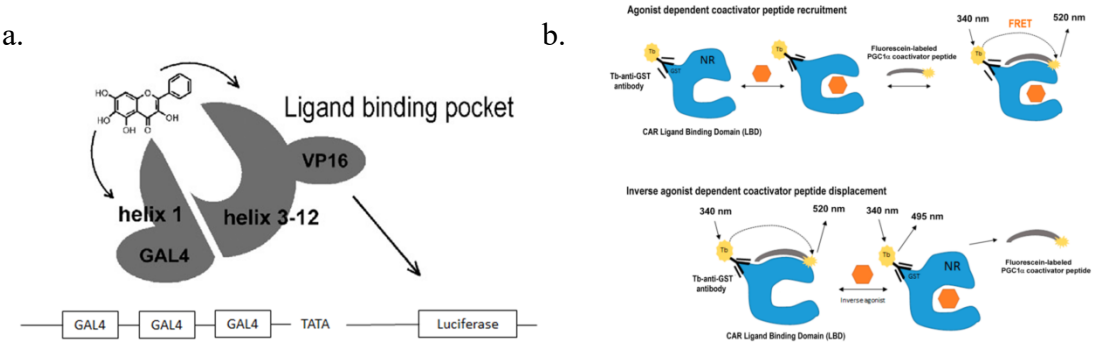


Figure 7. Scheme of the CAR Assembly Assay (a.) and scheme of the LanthaScreen™ TR-FRET CAR Coactivator Assay (b.).

*In vivo* models are useful methods to study the behavior of a ligand in a living organism. However, due to species differences and the intrinsic characteristics of animal models used in research, results should always be interpreted with caution. The outcomes from laboratory animals cannot be translated to humans, since each organism responds differently to drugs and the drug targets in humans and experimental animals are different.

In the 1990's, computer programs were developed to rationally develop or design molecules (*in silico*) that will potentially fit the biological target and eventually have a pharmacological effect (Danchin et al 1991). In pharmacology, these methods use computer software to analyze and interpret data obtained from different sources (medical, chemical, biological). These *in silico* techniques provide useful information about the models and the molecules developed. In our research, we used *in silico* methods to rationally develop potential ligands for the receptors we wanted to study and to predict the interaction of some ligands to the receptor LBD. These methods simplify the process of the search for new ligands.

## 2.3. Genetic variability in the study of nuclear receptors

Each individual responses differently to administered drugs, xenobiotics or to the environment stimuli and can express differently many genes, enzymes and proteins. This is called individual variability. The factors that determine this variability are genetic, environmental and individual (age, health conditions, and lifestyle).

Regarding nuclear receptors, genetic variability plays an important role since each receptor can present different isoforms and be expressed to a different proportion in each individual. For instance, from the receptors described in this doctoral work, all of them have been reported to have several transcription variants.

CAR has been reported to present many SNPs and two main isoforms, namely CAR1 and CAR3, which are the most relevant in humans (Auerbach et al 2003, Chen et al 2010, Lamba et al 2004a). These receptor isoforms represent around 80% and 20% of the total receptor expression. In the current doctoral thesis, I used both isoforms indistinctively for the testing of new CAR ligands, however, CAR3 variant is easily inducible and sensitive in cellular assays (Chen et al 2010). Interestingly, it has been reported that CAR presence in some animals varies between male and female individuals (Yoshinari et al 2001), but no studies to my knowledge reveal similar data in humans. In addition, several transcription variants and polymorphisms have been also reported for human CAR (Chen et al 2010).

Multiple variants and SNPs have also been stablished for hPXR (Hustert et al 2001, Lamba et al 2004b). It is logic to think that these variants modulate the expression of PXR target genes and determine how an individual will respond to a drug. These variants and polymorphisms are not exhaustively studied and are interesting fields of study in pharmacogenetics.

For the other receptors described in this work, the existence of isoforms has also been verified. For instance, in the case of FXR, 4 isoforms have been reported (α1, α2, β1, β2) (Zhang et al 2003). GR was described to have five isoforms (GRα, GRβ, $GR_Y$, GR-A and GR-P) (Oakley & Cidlowski 2011) of which the main are GRα (active)and GRβ(inactive). Lastly, VDR has been reported to have several SNPs but, to my knowledge, no isoforms have been described.

# 3. AIMS

The aims of my work are the following:

1) Discovery of new human CAR or other nuclear receptors ligands.
   a) Testing of steroid compounds related to known CAR ligands such as androstenol and androstanol.
   b) Testing of novel bile acid derivatives as new ligands of CAR, PXR, FXR and VDR.

2) Introduction of novel methods for studying direct and indirect CAR activation.
   a) Introduction of Time-Resolved Fluorescence Energy Transfer (TR-FRET) CAR coactivator method.
   b) Introduction of CAR assembly assay.
   c) Introduction of Elk1 reporter gene assay and ELISA assay with antibody against phosphorylated EGFR receptor.

3) Testing the indirect activation of human CAR.
   a) Testing the indirect activation of CAR with natural flavonoids.
   b) Testing the indirect activation of the receptor with leflunomide and teriflunomide.

# 4. LIST OF PUBLICATIONS RELATED TO THE DOCTORAL THESIS TOPIC

During my doctoral study, I published my results in several papers in international peer-reviewed journals with impact factor and I presented the data in several international and national scientific congresses and meetings.

My thesis is based on the following papers:

A.1. Carazo Fernandez A, Smutny T, Hyrsova L, Berka K and Pavek P (2015) Chrysin, baicalein and galangin are indirect activators of the human constitutive androstane receptor (CAR). *Toxicol Lett* **233**:68-77.

A.2. Carazo A and Pavek P (2015) The Use of the LanthaScreen TR-FRET CAR Coactivator Assay in the Characterization of Constitutive Androstane Receptor (CAR) Inverse Agonists. *Sensors (Basel)* **15**:9265-9276.

A.3. Carazo A, Hyrsova L, Dusek J, Chodounska H, Horvatova A, Berka K, Bazgier V, Gan-Schreier H, Chamulitrat W, Kudova E and Pavek P (2016) Acetylated deoxycholic (DCA) and cholic (CA) acids are potent ligands of pregnane X (PXR) receptor. *Toxicol Lett* **265**:86-96.

A.4. Smutny T, Nova A, Drechslerova M, Carazo A, Hyrsova L, Hruskova ZR, Kunes J, Pour M, Spulak M and Pavek P (2016) 2-(3-Methoxyphenyl)quinazoline Derivatives: A New Class of Direct Constitutive Androstane Receptor (CAR) Agonists. *J Med Chem* **59**:4601-4610.

A.5. Unpublished observations: Leflunomide and its major metabolite teriflunomide are activators of human CAR (hCAR)

# 5. AUTHOR'S CONTRIBUTION

My papers are detailed in the previous section, numbered from A.1. to A.5. In this section, I will detail my personal contribution to each paper.

- In paper number A.1., my contribution was:
    - Design of the experiments together with prof. PharmDr. Petr Pávek, PhD.
    - Execution of all experiments except Fig. 5 and Fig. 6.
    - Writing the draft of the paper and its final edition.

- In paper number A.2., my contribution was:
    - Design of the experiments together with prof. PharmDr. Petr Pávek, PhD.
    - Performance of all experiments.
    - Writing the draft of the paper and its final edition.

- In paper number A.3., my contribution was:
    - Design of the experiments together with prof. PharmDr. Petr Pávek, PhD.
    - Execution of all experiments except for Fig. 5 b) and Fig. 6.
    - Writing the draft of the paper and participating in its final edition.

- In paper number A.4., my contribution was:
    - Participated in the design of some of the experiments together with prof. PharmDr. Petr Pávek, PhD.
    - Performed experiments TR-FRET CAR (Fig. 1. e)) and inhibition of recombinant human CYPs 3A4, 2B6, 1A2, 2C9 and 2D6 (Figure 4).
    - Writing the parts of manuscript regarding the experiment I performed and analyzing the results.

- In paper number A.5., even though it is still under preparation, my contribution was:
    - Design of some of the experiments together with prof. PharmDr. Petr Pávek, PhD.
    - Performed all the experiments.
    - Writing the parts of manuscript and participating in the final version for submission to an international journal with impact factor.

# 6. OTHER PUBLICATIONS NOT RELATED TO THE DOCTORAL THESIS TOPIC

A.6. Karabanovich G, Nemecek J, Valaskova L, Carazo A, Konecna K, Stolarikova J, Hrabalek A, Pavlis O, Pavek P, Vavrova K, Roh J and Klimesova V (2016) S-substituted 3,5-dinitrophenyl 1,3,4-oxadiazole-2-thiols and tetrazole-5-thiols as highly efficient antitubercular agents. *Eur J Med Chem* 126:369-383.

A.7. Hyrsova L, Smutny T, Carazo A, Moravcik S, Mandikova J, Trejtnar F, Gerbal-Chaloin S and Pavek P (2016) The pregnane X receptor down-regulates organic cation transporter 1 (SLC22A1) in human hepatocytes by competing for ("squelching") SRC- 1 coactivator. *Br J Pharmacol* **173**:1703-1715.

A.8. Soukup T, Dosedel M, Pavek P, Nekvindova J, Barvik I, Bubancova I, Bradna P, Kubena AA, Carazo AF, Veleta T and Vlcek J (2015) The impact of C677T and A1298C MTHFR polymorphisms on methotrexate therapeutic response in East Bohemian region rheumatoid arthritis patients. *Rheumatol Int* **35**:1149-1161.

# 7. COMMENTARY ON THE PUBLISHED PAPERS IN INTERNATIONAL JOURNALS WITH IMPACT FACTOR RELATED TO THE DOCTORAL THESIS TOPIC

## A1. Chrysin, baicalein and galangin are indirect activators of the human constitutive androstane receptor (CAR)

Carazo Fernandez A, Smutny T, Hyrsova L, Berka K and Pavek P (2015) Chrysin, baicalein and galangin are indirect activators of the human constitutive androstane receptor (CAR). *Toxicol Lett* **233**:68-77. $IF_{2014}$: 3,262

The first project I was working on deals with the testing of several flavonoids widely present in plants on interaction with human CAR.

Baicalin, baicalein, chrysin and galangin are flavonoids present in *Sculletaria baicalensis* Georgi, *Chamomille* and *Passiflora* families and *Alpinia officinarum,* respectively. These plants have been used in traditional Chinese medicine (TCM) for its anti-oxidative, anti-inflammatory, anti-proliferative and anti-hypertensive properties, and for the treatment of viral diseases and improvement in diabetes mellitus type II (Heo et al 2001, Li et al 2011).

In my experiments, I used a novel approach to test direct CAR ligands (CAR assembly assay). This assay allows detection of direct CAR activation since the receptor is transfected in two parts and only upon presence of a ligand, both receptor parts come together forming the receptor and emitting luminescent signal. No significant activation of CAR was observed by these flavonoids using this method. As previous laboratories reported that these flavonoids enhance the expression of CAR target genes, we raised the question whether this activation was other than direct.

To confirm/deny this hypothesis, we performed complementary experiments using different techniques. With LanthaScreen™ TR-FRET (Time Resolved – Fluorescence Energy Transfer) CAR coactivation *in silico* assay, I clearly demonstrated that these compounds do not bind directly to CAR-LBD. This data confirmed our

speculation that CAR activation is unlikely direct. As RT-PCR (Real Time – Polymerase Chain Reaction) performed in human hepatocytes showed that all four flavonoids enhanced CYP2B6 expression, a key target gene of CAR, we raised the question whether these flavonoids would act as antiepileptic phenobarbital (PB) via phenobarbital-like indirect CAR activation.

PB is a well-known CAR indirect ligand. It was shown that PB-mediated CAR activation is exerted through inhibition of the epithelial growth factor receptor (EGFR). After PB binding to the EGFR, the EGF binding to the receptor is prevented, which consequently activates CAR through dephosphorylation (Mutoh et al 2013). Thus, we tried to determine if these flavonoids activate CAR receptor in a similar way. Experiments performed in A431 cells, elucidated a phenobarbital-like response by these flavonoids, being stronger for chrysin and baicalein than for the other two flavonoids. Next, we tested whether the signaling cascade was, as it is in phenobarbital, through ELK1 activation or inhibition. ELK1 is a downstream target transcription factor for ERK pathway and of EGFR receptor activation. In three different cell lines, I got similar results confirming that these flavonoids indirectly activates CAR via this mechanism.

In *in silico* assays (docking study), fitting of the flavonoids in the EGF receptor was studied. Our results show that especially baicalin, but also galangin and chrysin, significantly interact with the receptor.

This is the first research work confirming indirect (phenobarbital-like) CAR activation mechanism of a compound via inhibition of EGFR receptor.

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# Chrysin, baicalein and galangin are indirect activators of the human constitutive androstane receptor (CAR)

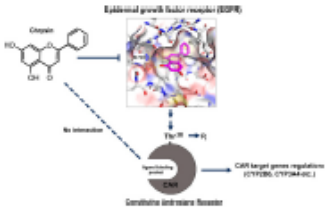
Alejandro Carazo Fernández [a], Tomas Smutny [a], Lucie Hyrsová [a], Karel Berka [b], Petr Pavek [a,*]

[a] Department of Pharmacology and Toxicology, Faculty of Pharmacy, Charles University in Prague, Heyrovského 1203, Hradec Kralove CZ500 05, Czech Republic
[b] Regional Centre of Advanced Technologies and Materials, Department of Physical Chemistry Palacky University in Olomouc, 17. listopadu 1131, Olomouc CZ779 00, Czech Republic

## HIGHLIGHTS

- Galangin, chrysin, baicalein and baicalin do not interact with human CAR.
- Galangin, chrysin, and baicalein significantly repress EGFR-Tyr1068 autophosphorylation and signaling.
- TR-FRET coactivator assay and CAR assembly assay detect direct interaction with CAR.
- This study demonstrates the needfor the testing of the direct CAR interaction of CAR activators.

## GRAPHICAL ABSTRACT

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## ABSTRACT

The constitutive androstane receptor (CAR) is a crucial transcriptional regulator of key xenobiotic-metabolizing enzymes such as cytochrome P450 CYP3A4, CYP2C9 and CYP2B6. The flavonoids chrysin, baicalein and galangin have been reported to activate CAR and interfere with EGFR signaling. Nevertheless, it is not known if these flavonoids are direct CAR ligands or indirect phenobarbital-like CAR activators via the inhibition of epidermal growth factor receptor (EGFR) signaling.

We analyze the interactions of chrysin, galangin and baicalein and its glycoside baicalin with human CAR. We have employed and validated methods that can study direct interaction with the CAR ligand binding pocket. Secondly, we determined if the compounds affect human EGFR signaling and interact with EGFR.

Employing a TR-FRET coactivator assay with recombinant CAR or CAR assembly assay, a consistent activation of CAR with flavonoids and phenobarbital was not observed. It was determined, however, that galangin, chrysin, and baicalein may slightly repress EGFR-Tyr1068 autophosphorylation after EGF treatment, phosphorylation of downstream transcription factor ELK1 and stimulate EGFP–CAR nuclear translocation in primary human hepatocytes.

These data suggest that flavonoids chrysin, galangin and baicalein are indirect human CAR activators. This study also demonstrates new approach how to test the direct CAR interaction with its ligands.



*Abbreviations:* CAR, constitutive androstane receptor; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor (ErbB-1, ERBB, HER1); ELK1, transcription factor, a member of ETS oncogene family; ERK, mitogen-activated protein kinases 1 and 3 (MAPK1 and 3); LBD, ligand binding domain; PGC1α, peroxisome proliferator-activated receptory, coactivator 1α; TR-FRET, time resolved fluorescence resonance energy transfer.

* Corresponding author. Tel.: +420 495067334.
*E-mail address:* petr.pavek@faf.cuni.cz (P. Pavek).

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0378-4274/

## 1. Introduction

The constitutive androstane receptor (CAR, NR1I3) is a ligand-activated transcription factor belonging to the nuclear receptor group NR1I. CAR has been extensively investigated in terms of controlling the gene expression of a broad spectrum of target genes which encode key phase I and phase II xenobiotic-metabolizing enzymes (DMEs) and drug transporters such as cytochrome P450 CYP3A4 and CYP2B6 enzymes, ABC efflux transporter MDR1 and UGT1A1 conjugation enzyme (Chai et al., 2013).

Recent studies suggest that CAR also plays important roles in the metabolism of glucose, lipids, fatty acids; in the endobiotic metabolism of bile acids, bilirubin and thyroid hormones; as well as in cell-cycle regulation, apoptosis and cell–cell interaction (Jiang and Xie, 2013; Wada et al., 2009). It has been proposed in several independent animal studies that CAR activation may ameliorate glucose homeostasis and insulin sensibility in the treatment of type 2 diabetes (Dong et al., 2009; Gao et al., 2009). In addition, CAR activation reduces levels of lipoproteins and inhibits the expression of lipogenic genes in what might be a promising therapeutic intervention in the treatment of obesity (Dong et al., 2009; Gao and Xie, 2012; Jiang and Xie, 2013; Molnar et al., 2013).

Baicalein (5, 6, 7-OH flavone) and baicalin (baicalein 7-O-glucuronide) have been found to be among the most significant flavones present in the root of *Scullelaria baicalensis* Georgi. The therapeutic potential of these compounds in the treatment of inflammation, cancer and virus-related diseases as well as to enhance neuroprotection and cardioprotection has been proposed (Li et al., 2011). Chrysin (5,7-OH flavone) is a flavone present naturally in the *Chamomile* and *Passiflora* families and in propolis; it has been reported to facilitate antioxidant activity and potential therapeutic activities in the treatment of diseases such as diabetes, hypertension and cancer. The flavonol galangin (3,5,7-OH flavone), abundant in *Alpinia officinarum* Hance and in propolis, has been shown to exert anti-oxidative, free radical scavenging, anti-inflammatory and anti-proliferative activities (Heo et al., 2001).

Baicalein, chrysin, galangin, but not baicalin (Fig. 1), have been recently proposed as CAR ligands. Baicalein was found to induce the expression of CAR target genes CYP3A4 and MDR1 (P-glycoprotein) as well as CYP2B6 transactivation in a gene reporter assay (Li et al., 2010; Yao et al., 2010). Chrysin was shown to up-regulate UGT1A1 expression and the CAR-mediated transactivation of CYP2B6 (Sugatani et al., 2004). In addition, chrysin induced the *Car* target gene *Cyp2b10* only in wild-type, but not in *Car* knockout mice (Yao et al., 2010). Similarly, galangin was found to strongly activate both wild-type or splicing variant SV25 of human CAR and mouse *Car* in vitro (Lau and Chang, 2013; Yao et al., 2010).

CAR was originally identified as a xenosensor in the binding of many environmental, natural, dietary and synthetic ligands. Numerous compounds have been proposed as agonists, antagonists as well as inverse agonists for human CAR (Hernandez et al., 2009; Molnar et al., 2013). In contrast to other nuclear or steroid hormone receptors, CAR can be activated in both ligand binding domain (LBD)-dependent and independent (indirect) interactions (Moore et al., 2000). However, little effort has been devoted to distinguish between indirect CAR activators (such as

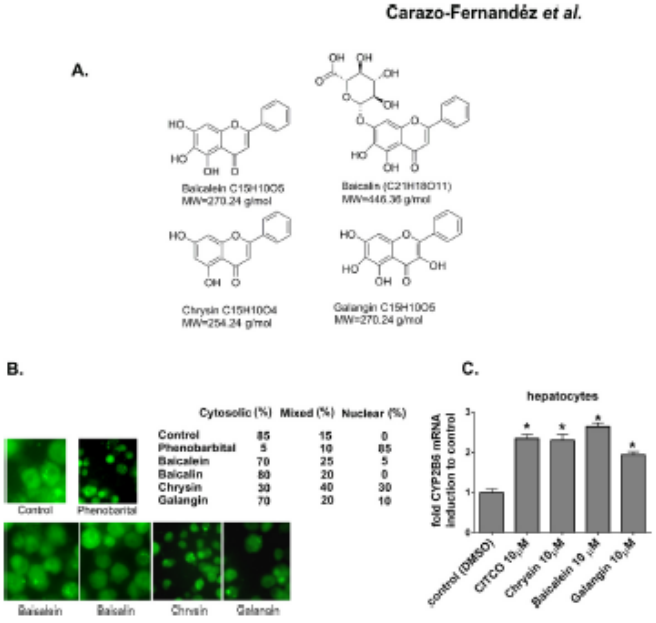


**Fig. 1.** (A) Chemical structures of flavones baicalein, its glucuronide baicalin, chrysin and flavonol galangin. (B) CAR translocation assay in primary human hepatocytes. Cryopreserved human hepatocytes were seeded at the density of $1 \times 10^6$ cells/well in 12-well collagen coated plates and were cultured for 24 h at 37 °C before transfection with pEGFP-hCAR construct. After 24 h, hepatocytes were treated with the vehicle control (0.1% DMSO), phenobarbital (1 mM), or galangin, chrysin and baicalein (30 μM) for 16 h. Then hepatocytes were washed twice with phosphate-buffered saline, and fluorescent microscopy analysis was employed for the cellular localization of EGFP-tagged CAR. One hundred EGFP-hCAR expressing hepatocytes were counted from each experiment and then averaged. Relative distribution of CAR (%) was classified as dominantly cytosolic, nuclear, or mixed (nuclear + cytosolic). (C) Chrysin, baicalein and galangin induce CYP2B6 mRNA expression in primary human hepatocytes. RT-PCR experiments in primary human hepatocytes (Primacyt) were performed with TaqMan probes for CYP2B6 and HPRT (housekeeping) genes after 16 h treatment with chrysin, baicalein and galangin at the concentration of 10 μM. CITCO was used as a ligand of human CAR (10 μM). Experiments were performed in triplicate samples and PCR reactions. *$p < 0.05$ – statistically significant difference compared to control (vehicle-treated) cells (ANOVA with a Bonferroni *post hoc* test).

acetaminophen, bilirubin and phenobarbital) and the ligands which interact within its ligand binding domain (Molnar et al., 2013; Moore et al., 2000). Currently the only high-affinity agonist for human CAR is 6-(4-chlorophenyl) imidazo[2,1-*b*]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl) oxime (CITCO) (Maglich et al., 2003). Recently, the indirect activation of CAR by phenobarbital has been explained based on the inhibition of the epidermal growth factor receptor (EGFR) binding of EGF, a process which prevents the activation of EGFR signaling and thus promotes the dephosphorylation of CAR at $Thr^{38}$. Dephosphorylated CAR then translocates to the nucleus, where it stimulates the transcription of target genes (Mutoh et al., 2013). Several studies in the past have shown that balcalein, baicalin, chrysin and galangin affect the ERK signaling cascade (see Section 4).

In the mechanistic study the goal was to investigate whether the flavonoids chrysin, baicalein, baicalin and galangin are direct or indirect activators of human CAR receptor. For this purpose, a TR-FRET based coactivator competitive assay and a CAR LBD assembly assay was used and validated. Stimulation of CAR nuclear translocation was studied in primary human hepatocytes. In addition, an EGFR phosphorylation ELISA along with an ELK1 activation luciferase gene reporter assay served to monitor the effects of the compounds on EGF-induced EGFR autophosphorylation and on EGFR signaling inhibition. Finally, chrysin and baicalin were also docked to EGFR to corroborate the interactions.

## 2. Materials and methods

### 2.1. Chemicals

Chrysin, baicalein, baicalin and galangin as well as phenobarbital and phenytoin (indirect activators of CAR), androstanol (3β-hydroxy-5α-androstane), clotrimazole, PK11195 (CAR antagonists), erlotinib, U0126, cell culture media, and fetal bovine serum (FBS) were purchased from Sigma–Aldrich (St. Louis, MO). CITCO (6-(4-chlorophenyl)-imidazo[2,1-b]thiazole-5-carbaldehyde-*O*-(3,4-dichlorobenzyl) oxime) was purchased from BIOMOL Research, Germany. Phenol red-free media was purchased from Life Technologies/GIBCO (Carlsbad, CA). The final concentration of DMSO in the culture media was 0.1% (v/v) in all experiments.

### 2.2. Cell lines

The A431 squamous carcinoma cell line which express epidermal growth factor (EGF) and display functional EGFR/Ras/Raf/MEK/ERK signaling was purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK) and was maintained in an antibiotic-free DMEM medium supplemented with 10% FBS supplemented with 1% nonessential amino acids (Sigma–Aldrich). The human hepatocellular carcinoma Huh-7(D12) and HepG2 cell lines were purchased from the ECACC and was maintained in an antibiotic-free DMEM medium supplemented with 10% FBS and 1% non-essential amino acids (in case of HepG2).

### 2.3. TR-FRET CAR coactivator binding assay

The LanthaScreen® TR-FRET CAR Coactivator Binding Assay (Life Technologies, now Thermo) was used with slight modifications of the manufacturer's protocol. The assay uses two fluorophores: a terbium-labeled anti-GST antibody interacting with glutathione-*S*-transferase tagged human CAR ligand-binding domain (LBD) and a fluorescein-labeled PGC1α coactivator peptide. The interaction of a ligand stimulates interactions of the components, resulting in a FRET emission shift from 495 nm to

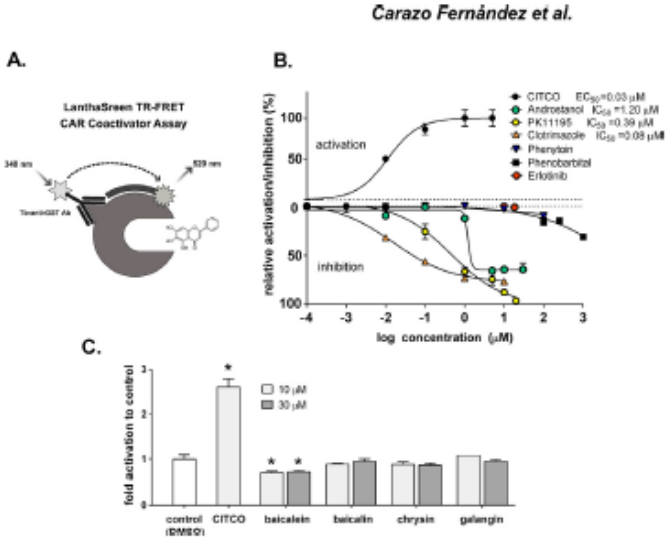


**Fig. 2.** Interaction of tested flavonoids with recombinant human CAR using the LanthaScreen® TR-FRET CAR Coactivator Binding assay. (A) Schematic of the TR-FRET-based CAR Coactivator Binding assay; (B) the TR-FRET CAR Coactivator Binding assay was performed to determine the ability of baicalein, baicalin, chrysin and galangin to directly bind to the CAR ligand binding domain. Phenobarbital, a prototype indirect CAR activator, and clotrimazole, PK11195 and androstanol as model inverse agonists/antagonists were used in validation experiments. Data are presented as the relative activation or inhibition to the control DMSO vehicle-containing samples, which were set to 1. Maximum activation with CITCO (10 μM) was set at 100%. Values below 1 represent the relative suppression of CAR constitutive activity (%) with the antagonists/inverse agonists of CAR; (C) baicalein, baicalin, chrysin and galangin do not activate CAR. The tested flavonoids were used at the 10 and 30 μM concentrations together with CITCO (10 μM) and tested according to manufacturer's protocol with the slight modification described in Section 2. The relative fold activation to control (DMSO vehicle-treated samples) was calculated and the data are presented as the means ± SD from three independent experiments ($n = 3$) performed in triplicate measurements. ANOVA with a Bonferroni *post hoc* test were used for statistical analysis, $^*p < 0.05$ – statistically significant difference compared to control (vehicle-treated) cells.

520 nm (Fig. 2A). The assay was performed in a 384-well plate format in 20 μl of reaction mixture. Incubation lasted 1 h at room temperature protected from light. After an incubation period, TR-FRET fluorescence was measured with a Synergy Biotek plate reader (Bio-tek, Winooski, VT) using an excitation wavelength of 340 nm with a filter detecting the fluorescent emission signals of terbium at 495 nm and fluorescein at 520 nm. Delay time was set at 100 μs and integration time 200 μs. The 520 nm/495 nm TR-FRET ratio was calculated. Data are presented as relative activation or inhibition to control (DMSO vehicle-containing sample), which was set at 1. TR-FRET assay was validated in the presence of 2% DMSO. Maximum activation with CITCO was set to 100% activation. We were able to reproductively detect activity of 5 nM CITCO. The 520 nm/495 nm TR-FRET ratio of samples without CAR LBD was taken as background and was subtracted. Values below 1 represent the inhibition of CAR-PGC1α interaction and suppression of CAR constitutive activity with antagonists/inverse agonists of CAR. The experiments were performed at least three times in triplicates. Data are presented as the means and S.D. from three independent experiments ($n = 3$).

### 2.4. CAR LBD assembly assay

The CAR LBD assembly assay was performed with the afore-mentioned described constructs (Kobayashi et al., 2010). The LBD assembly assay is based on two hybrid expression constructs encoding C (151-349 aa, helices 3-12, pCAR-C/VP16) and N (103-150 aa, helix 1, pCAR-N/GAL4) terminal parts of human CAR LBD that are co-transfected together with the pGL5-luc luciferase reporter plasmid (Promega) containing UAS binding sites. A ligand promotes connection of the helix 1 to CAR LBD helices 3-12 resulting in luciferase transactivation (Fig. 3A).

Transient transfection assays were carried out in the Huh-7 cells using TransFectin™ transfection reagent (Bio-Rad Hercules, CA). Cells were seeded into 48-well plates ($7 \times 10^5$ cells per well) and transfected with a pGL5-luc luciferase reporter construct (80 ng/well), expression constructs pCAR-C/VP16 and pCAR-N/GAL4 (160 ng/well) and the *Renilla reniformis* luciferase transfection control plasmid (pRL-TK) (30 ng/well) 24 h later. Cells were maintained in a phenol red-free medium (200 μl) and treated with the tested compounds (in a range from 5 up to 30 μM); reference compounds CITCO (1 or 10 μM), phenytoin (100 μM), phenobarbital (500 and 1000 μM), clotrimazole (10 and 30 μM), PK111195 (20 μM) and androstanol (10 μM) for 24 h. Luminescence activity in the cell lysate was measured using a commercially available luciferase detection system (Dual Luciferase Reporter Assay, Promega, Madison, WI, USA). The data are presented as means and S.D. and expressed as the fold-change of firefly luciferase activity normalized to *Renilla* luciferase activity in each sample and relative to the vehicle (DMSO 0.1%)-treated controls, which were set at 1. Statistical analyses were performed using at least three independent assays ($n = 3$) performed in triplicates. Experiments with the empty expression construct were perform to analyze the effect of tested compounds on the luciferase reporter construct.

### 2.5. EGFR binding assay

The phosphorylated EGFR at Tyr1068 was measured by the ELISA method with a phosphorylation-specific antibody according to manufacturer's protocol (RD Systems, Inc. Minneapolis, MN).

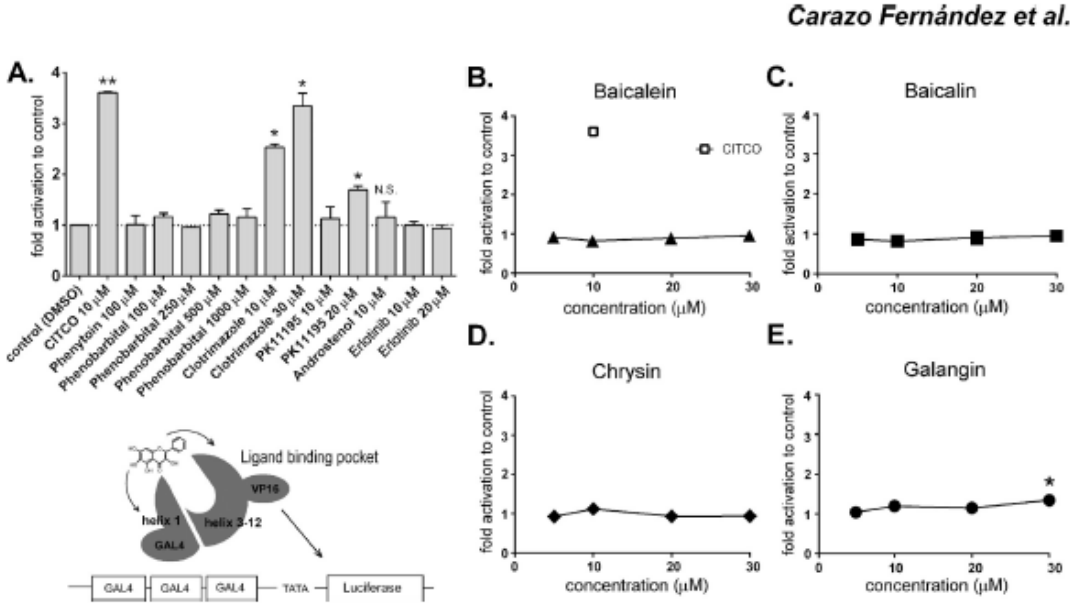


**Fig. 3.** Interaction of tested flavonoids with CAR in CAR ligand binding domain assembly assay in Huh-7 cells. (A) The validation data and schematic diagram of the CAR ligand binding domain assembly assay. CITCO (a direct CAR agonist, 10 μM), phenobarbital and phenytoin, prototype indirect CAR activators, and clotrimazole (10 and 30 μM), PK11195 (10 μM) and androstanol (10 μM) were used as the model inverse agonists/antagonists in the validation experiments. The relative fold activation to control (DMSO vehicle-treated samples) was calculated and the data are presented as the means ± SD from three independent experiments ($n = 3$). (B) Baicalein, (C) baicalin, (D) chrysin and (E) galangin were tested at the concentrations 5, 10, 20 and 30 μM. The data are presented as means and S.D. from three independent experiments ($n = 3$). Data are expressed as the fold-change of firefly luciferase activity normalized to *Renilla* luciferase activity in each sample and relative to the vehicle (DMSO 0.1%)-treated controls, which were set at 1. $^*p < 0.05$, $^{**}p < 0.01$ – statistically significant difference compared to control (vehicle-treated) cells (ANOVA with a Bonferroni *post hoc* test). N.S. – statistically non-significant difference.

A431 cells were seeded ($2 \times 10^5$ cells per well) in 96-well plates and stabilized for 24 h. After this time, cells were pretreated for 1 h with serum-free Opti MEM™ medium, after which cells were treated with the tested compounds chrysin, baicalin, baicalein and galangin (at concentrations of 30 μM) and reference compounds CITCO (1 μM and 10 μM), erlotinib (10 μM, an EGFR inhibitor), as well as phenobarbital (an indirect CAR activator, 1 mM) for 30 min. After this cells were treated with EGF (50 ng/ml) added into each media for 10 min. The cells were then fixed with 4% formaldehyde and incubated simultaneously with two primary antibodies (a phosphor-specific antibody and a normalization antibody for the recognition of the EGFR protein regardless of phosphorylation status). Two secondary antibodies and two spectrally different fluorogenic substrates were used in the recognition of the primary antibodies. The fluorescence of the phosphorylated protein was normalized with that of the pan-EGFR protein. After background subtraction, the data were expressed as a relative activation and related to the EGF-treated cells, which were set at 100%. Statistical analysis was performed using at least three independent assays performed in triplicates.

### 2.6. *ELK1* trans-reporting assay

The activation of the EGFR/Ras/Raf/MEK/ERK mitogen-activated protein kinase (MAPK/ERK) signaling cascade was monitored using the PathDetect ELK1 *trans*-Reporting System (Agilent Technologies, Inc., Santa Clara, CA). In the assay, the phosphorylation of ELK1 transcription factor via ERK kinases, downstream kinases in the EGFR-induced ERK mitogen-activated protein kinase (MAPK) signaling pathway, was analyzed using a luciferase gene reporter construct. The ELK1 transcription factor assay was used, as it is a highly specific downstream factor activated by the ERK mitogen-activated protein kinase (MAPK) signaling pathway, although also p38 and JNK kinases have been reported to phosphorylate the factor (Besnard et al., 2011).

Huh-7, HepG2 or A4321 cells were seeded into 48-well plates ($1 \times 10^5$ cells per well) and were maintained to stabilize for 24 h. Then the cells were transfected with a pGL5-luc luciferase reporter construct (150 ng/well), an expression pFA2-ELK1 trans-Reporter Activator Plasmid (100 ng/well), and the *Renilla reniformis* luciferase transfection control plasmid (pRL-TK) (30 ng/well) 24 h later. After 24 h, the media were removed and the cells were washed with PBS and treated with EGF (50 ng/mL) in combination with the tested compounds chrysin, baicalein, baicalin, and galangin (at a concentration of 30 μM) or with the reference compounds U0126 (10 μM, a MEK1 and 2 inhibitor of the ERK MAPK pathway), erlotinib (10 μM, an EGFR inhibitor), phenobarbital (an indirect CAR activator, 1 mM) and CITCO (10 μM, direct CAR activator) for 24 h. Luminescence activity in the cell lysate was measured using the Dual Luciferase Reporter Assay (Promega). The activity of the cells transfected with an empty expression vector was subtracted. The data are expressed as a relative activation of firefly luciferase activity normalized to *Renilla* luciferase activity in each sample relative to the EGF-treated controls, which were set at 100%. Statistical analyses were performed using at least three independent assays ($n = 3$) performed in triplicates.

### 2.7. Molecular modeling

3D structures of compounds were prepared and all hydrogens were added with Marvin and MarvinSketch 14.7.7 program (ChemAxon, http://www.chemaxon.com). 3D structures of ligands were optimized and all hydrogens were added within the MarvinSketch 14.7.7 program. All nonaromatic and nonring bonds were set as rotatable within AutoDock Tools 1.6.6 program (Sanner, 1999). The crystal structure for EGFR with erlotinib (PDB ID: 1M17) was used as the protein docking template with a docking grid of 14 Å around the center of the ligand in the crystal structure, which was deleted prior to docking. Polar hydrogens were added to receptor or selected for all ligands with the AutoDock Tools program prior to docking with the Autodock Vina program (Trott and Olson, 2010).

### 2.8. CAR translocation assay in primary human hepatocytes and induction of CYP2B6 mRNA

Induction qualified plateable cryopreserved human hepatocytes (Cat. Code HMCPIS, Life Technologies) or Cryopreserved Plateable Human Hepatocytes for Induction Cell Specification (Cat. Code CHHP-I1 Lot 140-130614-2, Male, 54 years) were obtained from Life Technologies (Durham, NC) or Primacyt (Swerin, Germany), respectively. Hepatocytes were seeded at $1 \times 10^6$ cells/well in the 12-well collagen coated plates, respectively. Hepatocytes were cultured for 24 h at 37 °C before transfection with pEGFP-hCAR construct (Kanno et al., 2007) with Transfectin™ (Biorad, Hercules, CA). After 24 h, the hepatocytes were treated with the vehicle control (0.1% DMSO), phenobarbital (1 mM), or selected compounds (30 μM) for 16 h. Then hepatocytes were washed twice with phosphate-buffered saline, and fluorescent microscopy analysis was employed for the cellular localization of EGFP-tagged CAR using Nikon Eclipse microscope. Quantitative distribution of CAR was classified as dominantly cytosolic, dominantly nuclear, or mixed (nuclear+cytosolic). One hundred fluorescent EGFP-hCAR expressing hepatocytes were counted from each experiment and then averaged. Total RNA isolation and semi-quantitative real-time RT-PCR (qRT-PCR) analyses of CYP2B6 mRNA expression in primary human hepatocytes were performed after 16 h treatment by employing commercial assays (hCYP2B6_Q2 and hHPRT_Q3 for HPRT housekeeping gen) from Generi-Biotech (Hradec Kralove, Czech Republic) as described elsewhere (Krausova et al., 2011). All experiments (treatment and PCR) were performed in triplicate.

### 2.9. Statistical analysis

A one-way analysis of variance followed by a Dunnett's multiple comparison *post hoc*; a Bonferroni *post hoc* test or Student's *t*-test was used for the statistical analysis of differences between two or more experimental groups using GraphPad Prism software ver. 6 (GraphPad Software Inc., San Diego, CA). $EC_{50}$/$IC_{50}$: the concentration required to achieve half-maximum activation/inhibition was determined according to Hill's equation by nonlinear regression analysis from at least 4-point curves performed in triplicate.

## 3. Results

### 3.1. Galangin, baicalein, chrysin and phenobarbital stimulate nuclear translocation of EGFP-CAR

In normal hepatocytes, CAR is mainly localized in the cytoplasm and translocates to the nucleus only after exposure to CAR activators. In opposite, CAR is spontaneously accumulated in nucleus in tumor immortalized cell lines.

In first series of experiments, we studied nuclear translocation of EGFP-tagged CAR in primary human hepatocytes. As expected, approximately 85% CAR was dominantly expressed in the cytoplasm in control vehicle-treated hepatocytes, while more than 85% CAR translocated to the nucleus after being treated with phenobarbital (1 mM), a prototypical CAR activator, for 16 h (Fig. 1B). Galangin, baicalein, and chrysin treatment resulted in moderate stimulation of EGFP-hCAR nuclear translocation (control

10%) (Fig. 1B). These data confirm published data that galangin, baicalein and chrysin are human CAR activators.

In RT-PCR experiments, baicalein, chrysin and galangin induced CYP2B6 mRNA gene expression, a target gene mainly regulated by CAR, in primary human hepatocytes after 16 h treatment (Fig. 1C). Nevertheless, since CYP2B6 is also regulated by PXR receptor and chysin and baicalein interact with PXR, we cannot exclude an effect of PXR activation in the experiments.

### 3.2. Chrysin, balcalein, baicalin and galangin do not activate CAR through its ligand binding domain

Firstly we analyzed if the tested flavonoids interact with CAR LBD in a TR-FRET-based coactivator assay (schematic diagram in Fig. 2A).

In the validation experiments, we observed no direct activation of CAR by phenobarbital or phenytoin (Fig. 2B), a result which corresponds with previous reports (Molnar et al., 2013; Moore et al., 2000). On the other hand some inhibition of CAR by phenobarbital was observed at 1000 μM concentration. Known inhibitors or inverse agonists androstanol, PK11195 and clotrimazole significantly inhibited CAR in a dose-dependent manner (Fig. 2B). CITCO, a prototype agonist ligand, significantly stimulated the interaction of CAR LBD with the coactivator peptide in a dose-dependent manner with the value $EC_{50}$ = 0.03 μM (Fig. 2C).

In testing the flavonoids, it was found that none of them activated CAR in the assay. Rather, some slight inhibition was observed, with the finding being statistically significant in the case of baicalein ($p < 0.05$, Fig. 2C). Erlotinib had no effect on CAR activation.

To confirm this data, a CAR LBD assembly assay was used in the next experiments (Fig. 3). CITCO, but not phenobarbital and phenytoin, interacted with the assembly of CAR LBD (Fig. 3A) (Burk et al., 2005; Kobayashi et al., 2010; Maglich et al., 2003; Moore et al., 2000). Importantly, PK11195 and clotrimazole, supposed to be inhibitors or inverse agonists of CAR (Li et al., 2008; Maglich et al., 2003) significantly ($p < 0.05$) stimulated interactions of CAR LBD fusion proteins. Androstanol also activated the gene reporter construct in the experiments, although the effect was not statistically significant (Fig. 3A). Tested flavonoid had no effect on the luciferase reporter construct if the cells were co-transfected with the empty GAL4 expression construct. Erlotinib had no effect on CAR activation. We observed no significant effect on Huh-7 cells viability as determined with the CellTiter 96® $AQ_{ueous}$ One Solution Cell Proliferation assay (Promega) with MTS tetrazolium salt (unpublished data). These data indicate that the CAR assembly assay does not discriminate between agonists and antagonists, but detects all ligands of the CAR ligand binding domain formed.

Consistent with the data in Fig. 2C, it was found that baicalein, baicalin and chrysin do not activate CAR in the assay, again suggesting no direct interactions of the compounds with CAR LBD (Fig. 3B–D). Slightly (1.4-fold), but significantly ($p < 0.05$), galangin stimulated CAR LBD formation in the assay at 30 μM concentration (Fig. 3E). However, such similar activation was not seen in TR-FRET based assay (Fig. 2C). No effect of either the tested flavonoids or the known ligands in control experiments with empty expression constructs was observed.

These data demonstrate the validity of the methods used and suggest no interactions of baicalein, baicalin, galangin and chrysin with human CAR in two independent assays.

### 3.3. Chrysin inhibits EGFR receptor autophosphorylation stimulated by EGF in A431 cells

In the next series of experiments, we examined whether the tested flavonoids inhibit EGFR autophosphorylation at Tyr1068. After EGF binding, the GRB2 adaptor protein binds activated EGFR at phosphosite Tyr1068, and triggers mitogen-activated protein kinase (MAPK) pathway activation (Rojas et al., 1996). It was found that phenobarbital, baicalin, chrysin, galangin and baicalein suppressed the EGFR phosphorylation by about 20–45% at a concentration of 30 μM or 1 mM, respectively (Fig. 4). The inhibition of EGFR phosphorylation by phenobarbital was modest but reproducible in all the experiments ($n = 5$) (Fig. 4) in comparison with reported data (Mutoh et al., 2013). Interestingly, we also observed that CITCO significantly attenuated EGFR Tyr1068 autophosphorylation in the experiments if the A431 cells were treated with a 10 μM concentration. Erlotinib, a model EGFR receptor antagonist, abolished the EGFR Tyr1068 phosphorylation in the experiments (Fig. 4).

These data indicate significant interactions of baicalin, chrysin and galangin with the EGFR receptor resulting in partial EGFR Tyr1068 autophosphorylation decrease after treatment with EGF.

### 3.4. Flavonoids suppress ELK1 phosphorylation by EGF in Huh-7 and HepG2 cells

Next, it was analyzed whether the effects of the tested flavonoids on EGFR is reproducible at the level of a down-steam transcription factor ELK1 phosphorylation. Consistently with data in Fig. 4, we observed that baicalein, galangin, chrysin and baicalin as well as phenobarbital, erlotinib and MEK inhibitor U0126 significantly inhibit ELK1 phosphorylation after EGF treatment in Huh-7 and HepG2 cells (Fig. 5A and B). In contrast,

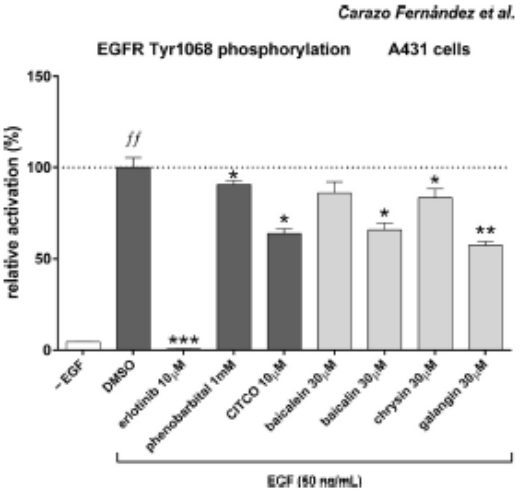


**Fig. 4.** Interaction of tested flavonoids with the EGFR receptor. The phosphorylated EGFR at Tyr1068 and total EGFR expression were measured using the ELISA method with a phosphorylation-specific and non-specific antibodies according to manufacturer's protocol in A431 cells. Cells were pretreated with chrysin, baicalin, baicalein and galangin (at concentrations of 30 μM), CITCO (10 μM), erlotinib (10 μM, an EGFR inhibitor), and phenobarbital (an indirect CAR activator, 1 mM) for 30 min and then treated with EGF (50 ng/ml) for 10 min. Two secondary antibodies and two spectrally different fluorogenic substrates were used for the recognition of the primary antibodies. The fluorescence of the phosphorylated protein was normalized with that of the pan-EGFR protein. Following background subtraction, the data are expressed as a relative activation and related to the EGF-treated cells, which were set at 100%. Statistical analysis (ANOVA with a Dunnett's *post hoc*) were performed from three independent assays ($n = 3$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ – statistically significant difference compared to control (vehicle- and EGF-treated treated) cells; $^{ff}p < 0.01$ – statistically significant difference compared to vehicle-treated cells without EGF treatment (–EGF).

in non-hepatic A431 cell line, phenobarbital, baicalein, baicalin and chrysin had no significant effect on ELK1 activation (Fig. 5C). A direct CAR agonist CITCO did not significantly suppress ELK1 activation in the experiments although we observed inhibition of EFGR autophosphorylation (Figs. 4 and 5).

These data indicate that tested flavonoids interfere with the EGFR-induced MAPK signaling pathway in hepatocyte-derived cell lines.

### 3.5. Docking of galangin, baicalin, erlotinib and phenobarbital into EGFR binding pocket

Molecular docking showed the potential interactions of all tested molecules within the binding pocket of EGFR receptor. Crystal structure (PDBID: 1M17) reveals that the binding groove is formed by two planar hydrophobic pockets. These planar pockets are formed between triangle of nonpolar amino acids – L718, M766 and M793. Redocking of erlotinib back into the EGFR crystal structure shows three interaction patterns: (i) the fulfilment of the hydrophobic pocket between M766 and L718 by a tilted *m*-ethynylphenyl ring; (ii) hydrogen bonding of quinazolin N1 hydrogen-bond acceptor with M793 mainchain; and (iii) two 2-methoxyethoxy chains exposed to the solvent (Fig. 6A). Fulfilment of only one interaction pattern as in phenobarbital (Fig. 6B) transmits into its low affinity towards EGFR receptor. Better affinity can be achieved by the fulfilment of two patterns as in the case of chrysin (Fig. 6C), baicalin (Fig. 6D) and galangin (unpublished observations). And the strongest affinity was found in the case of baicalin (Fig. 6D), where all three interaction patterns can be identified.

## 4. Discussion

CAR is emerging as a promising new therapeutic target for metabolic diseases such as type 2 diabetes mellitus, dyslipidemia and obesity. Both ligand binding domain (LBD)-dependent and indirect activation of human CAR has been described as releasing CAR from its cytoplasmic complex and translocating the receptor into the nucleus to transactivate target genes. Unlike other nuclear receptors, CAR exerts LBD-independent mechanisms of activation and false nuclear localization in tumor cell lines, an effect which hinders the discovery of new therapeutic ligands either of natural or synthetic origin. In addition to endobiotic metabolism, CAR is a mediator of clinically significant drug–drug interactions which modulate the efficacy of co-administered drugs through the induction of xenobiotic metabolizing enzymes (Chai et al., 2013). Thus knowledge of interactions of CAR with drugs, natural compounds and dietary supplements may also help us to avoid failures in pharmacotherapy due to the induction of drug metabolism.

At present, natural compounds such as artemisinin and its derivatives arteether and artemether; pyrethroids (e.g., permethrin, cypermethrin); flavonoids such as chrysin, baicalein and galangin, and diallyl sulfide derived from garlic, have been reported as activators of CAR (Burk et al., 2005, 2012; Molnar et al., 2013; Sueyoshi et al., 2011; Yao et al., 2011). In addition, numerous synthetic compounds have been demonstrated to activate CAR such as carbamate benfuracarb, organochlorines (e.g., methoxychlor), CITCO, FLB1, octicizer, thiazolidin-4-ones, 6-arylpyrrolo[2,1-*d*][1,5]benzothiazepine derivatives, and sulfonamides; and drugs (e.g., artemisinin derivatives, nevirapine, nicardipine, efavirenz, carbamazepine) (Anderson et al., 2011; Burk et al., 2012; Faucette et al., 2007; Kublbeck et al., 2011a,b; Lynch et al., 2013; Molnar et al., 2013; Yao et al., 2011).

However, only marginal attention has been paid to the molecular mechanism of CAR activation by these compounds (Burk et al., 2012). Baicalein, chrysin and galangin have been reported to be strong activators of human CAR (Li et al., 2010; Yao et al., 2010). However, direct interaction of the flavonoids with the CAR LBD domain has yet not been systematically studied.

In this study it was demonstrated using recombinant human CAR that baicalein, baicalin, galangin and chrysin do not interact with the CAR ligand binding pocket although some of them may promote nuclear translocation of CAR in primary human hepatocytes (Figs. 1B, 2B and 3A). The known human CAR agonist CITCO significantly activated CAR in a dose-dependent manner, validating the used assays (Figs. 2B and 3A). The established antagonists/inverse agonists of human CAR receptor consistently displayed significant dose-dependent interactions with CAR in the assays (Figs. 2B and 3A). Importantly, phenobarbital and

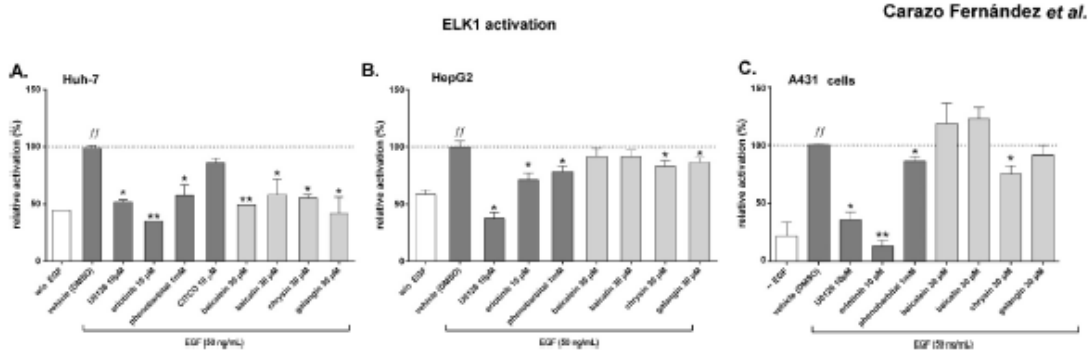


**Fig. 5.** Activation of ELK1 transcription factor by tested flavonoids in A431 cell line.
The activation of the EGFR-induced mitogen-activated protein kinase (MAPK/ERK) signaling cascade was monitored using the PathDetect ELK1 *trans*-Reporting System. Huh-7 (A), HepG2 (B) and non-hepatic A431 (C) cells were transfected with a pGL5-luc luciferase reporter construct (150 ng/well), an expression pFA2-ELK1 trans-Reporter Activator Plasmid (100 ng/well), as well as the *Renilla reniformis* luciferase transfection control plasmid (pRL-TK) (30 ng/well). After 24 h, media were removed, cells were treated with EGF (50 ng/mL) in combination with the tested compounds chrysin, baicalein, baicalin, and galangin (at a concentration of 30 μM) or the reference compounds U0126 (10 μM, a MEK1 and 2 inhibitor of the ERK MAPK pathway), erlotinib (10 μM, an EGFR inhibitor), phenobarbital (an indirect CAR activator, 1 mM) for 24 h. Luminescence activity in the cell lysate was measured using a Dual Luciferase Reporter Assay. The data are presented as a relative activation of firefly luciferase activity normalized to *Renilla* luciferase activity in each sample and relative to the EGF-treated controls, which were set at 100%. $^{*}p < 0.05$, $^{**}p < 0.01$ – statistically significant difference compared to control (vehicle- and EGF-treated treated) cells; $^{//}p < 0.01$ – statistically significant difference compared to vehicle-treated cells without EGF treatment (–EGF) (ANOVA with a Dunnett's *post hoc*).

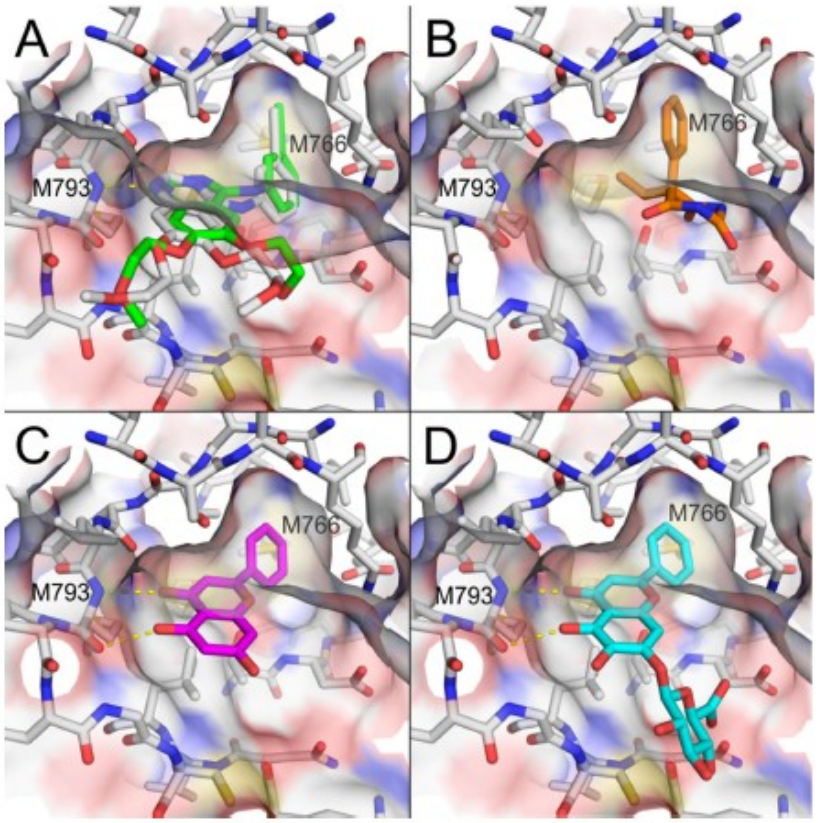


**Fig. 6.** Molecular docking into the EGFR receptor (PDBID: 1M17). (A) Structure of the docked binding pose of erlotinib (green) compared to the crystal structure (white) shows successful overlap between docked pose and experimental one with hydrogen bonding to the main chain of M793 backbone. (B) Phenobarbital (orange) have the lowest affinity as it fills only the hydrophobic pocket; (C) chrysin (magenta) forms two hydrogen bonds with M793 – one as an acceptor and second as a donor; and (D) baicalin (cyan) have the strongest affinity towards the EGFR receptor as it has in addition to chrysin's hydrogen bonding pattern also solvent exposed glucuronic moiety. For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

phenytoin, prototype indirect activators of human and rodent CAR/*Car* (Moore et al., 2000; Wang et al., 2004), did not significantly activate CAR in our experiments even in high milimolar concentrations (Figs. 2B and 3A). Another interesting finding was that phenobarbital in supramicromolar concentration slightly inhibited CAR in the TR-FRET assay (Fig. 2B). To our knowledge this is the first report that has used the TR-FRET-based coactivator assay and CAR LBD assembly assay to characterize direct CAR ligands or to distinguish them from indirect CAR activators. In addition, this is the first report on the interactions of CAR ligands with human EGFR and its signaling.

Since, both CAR and EGFR signaling have numerous cellular and tissue functions under both normal and pathological conditions, knowing the precise molecular nature of CAR activation of known CAR activators is critical in the evaluation of their pharmacological potential. At present, most of current knowledge about CAR comes from animal studies using the rodent specific *Car* ligand 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) or using *Car* knockout or transgenic mice (Chai et al., 2013; Molnar et al., 2013). There is thus an urgent need for a nontoxic and specific high-affinity human CAR agonist that does not affect other nuclear receptors or additional signaling pathways.

In our study we also examined whether chrysin, baicalin, baicalein, or flavonol galangin inhibit human EGFR Tyr1068 autophosphorylation and ERK/MAPK cascade downstream transcription factor ELK1. Several studies in the past have shown that chrysin exerts an inhibition effect on the ERK signaling cascade in different cells and thus suppresses the levels of active phosphorylated ELK1 (Gao et al., 2013; Pichichero et al., 2011). Similarly, baicalein has been reported to have an inhibitory effect on ERK1/2 phosphorylation in both *in vivo* and *in vitro* (Agarwal et al., 2009; Gao et al., 2013; Chen et al., 2013; Liang et al., 2012; Zhou et al., 2013). On the other hand opposite effects have also been reported for baicalein, baicalin and galangin, suggesting cell- or tissue-specific effects (Chen et al., 2006; Lei et al., 2012; Liu et al., 2013; Zhou et al., 2009). These data together with the recent findings of the research group of Dr. Masahiko Negishi defining the indirect activation of CAR via EGFR inhibition (Mutoh et al., 2013) urge for a comprehensive study of the molecular mechanism of their interactions with CAR.

Finally, we studied if tested flavonoids inhibit human EGFR receptor autophosphorylation at Tyr1068 involved in ERK signaling and the activation of the ELK1 transcriptional factor, a downstream effector protein of the EGFR/Ras/Raf/MEK/ERK pathway in the nucleus. We found that the tested flavonoids inhibit human EGFR receptor autophosphorylation at Tyr1068. We confirmed that phenobarbital interferes with human EGFR in A431 cells, although the effect was weak even at a 1 mM concentration (Fig. 4). When we analyzed the effects of tested compounds on ELK1 activation in hepatic Huh-7 and HepG2 cells, we found that galangin, chrysin (Huh-7, HepG2), and baicalein and baicalin (Huh-7) significantly inhibited ELK1 activation (Fig. 5A and B). Phenobarbital displayed significant inhibitory effect comparable with U0126 or erlotinib in the experiments suggesting significant inhibition of the signaling after 24 h treatment (Fig. 5A and B). Molecular docking showed hypothetical interactions of all tested flavonoids within the binding pocket of EGFR receptor.

These results suggest that in hepatic cells baicalein, chrysin and galangin interact with EGFR signaling to indirectly activate CAR translocation into nucleus. We also confirmed that indirect CAR activator phenobarbital affects ERK signaling although we found only weak interaction with human EGFR in model non-hepatic A431 cells. These observations contradict the data presented by Mutoh et al. (2013) who showed a strong inhibition of EGF binding to GST-tagged mouse *Egfr* with a phenobarbital concentration of 100 μM. Further experiments should address whether other phosphorylation sites of EGFR (such as Tyr845, 992, 1148 and 1173, Ser1046 and Ser1047 phosphosites) that interact with other adaptor proteins are responsive to human EGFR signaling inhibition with phenobarbital.

## 5. Conclusions

Taken together, these facts suggest that these flavonoids do not directly activate the CAR nuclear receptor and that they interact with the EGFR signaling. This study also demonstrates new approach how to test the direct CAR interaction with its ligands using TR-FRET CAR coactivator assay and CAR assembly assay. CAR activation is associated with many beneficial effects on the metabolism of glucose and lipids. Baicalin, baicalein, galangin and chrysin have been reported to ameliorate high glucose and lipids levels in various experimental models, with various molecular mechanisms having been proposed for the activities (El-Bassossy et al., 2013; Guo et al., 2009; Kumar and Alagawadi, 2013; Sivakumar et al., 2010; Waisundara et al., 2009). A more detailed characterization of the molecular effects of the studied flavonoids would help us consider their clinical applications in the treatment of highly prevalent illnesses such as obesity, hypercholesterolemia and type 2 diabetes. In addition, knowledge of CAR activation may avoid drug–drug interactions due to xenobiotic-metabolizing enzymes induction.

## Conflict of interest

The authors declare that there are no conflicts of interest.

## Transparency document

The Transparency document associated with this article can be found in the online version.

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## A.2. The use of the LanthaScreen TR-FRET CAR coactivator assay in the characterization of constitutive androstane receptor (CAR) inverse agonists

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The purpose of this work was to validate the LanthaScreen™ TR-FRET CAR coactivator assay and to optimize its use for the testing of direct activation of CAR receptor by selected compounds in a highly sensitive cell-free based mode. The advantage of this assay lies in that it is a quick and efficient method to measure the direct interaction between CAR-LBD and a given ligand. Therefore, agonists, antagonists and inverse agonists are able to bind to the CAR LBD providing valuable information about affinity without regards to potential interfering effects as it may happen in cell-based experiments.

TR-FRET is a cell-free based method that uses a fluorophore bind to human CAR-LBD. Upon the presence of a ligand, the CAR-LBD changes its conformation and binds the fluorescent labeled peptide derived from PGC-1α coactivator. After the addition of an appropriate buffer and ligands, compounds interact and generate the signal. The quantification of the results is calculated after the incubation time, measuring the plate with two different emission wavelengths (490 nm and 520 nm), after being excited with the same wavelength (340 nm).

First, I optimized the protocol of the assay. For this purpose, we performed several experiments with reference ligands to determine the optimal conditions for the assay. Based on the available protocol, we performed several modifications and optimization. I found that incubation time could be shorten from initial 4 hours to only 1 hour reducing the time requirement of the assay. Next, we studied the affinity for well stablished CAR ligands (agonists, inverse agonists and antagonists). In all experiments, remarkable sensibility was achieved and our modifications from the original protocol proved valid.

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*Article*

# The Use of the LanthaScreen TR-FRET CAR Coactivator Assay in the Characterization of Constitutive Androstane Receptor (CAR) Inverse Agonists

**Alejandro Carazo and Petr Pávek ***

Department of Pharmacology and Toxicology, Faculty of Pharmacy, Charles University in Prague, Heyrovského 1203, Hradec Kralove CZ500 05, Czech Republic; E-Mail: carazo@faf.cuni.cz

* Author to whom correspondence should be addressed; E-Mail: petr.pavek@faf.cuni.cz; Tel.: +420-495-067-334.

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**Abstract:** The constitutive androstane receptor (CAR) is a critical nuclear receptor in the gene regulation of xenobiotic and endobiotic metabolism. The LanthaScreen™ TR-FRET CAR coactivator assay provides a simple and reliable method to analyze the affinity of a ligand to the human CAR ligand-binding domain (LBD) with no need to use cellular models. This *in silico* assay thus enables the study of direct CAR ligands and the ability to distinguish them from the indirect CAR activators that affect the receptor via the cell signaling-dependent phosphorylation of CAR in cells. For the current paper we characterized the pharmacodynamic interactions of three known CAR inverse agonists/antagonists—PK11195, clotrimazole and androstenol—with the prototype agonist CITCO (6-(4-chlorophenyl)imidazo[2,1-*b*][1,3] thiazole-5-carbaldehyde-*O*-(3,4-dichlorobenzyl)oxime) using the TR-FRET LanthaScreen™ assay. We have confirmed that all three compounds are inverse agonists of human CAR, with $IC_{50}$ 0.51, 0.005, and 0.35 μM, respectively. All the compounds also antagonize the CITCO-mediated activation of CAR, but only clotrimazole was capable to completely reverse the effect of CITCO in the tested concentrations. Thus this method allows identifying not only agonists, but also antagonists and inverse agonists for human CAR as well as to investigate the nature of the pharmacodynamic interactions of CAR ligands.

**Keywords:** TR-FRET; terbium; constitutive androstane receptor; pharmacodynamics interactions

## 1. Introduction

The LanthaScreen™ Nuclear Receptor Assay is commercially provided by Invitrogen (now a division of Thermo) for several nuclear receptors, including FXR, LXRα, PPARα and PXR. The LanthaScreen™ Constitutive Androstane Receptor Coactivator Assay is a TR-FRET based assay with terbium and fluorescein fluorophores to detect ligands of the Constitutive Androstane Receptor (CAR) nuclear receptor. In contrast to other nuclear receptor LanthaScreen™ assays, which are frequently reported in literature, to our knowledge no paper has been published on the characterization, validation or use of the TR-FRET CAR Coactivator Assay until quite recently, when we published the first article demonstrating the value of the assay in the characterization of indirect CAR ligands [1].

The constitutive androstane receptor (CAR, NR1I3) is a ligand-activated transcription factor with high constitutive activity belonging to the nuclear receptor group NR1I. CAR has been demonstrated to regulate the gene expression of major phase I and phase II xenobiotic-metabolizing enzymes (DMEs) and drug transporters such as cytochrome P450 CYP3A4 and CYP2B6 enzymes, the ABC efflux transporter MRP2 and the conjugation enzyme UGT1A1 [2].

CAR has also recently been shown to play important roles in the metabolism of glucose, lipids, and fatty acids as well as in the endobiotic metabolism of bile acids, bilirubin and thyroid hormones. In addition, CAR has been studied as an important factor controlling cell-cycle regulation, apoptosis and cell-cell interaction [3,4]. Human CAR displays unique properties in comparison with other nuclear receptors including high constitutive activity, both direct (ligand-binding domain (LBD)-dependent) and LBD-independent activation, and spontaneous nuclear localization in tumor cell lines. For these reasons, there are currently only few specific and nontoxic ligands of human CAR that can be considered for clinical investigation or for further characterization of human CAR [1,4].

CAR is formed from three domains: a highly conserved DNA-binding domain, a hinge region, and a divergent ligand binding/dimerization/transcriptional activation domain [5]. Both the ligand-binding domain (LBD)-dependent and the independent activation of human CAR have been shown to release CAR from its cytoplasmic tethering complex into the nucleus. Dephosphorylation-induced translocation of CAR to the nucleus has been recently found as the key step toward indirect activation via EGFR-dependent signaling inhibition with phenobarbital [6–8].

As indicated earlier, we have recently shown the LanthaScreen™ TR-FRET CAR Coactivator Assay as a suitable method to distinguish indirect CAR activators [1]. The procedure is based on the detection of the energy transfer between terbium bound to the recombinant GSH-tagged human CAR ligand binding domain (LBD) and fluorescein-labeled PGC1α coactivator interacting with CAR LBD. The interaction between CAR LBD and its coactivator PGC1α is triggered by an agonist or released by an inverse agonist or antagonist [1]. The assay provides a simple and reliable method to analyze the affinity to the CAR-LBD with no need to use cell lines, thus eliminating cell signaling [8], cellular transport and metabolism as confounding factors.

For the current paper we analyzed the pharmacodynamic interactions of the known CAR agonist CITCO (6-(4-chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde-*O*-(3,4-dichlorobenzyl)oxime) [9] with three antagonists/inverse agonists—clotrimazole [10], PK11195 [11] and androstenol [12]—using the LanthaScreen™ CAR Coactivator TR-FRET Assay. Our main goals were to (i) further optimize the protocol of the assay; (ii) apply the method in the study of agonist–antagonist/inverse agonist

pharmacodynamic interactions; and (iii) characterize and compare three known inverse agonists/antagonists in the cell-free assay.

## 2. Experimental Section

### *2.1. Chemicals*

CITCO (6-(4-Chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime) was purchased from BIOMOL Research, Germany. Clotrimazole (1-(o-Chlorotrityl)imidazole, 1-(o-Chloro-α,α-diphenylbenzyl)imidazole, 1-[(2-Chlorophenyl)diphenylmethyl]-1H-imidazole), androstenol (5α-androst-16-en-3α-ol) and PK11195 (1-(2-Chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinolinecarboxamide) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

### *2.2. LanthaScreen TR-FRET Coactivator Assay*

The LanthaScreen™TR-FRET CAR Coactivator Binding Assay (Cat. No. PV4836, now produced by Thermo) was used with slight modifications of the manufacturer's protocol. The assay uses two fluorophores: a terbium-labeled anti-GST antibody interacting with glutathione-S-transferase (GST) tagged human CAR ligand-binding domain (LBD) and a fluorescein-labeled PGC1α coactivator peptide (Figure 1). The interaction of an agonist ligand stimulates the interactions of the components, resulting in energy transfer to the acceptor fluorophore and a FRET (Fluorescence or Förster Resonance Energy Transfer) emission shift from 495 nm to 520 nm when fluorophores are within close proximity to one another. Thus, this energy transfer is detected by an increase in the fluorescence emission of the acceptor (fluorescein) and a decrease in the fluorescence emission of the donor (terbium). To quantify the process, TR-FRET is expressed as a ratio of the intensities of the acceptor and donor fluorophores.

Since the CAR nuclear receptor has high constitutive activity independent of a ligand, CAR LBD partly interact with the fluorescein-labeled PGC1α coactivator peptide in the absence of ligands. When an inverse agonist or antagonist is bound to CAR LBD, helix 12 of CAR LBD adopts a conformation that precludes fluorescein-labeled PGC1α coactivator peptide binding, and a decrease in TR-FRET is observed.

The assay was performed in a 384-well plate (black round-bottom plates purchased from Corning™) format in 20 μL of reaction mixture. Incubation time was optimized from 1 to 4 h at room temperature and protected from light. The reaction mixture was composed according to the manufacturer's recommended final concentrations (fluorescein-labeled PGC1 α coactivator 125 nM, Tb-labeled GST antibody 5 nM, CAR LBD GST-tagged protein 5 nM) [13].

According to the recommended protocol of the manufacturer, CAR-LBD is added to the solution of test compounds, followed by the addition of a mixture of the fluorescein-coactivator peptide and terbium-labeled anti-GST antibody. In the study we also tested to determine if the order of adding the constituents in the mixture may have an influence on the sensitivity and reproducibility of the assay.

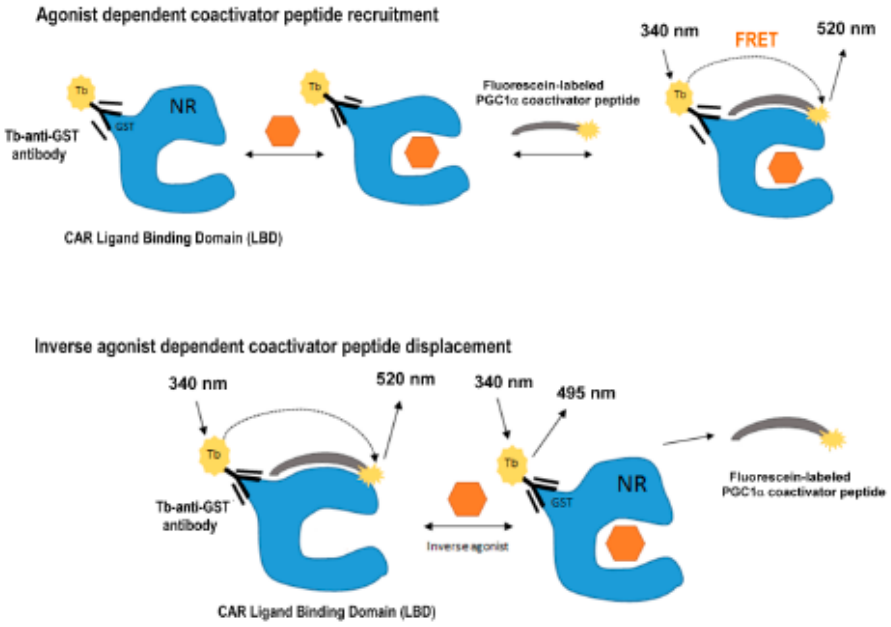


**Figure 1.** Principle of the TR-FRET (Time-Resolved Fluorescence Resonance Energy Transfer) LanthaSceen™ CAR Coactivator Assay.

After an incubation period of from 1 to 4 h, TR-FRET fluorescence was measured with a Synergy 2 Biotek plate reader (Bio-tek, Winooski, VT) using an excitation wavelength of 340 nm with filters detecting the fluorescent emission signals of terbium at 495 nm (10 nm bandwidth) and fluorescein at 520 nm (20 nm bandwidth). The terbium was excited using a 340-nm excitation filter with a 30-nm bandwidth and fluorescein was excited with the first emission peak of terbium centered at 495 nm. Energy transfer to fluorescein emission (520 nm) is then measured in the silent region between the first two terbium emission peaks centered at 490 and 545 nm (see Graphical Abstract). In this way, less than 1% of the total $Tb^{3+}$ emission is detected as bleed-through [14]. Delay time was set at 100 µs and integration time 200 µs. Gains set to 100 were the use constant in all the experiments. The 520 nm/495 nm TR-FRET ratio was then calculated. TR-FRET data are presented as 520 nm/495 nm TR-FRET ratio (Figure 2) or as relative activation or inhibition of CAR to control (DMSO vehicle-containing sample) without CAR LBD (set to zero, background) and to CITCO (10 µM)-treated samples (set at 100% activation) (Figures 3–5). Values below the baseline value of the sample with CAR LBD and treated with vehicle (0.1% DMSO) represent the inhibition of CAR-PGC1α interaction and suppression of CAR constitutive activity with CAR antagonists/inverse agonists. The values above the baseline represent activation of CAR LBD-PGC1α interaction and agonistic activity of the tested ligand. The experiments were performed at least three times in quadruplicates. Data are presented as the means and S.D. from three independent experiments ($n = 3$). The Z-factor was always higher than 0.5 in our experiments.

In the agonist assay (upper panel), a ligand binds the Constitutive Androstane Receptor (CAR) ligand binding domain (LBD) labeled with the terbium bound anti-GST antibody. Binding of the agonist causes conformational changes of CAR LBD around helix 12 resulting in an increased affinity of the fluorescein-labeled PGC1α coactivator peptide. The close proximity of terbium (donor) and fluorescein

(acceptor) causes energy transfer to the fluorescein and TR-FRET in emission at 520 nm after excitation at 340 nm.

In the case of the inverse agonist mode (lower panel), CAR LBD labeled with terbium through the anti-GST antibody partly interacts with the fluorescein-labeled PGC1α coactivator peptide causing constitutive ligand-independent activity of CAR. Binding of an inverse agonist to the CAR LBD produces conformational changes decreasing the affinity of the PGC1α coactivator. The close proximity of the terbium (donor) and fluorescein (acceptor) and the resultant energy transfer TR-FRET is thus disrupted; emission decreases at 520 nm.

### *2.3. CAR LBD Assembly Assay*

The CAR LBD assembly assay was performed according to the protocol we described in our latest report [1]. The CAR LBD assembly assay is based on two hybrid expression constructs encoding C (151–349 aa, helices 3–12, pCAR-C/VP16) and N (103–150 aa, helix 1, pCAR-N/GAL4) terminal parts of human CAR LBD that are co-transfected together with the pGL5-luc luciferase gene reporter plasmid (Promega) containing GAL4 binding sites. When the CAR LBD interacts with a ligand (both agonist and antagonist), connection of the helix 1 to CAR LBD helices 3–12 promotes firefly luciferase activation. Thus, the assay monitor interaction of CAR LBD with ligands rather than its activation or deactivation. Experiments have been done in HepG2 cells with CITCO (1 μM) as an agonist and with serial dilutions (range 0.1–30 μM) of CAR antagonists clotrimazole, PK11195 and androstenol, respectively. $IC_{50}$ has been calculated for each compound from at least five data points.

### *2.4. Data Analysis*

Dose-response curves were generated by plotting the emission TR-FRET ratio *vs*. the log of a ligand (in μM). To determine the half maximal inhibitory concentration ($IC_{50}$) value, the data were fitted using an equation for a sigmoidal dose response inhibition (with a varying slope) using the software GraphPad™ PRISM version 6.05. Z-factors were calculated using the method of Zhang *et al*. [15].

## 3. Results and Discussion

### *3.1. Results*

#### 3.1.1. Optimization of TR-FRET Mixture Composition and Incubation Times

In the first experiments, we determined whether modifications in the procedure provided by the manufacturer would have an influence on the sensitivity of the assay for an agonist as well as on the reduction of non-specific background. We altered the order in which the reagents were added to the reaction mixture and compared the results with the signals obtained with the standard procedure. One of the modifications was to add the ligand (CITCO), then a fluorescein-labeled PGC1α coactivator and terbium-labeled anti GSH antibody and finally the CAR-LBD solution. Another modification consisted of adding CAR-LBD and fluorescein-/terbium-labeled reagents solutions before the agonist. As shown in Figure 2, both modifications proved to be effective (high fold-activation by the ligand) but the results showed less sensitivity than the signals obtained by the standard procedure (the fold activation by CITCO

10 μM was 2.26; 1.94; and 2.56, respectively, compared to vehicle control). We also found that the standard protocol recommended by manufacturer results in a relatively lower background (the sample without CAR LBD, 2.5-fold lower) in comparison with the control sample with vehicle (DMSO 0.1%), CAR LBD, Tb-labeled anti-GSH antibody and fluorescein-labeled PGC1α coactivator (Figure 2C).

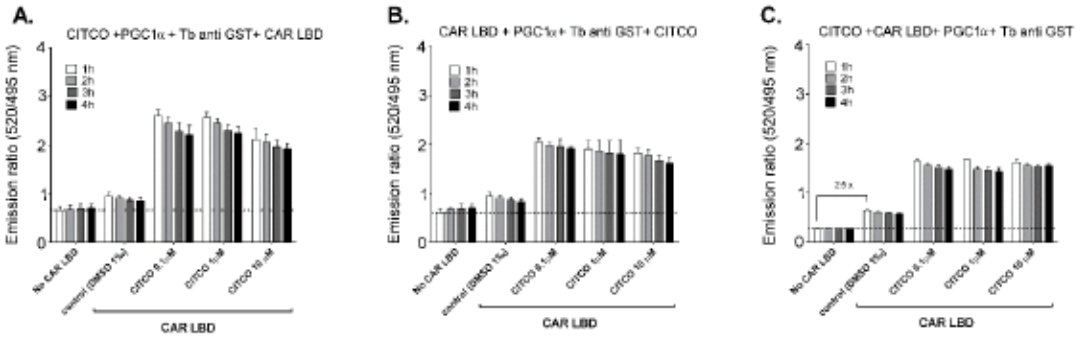


**Figure 2.** *Optimization of the TR-FRET* (Time-Resolved Fluorescence Resonance Energy Transfer) *LanthaScreen™ Assay*. Our TR-FRET (Time-Resolved Fluorescence Resonance Energy Transfer) experiments were slightly modified to study whether a protocol alteration influenced the response to the CAR agonist. In (**A**), the reaction mixture was composed in the following order: CITCO, the fluorescein-labeled anti-PGC1α coactivator, Tb-labeled GST antibody and CAR LBD; in (**B**), the reaction mixture was composed in the following order: CAR LBD, the fluorescein-labeled anti-PGC1α coactivator, Tb-labeled GST antibody and CITCO; and in (**C**), the standard protocol was followed (the order of CITCO, CAR LBD, the fluorescein-labeled anti-PGC1α coactivator and Tb-labeled GST antibody). The dotted line represents background nonspecific fluorescence in the absence of CAR LBD.

Secondly, we tested to see if incubation time had an effect on the outcomes of the experiments. We observed no significant effect of incubation time in all three experimental setups (Figure 2), although a slightly higher FRET ratio was observed for the incubation time of 1-h. These data confirm published results [13].

Thus these results confirm that the recommended experimental protocol and 1-h incubation time are optimal for further experiments.

### 3.1.2. PK11195 Shows Competitive Inhibition for CAR

PK1195 (1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinolinecarboxamide) is a known antagonist for human CAR, acting as a direct ligand for the receptor [11]. PK11195 has been shown to act as an antagonist of CITCO as well as an inverse agonist of constitutive activity in vehicle-treated samples in gene reporter assays and in RT-PCR experiments [11,16]. In our experiments, we confirmed that PK11195 is an inverse agonist with $IC_{50}$ 0.51 μM (Table 1) to reduce CAR constitutive activity (Figure 3). We also observed concentration-dependent antagonistic competition of PK11195 with CITCO for the CAR LBD, as shown in Figure 3. Significantly, PK11195 at the concentration of 20 μM

almost completely abolished the constitutive (CITCO-independent) interaction of CAR LBD with PGC1α (Figure 3).

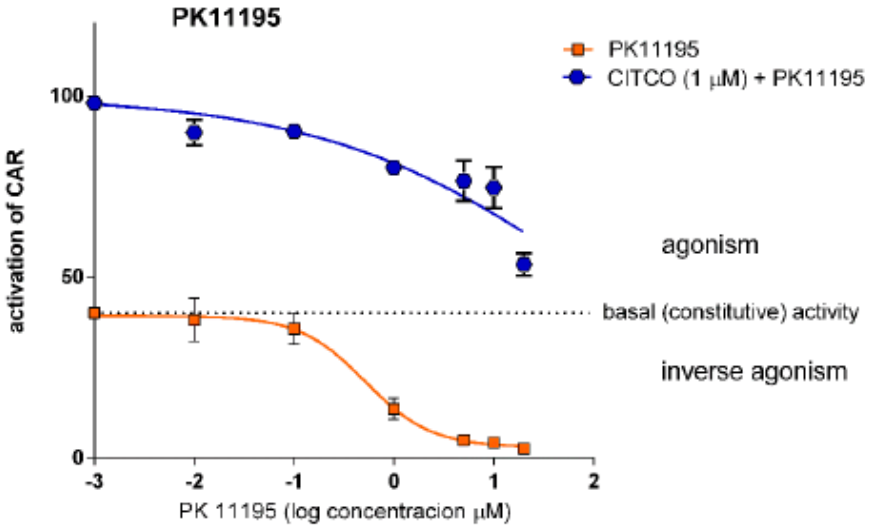


**Figure 3.** *Effect of PK11195 on CAR LBD activity in the TR-FRET LanthaScreen™ CAR Coactivator Assay*. PK11195 in a serial dilution was tested in inverse agonistic or antagonistic modes together with the prototype CAR agonist CITCO (1 μM concentration) using the TR-FRET assay. Data are presented as the relative activation to background activity (no CAR LBD in the reaction mixture, set to 0%) and to the effect of CITCO (1 μM) set as 100% activation. The dotted line represents the constitutive activity of CAR LBD (vehicle-treated samples). Data are presented as the means and S.D. from three independent experiments ($n$ = 3). Dose response curves were fitted using a sigmoidal dose response equation with a variable slope employing the software GraphPad PRISM ver. 6.06.

In the experiments agonist CITCO was always added into TR-FRET reaction mixture before PK11195. But no significant difference was observed in the TR-FRET fluorescence signals when PK11195 was added before CITCO (*unpublished data*).

### 3.1.3. Androstenol does not Show Significant Competitive Inhibition for CAR LBD

Unlike PK11195, androstane metabolites are weak inverse agonists of human CAR [12]. Androstenol (5α-androst-16-en-3α-ol) did not show any significant inhibition for CITCO-mediated CAR LBD interaction with PGC1α in our TR-FRET assays (Figure 4). The consistent antagonistic effect of androstenol on CITCO was only observed at the 30-μM concentration (Figure 4). In agreement with previous reports, androstenol decreased constitutive activity of CAR with $IC_{50}$ 0.345 μM (Table 1) in our TR-FRET experiments, a result which is in accordance with published data [13]. Again, as in the case of PK11195, no statistical difference was observed between the protocols when CITCO was added before androstenol or the other way round.

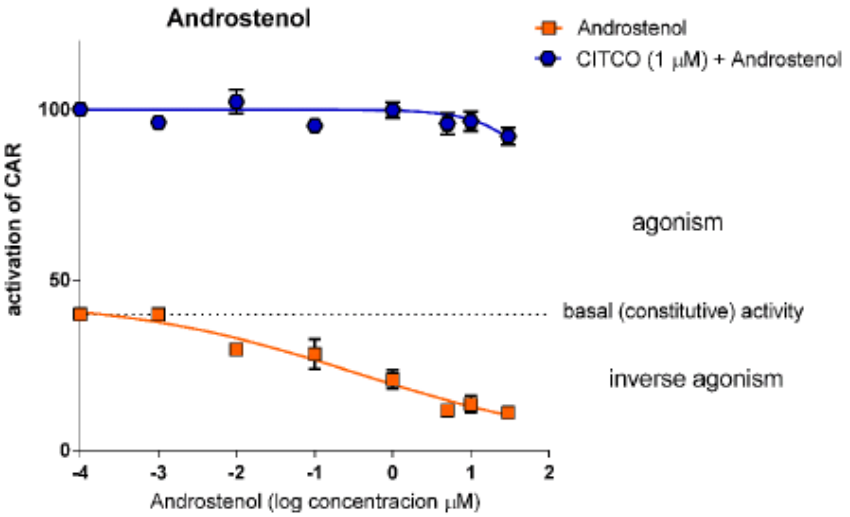


**Figure 4.** *Effect of androstenol on CAR LBD activity in the TR-FRET LanthaScreen™ CAR Coactivator Assay.* Androstenol in a serial dilution was tested in inverse agonistic or antagonistic modes together with the prototype CAR agonist CITCO (1 μM concentration) using the TR-FRET assay. Data are presented as the relative activation to background activity (set to 0%) and to the effect of CITCO (1 μM) set as 100% activation. The dotted line represents constitutive activity of CAR LBD (vehicle-treated samples). Data are presented as the means and S.D. from three independent experiments ($n = 3$).

3.1.4. Clotrimazole is a Potent Antagonist of Human CAR

Clotrimazole, an antifungal azole drug, has been reported as a human CAR antagonist [10]. In our work a significant inhibition of the CAR LBD constitutive activity was demonstrated already in concentrations of 0.01 μM and 10 μM clotrimazole almost completely inhibited the interaction of CAR LBD with the PGC1α coactivator in the TR-FRET assay (Figure 5). The $IC_{50}$ for clotrimazole was 0.005 μM (Table 1), which indicates high affinity to CAR LBD at low concentrations of clotrimazole. When combined with CITCO (either 1 or 10 μM), both concentration-dependent curves show the same profile (Figure 5) (*unpublished data for 10 μM CITCO*). Notably, at the 30-μM concentration, we observed that clotrimazole completely abrogated the effect of CITCO on CAR LBD interaction with PGC1α as well as further decreased the interaction below the baseline of CAR constitutive activity (Figure 5). These data demonstrate that clotrimazole is a highly potent antagonist and inverse agonist of human CAR that is able to completely inhibit CAR coactivation with PGC1α in low micromolar concentrations.

These data indicate that the tested inverse agonists/antagonists inhibit CAR coactivation with PGC1α in the TR-FRET assay in the following order: clotrimazole > PK11195 > androstenol. Clotrimazole was the only compound to reverse the effect of CITCO in the tested concentrations. Clotrimazole and PK111195 at the concentrations of 20 and 30 μM abolished constitutive activity of CAR and completely disrupted the interaction of CAR LBD with PGC1α (Figures 3 and 5), yielding background FRET ratio activities.

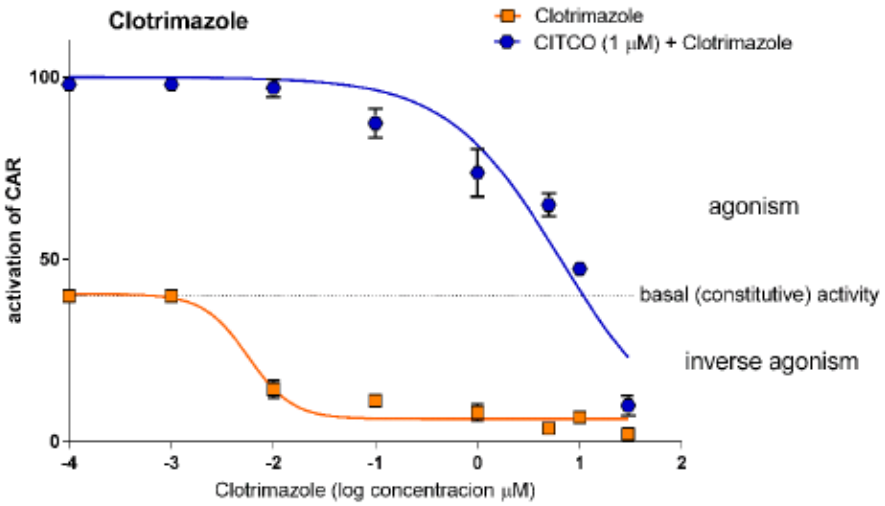


**Figure 5.** *Effect of clotrimazole on CAR LBD activity in the TR-FRET LanthaScreen™ CAR Coactivator Assay*. Clotrimazole in a serial dilution was tested in inverse agonistic or antagonistic modes together with the prototype CAR agonist CITCO (1 μM concentration) using the TR-FRET assay. Data are presented as the relative activation to background activity (set to 0%) and to the effect of CITCO (1 μM) set as 100% activation. The dotted line represents constitutive activity of CAR LBD (vehicle-treated samples). Data are presented as the means and S.D. from three independent experiments ($n = 3$).

3.1.5. Clotrimazole is a Potent Antagonist of Human CAR

To confirm our TR-FRET CAR Coactivator assay data, CAR LBD assembly assay has been employed/performed. The assay directly monitors interaction of a ligand with the CAR LBD producing a firefly luciferase gene reporter vector activation. We observed similar IC50s for clotrimazole and PK11195 antagonist in the assay in comparison with TR-FRET CAR coactivation assay (Table 1). On the other hand, androstenol exerted a weaker inhibition of the activity of CITCO and the data we obtained were not reliable to obtain kinetic data of the curve.

**Table 1.** Parameters of tested compounds to interact with human CAR LBD.

| | **$IC_{50}$ (μM)(95% Conf. Interv.)/R Square** | | |
|---|---|---|---|
| | **TR- FRET CAR Assay** | | **CAR LBD Assembly Assay** |
| Compound | No CITCO | CITCO 1 μM | CITCO 1 μM |
| PK11195 | 0.51 (0.36–0.72)<br>0.998 | 93.63 (9.24–948.3)<br>0.858 | 56.61 (36.68–87.37)<br>0.905 |
| Androstenol | 0.345 (0.01–670.8)<br>0.968 | 312.6 (152.8–676.8)<br>0.318 | N.A. |
| Clotrimazole | 0.005 (0.0008–0.039)<br>0.968 | 6.15 (3.42–12.41)<br>0.932 | 11.37 (2.59–49.71)<br>0.865 |

N.A.—not available, not reliable fitting in the concentration range.

### *3.2. Discussion*

The use of the TR-FRET assay to analyze the ligands of a nuclear receptor was first reported by Parks *et al*. [17], who developed an europium-based assay with biotinylated Farnesoid X receptor (FXR) LBD labeled with streptavidin-conjugated allophycocyanin (APC) dye and the 5′-biotinyled SRC1 coactivator peptide fragment (AA 676 to 700) labeled with streptavidin-conjugated europium chelate. The first TR-FRET europium-based assay to detect mice and human CAR nuclear receptor ligands was described by Moore *et al*. In this assay, biotinylated mouse and human CAR LBDs labeled with the steptavidin-conjugated fluorophore APC dye interacted with the peptide LXXLL motif of SRC-1 coactivator (AA 676–700) labeled with europium chelate [9,10]; this process was patented in 2011 [18].

In contrast to standard FRET assays, TR-FRET assays use a long-lifetime lanthanide chelate as the donor fluorophores [14]. Lanthanide chelates have extremely-long excited-state lifetime, on the order of a millisecond when the molecule spends in the excited state after accepting a photon [14]. The delay time of TR-FRET measurement is usually 50 to 100 microseconds after a flashlamp excitation. Therefore, TR-FRET eliminates interference from autofluorescent compounds as well as from scattered light, since the interference is in the nanosecond timescale. The most common lanthanides used in TR-FRET assays are terbium ($Tb^{3+}$) and europium ($Eu^{3+}$). Terbium-based TR-FRET assays can be conducted with common, cheap and easy-to-work fluorophores, such as fluorescein, as the acceptor in fluorescein-labeled molecules; this is in stark contrast to europium-based systems, which employ APC as the acceptor in biotinylated molecules. Thus the main advantage of the TR-FRET assay is overcoming interference from compound autofluorescence and light scatter from precipitated compounds. In addition, considering FRET as a ratio of the intensities of the acceptor and donor fluorophores, differences in assay volumes between wells can be eliminated and quenching effects due to colored compounds can be corrected using this approach.

The LanthaScreen™ time-resolved Förster resonance energy transfer (TR-FRET) technology was introduced by Invitrogen (now a division of Thermo) in 2007. The assay facilitates the discovery and evaluation of compounds that bind to human CAR LBD based on the recruitment or displacement of the coactivator-based fluorescein-labeled peptide PGC1α. The interaction of CAR LBD with PGC1α is controlled by a conformational change in the CAR receptor LBD around helix 12 that takes place upon ligand binding. Either the constitutive association of CAR LBD with a fluorescein-labeled coactivator peptide may be disrupted by inverse agonists, or the interaction between the receptor and fluorescein-labeled PGC1α coactivator peptide is augmented by agonists. This interaction is detected by monitoring the FRET signal between a terbium-labeled anti-GST antibody bound to CAR LBD and the fluorescein-labeled peptide PGC1α.

As far as we know, ours is the only research group reporting on the LanthaSceen™ CAR Coactivator Assay at the present time. In another recent publication, we demonstrated the applicability of the assay for distinguishing indirect CAR activators among the natural flavonoids, which activate CAR via posttranslational modification [1]. For the current work, using the TR-FRET LanthaSceen™ assay we studied three well-known inverse agonists/antagonists of human CAR. We found that clotrimazole is the most potent antagonist and inverse agonist of human CAR, followed by PK11195 and androstenol (Table 1). Clotrimazole was the only compound to reverse the effect of CITCO in the tested concentrations. Another interesting finding of the study is that at the concentration of 30 μM clotrimazole

and PK111195 abolish the constitutive activity of CAR and completely disrupt the interaction of CAR LBD with PGC1α (Figures 3 and 5).

The assembly assay method provides reliable information about the affinity of a studied substance to bind to CAR-LBD. In this regard, clotrimazole showed the most significant activity to revert the CAR activation by CITCO 1μM in a dose-dependent manner ($IC_{50}$ = 11.4 ± 1.7 μM), while PK11195 showed less potent inhibitory effect ($IC_{50}$ = 56.6 ± 1.2 μM). Androstenol displayed the weakest inhibition even in the highest studied concentration (30 μM) against CITCO 1 μM. These data are in accordance to the data obtained by the TR-FRET technology. Thus, both assays prove to be sensible, reliable and interchangeable methods to study ligand interaction and competition for CAR-LBD.

## 4. Conclusions

In this article, we communicate our results demonstrating that the TF-FRET CAR coactivator assay is a suitable, rapid (2 h), and convenient (mix-and-measure mode) tool for the interaction testing of human CAR and its ligands without the confounding effect of cellular signaling and cell-dependent posttranslational modification of CAR. Further, the assay can be adapted for a high-throughput screening format (384-well plate). Our current studies also show for the first time the pharmacodynamic interactions of a CAR agonist and an inverse agonist/antagonist *in silico*.

## Acknowledgments

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## Author Contributions

P.P. and A.C. conceived and designed the experiments; A.C. performed the experiments.

## Conflicts of Interest

The authors declare no conflict of interest.

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## A.3. Acetylated deoxycholic (DCA) and cholic (CA) acids are potent ligands of pregnane X (PXR) receptor

Carazo A, Hyrsova L, Dusek J, Chodounska H, Horvatova A, Berka K, Bazgier V, Gan-Schreier H, Chamulitrat W, Kudova E and Pavek P (2016) Acetylated deoxycholic (DCA) and cholic (CA) acids are potent ligands of pregnane X (PXR) receptor. *Toxicol Lett* **265**:86-96. $IF_{2015}$:3,522

In this paper, we aimed to study the effect of a set of newly synthetized bile acids on several nuclear receptors. Based on parent bile acids cholic acid (CA), deoxycholic acid (DCA), chenodeoxycholic acid (CDCA) and lithocholic acid (LCA), and knowing that bile acids are metabolized in the organism through acetylation and oxidation processes by liver or gut microflora metabolism, we synthesized a set of bile acids derivates and tested them for activity in PXR, FXR and VDR assays. Previously, LCA was shown to be a strong PXR and VDR ligand, whereas CDCA specifically activates FXR (Fiorucci et al 2010).

In HepG2 cells, we performed luciferase gene reporter assays to study the activity of these derivates on the receptors. Our results showed that derivates 3,7,12-triacetate CA and 3,12-diacetate DCA are strong PXR activators. For 3,12-diacetate DCA, the activation was stronger than PXR ligand LCA and similar to reference activator rifampicin. This activation showed to be dose-dependent with a $EC_{50}$ of 32.1 μM for 3,12-diacetate DCA. No other derivate showed significant activity for any of the receptors studied including CAR.

In TR-FRET PXR assays, 3,12-diacetate DCA showed high affinity for the PXR-LBD. In HepaRG cells, the compound was able to enhance the expression of PXR target genes (MDR1, CYP3A4, CYP2B6). Next, in molecular docking, 3,12-diacetate DCA strongly interacted with PXR-LBD, showing a higher affinity than DCA itself.

Next, we wanted to study the eventual presence of these derivates in human and mice bile samples. HPLC/MS-MS method was used to study the presence of these compounds in the samples, but no evidence was obtained. This could be either due to

the low physiological concentration of the compounds in the samples below detection limit or due to the fact that these compounds are not present in liver or bile.

All of the bile acids (parent and derivates) were tested also in CAR assays, but no activation was reported (unpublished observations).

In conclusion, we establish that rationally developed bile acid derivates may activate several nuclear receptors, and that the presence of these derivates in the organism remains to be assessed. Whether intestinal microflora is able to produce these derivates is yet to be confirmed.

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Full Length Article

# Acetylated deoxycholic (DCA) and cholic (CA) acids are potent ligands of pregnane X (PXR) receptor

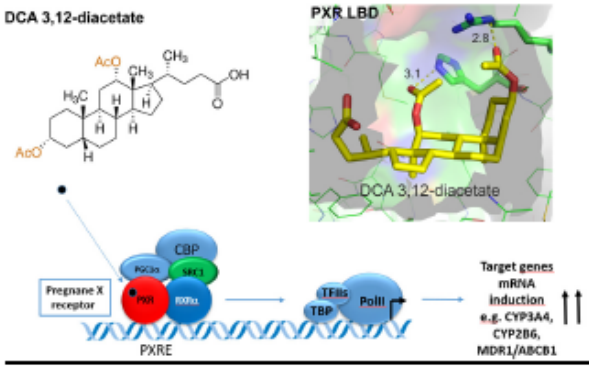
Alejandro Carazo[a], Lucie Hyrsova[a], Jan Dusek[a], Hana Chodounska[b], Alzbeta Horvatova[a], Karel Berka[c], Vaclav Bazgier[c], Hongying Gan-Schreier[d], Waleé Chamulitrat[d], Eva Kudova[b], Petr Pavek[a,*]

[a] Department of Pharmacology and Toxicology, Faculty of Pharmacy, Charles University in Prague, Heyrovského 1203, Hradec Kralove CZ500 05, Czechia
[b] Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Flemingovo náměstí 2, CZ160 00 Praha, Czechia
[c] Regional Centre of Advanced Technologies and Materials, Department of Physical Chemistry, Palacky University in Olomouc, 17. listopadu 1131, Olomouc CZ779 00, Czechia
[d] Department of Internal Medicine IV, Gastroenterology and Infectious Diseases, Im Neuenheimer Feld, Heidelberg, Germany

## HIGHLIGHTS

- Acetylated deoxycholic (DCA) and cholic (CA) acids are potent ligands of PXR.
- Acetylated DCA and CA enhance PXR target genes expression.
- Dehydrogenation or acetylation of DCA, CA, lithocholic (LCA) or chenodeoxycholic (CDCA) do not lead to increased affinity to FXR or VDR.
- Acetylated DCA and CA were not found in bile.

## GRAPHICAL ABSTRACT

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## ABSTRACT

The Pregnane X (PXR), Vitamin D (VDR) and Farnesoid X (FXR) nuclear receptors have been shown to be receptors of bile acids controlling their detoxification or synthesis. Chenodeoxycholic (CDCA) and lithocholic (LCA) acids are ligands of FXR and VDR, respectively, whereas 3-keto and acetylated derivates of LCA have been described as ligands for all three receptors.

In this study, we hypothesized that oxidation or acetylation at position 3, 7 and 12 of bile acids DCA (deoxycholic acid), LCA, CA (cholic acid), and CDCA by detoxification enzymes or microbiome may have an effect on the interactions with bile acid nuclear receptors. We employed reporter gene assays in HepG2 cells, the TR-FRET assay with recombinant PXR and RT-PCR to study the effects of acetylated and

*Abbreviations:* BA, bile acid; CA, cholic acid; CDCA, chenodeoxycholic acid; CYP, cytochrome P450; DCA, deoxycholic acid; 6-ECDCA, 6α-ethyl-chenodeoxycholic acid, obeticholic acid; FXR, farnesoid X receptor; LBD, ligand binding domain; LCA, lithocholic acid; PXR, pregnane X receptor; TR-FRET, time-resolved fluorescence energy transfer; VDR, vitamin D receptor.

* Corresponding author.
*E-mail address:* petr.pavek@faf.cuni.cz (P. Pavek).

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Nuclear receptors
FXR

keto bile acids on the nuclear receptors activation and their target gene expression in differentiated hepatic HepaRG cells.

We demonstrate that the DCA 3,12-diacetate and CA 3,7,12-triacetate derivatives are ligands of FXR and DCA 3,12-diacetate induces FXR target genes such as CYP3A4, CYP2B6 and ABCB1/MDR1.

In conclusion, we found that acetylated DCA and CA are potent ligands of FXR. Whether the acetylated bile acid derivatives are novel endogenous ligands of FXR with detoxification or physiological functions should be further studied in ongoing experiments.



## 1. Introduction

Three nuclear receptors of the nuclear receptor superfamily – the Pregnane X receptor (PXR, NR1I2), Farnesoid X (FXR, NR1H4) and Vitamin D receptor (VDR, NR1I1) – have been recently established as bile acid receptors in the liver and in the intestine and their role in bile acid (BA) synthesis regulation or detoxification has been clearly documented (Ishizawa et al., 2008; Makishima et al., 2002, 1999; Ridlon and Bajaj, 2015; Staudinger et al., 2001b; Wang et al., 1999; Xie et al., 2001). 3-Keto LCA has been found as a potent ligand for the VDR, FXR and PXR (Adachi et al., 2005; Makishima et al., 1999; Staudinger et al., 2001b), and the LCA acetate and LCA acetate methyl ester as highly potent VDR ligands (Adachi et al., 2005; Makishima et al., 1999; Staudinger et al., 2001b). It was also proposed that gut microbiome may produce endocrine molecules from steroids to activate nuclear receptors and several oxidized bile acid derivatives such as 7-oxo CA, 7-oxo DCA and 7-oxo CDCA have been identified as products of prokaryotic hydroxysteroid dehydrogenases (Ridlon and Bajaj, 2015).

We hypothesized that dehydrogenation (oxidation) or acetylation of bile acids may increase affinity to these nuclear receptors and that liver biotransformation enzymes or intestinal microflora may modify bile acids at position 3α, 7α and 12α of DCA, LCA, CA, and CDCA to derivatives more avidly interacting with the bile acid receptors (Ridlon and Bajaj, 2015). Therefore, to study structure-activity relationships (SAR) of dehydrogenated and acetylated bile acids with PXR, VDR and FXR receptors, we synthetized a series of dehydrogenated (keto) and acetylated derivatives of DCA, LCA, CA, and CDCA (Fig. 1). Some of these compounds are known products of gut microbiome bile salt 3α-, 7α, 12α-hydroxysteroid dehydrogenases or deconjugation enzymes as well as potential products of cytochrome P450-mediated biotransformation (compounds underlined in Fig. 1) (Deo and Bandiera, 2009; Ridlon and Bajaj, 2015; Ridlon et al., 2006).

The PXR has been identified as a "master" xenobiotic sensor regulating the expression of a wide variety of genes involved in the transport, metabolism and elimination of xenobiotics along with a number of endogenous substances. In addition, PXR has a function in regulating several cellular signaling pathways related to physiological processes (Banerjee et al., 2015). In the case of PXR, mainly lithocholic acid and its 3-keto derivative have been found to activate both human and mouse PXR (Krasowski et al., 2005; Staudinger et al., 2001b; Xie et al., 2001). 3-Keto LCA was found to be an even more potent ligand of PXR than LCA; whereas CDCA, DCA and CA only mildly activate PXR (Krasowski et al., 2005; Staudinger et al., 2001b). Therefore, PXR has been established as the receptor of LCA responsible for the detoxification of the highly hepatotoxic and a potentially enteric carcinogenic bile acid via induction of its metabolism (Staudinger et al., 2001b; Xie et al., 2001).

The FXR is localized mainly in the liver, intestine (ileum) and kidneys. FXR regulates the enterohepatic circulation and metabolism of bile acids, and it also modulates liver regeneration, inflammation and growth (Ali et al., 2015). Chenodeoxycholic acid (CDCA), and to a lesser extent lithocholic (LCA) and deoxycholic acid (DCA), are natural ligands of human FXR and able to transactivate the receptor, whereas cholic acid (CA) has weak effect on FXR activation (Makishima et al., 1999; Parks et al., 1999; Wang et al., 1999). Among keto-bile acids, 7-keto and 3,7-keto LCA are known to activate FXR, although with lower potency (Wang et al., 1999). Conjugates of CDCA, LCA, and DCA with taurine and glycine and to a lesser extent CA conjugates can also activate FXR when transported into cells by a conjugate transporter such as ABST (IBAT, SLC10A2) (Makishima et al., 1999; Parks et al., 1999). Ligands of FXR are considered therapeutically useful for the treatment of liver disorders including various forms of cholestasis and fatty liver (steatosis) disease (Ali et al., 2015).

The VDR mediates the effect of the vitamin D active form 1,25-dihydroxyvitamin $D_3$ (1,25-$(OH)_2$vit$D_3$). VDR is primarily associated with calcium and phosphate homeostasis, but it is also an important regulator of cell growth and differentiation, cell death and immunity (Dusso et al., 2005). It has been shown that VDR also functions as a receptor for the secondary bile acid lithocholic acid (LCA). VDR is the most sensitive receptor to activation by LCA and its metabolites in comparison with other nuclear receptors. Activation of VDR by LCA or by vitamin D induces expression of CYP3A4, a cytochrome P450 enzyme that detoxifies LCA in the liver and intestine (Makishima et al., 2002). Interestingly, derivatives of LCA such as 3-keto LCA (Makishima et al., 2002), LCA propionate and methylester LCA acetate display significantly higher affinity and potency to activate VDR (Adachi et al., 2005; Ishizawa et al., 2008).

In the current work, we attempted to determine if dehydrogenation or acetylation at position 3, 7 and 12 of unconjugated DCA, LCA, CA and CDCA has effects on interactions with the bile acid receptors PXR, FXR and VDR. We investigated interactions of synthetized acetylated, diacetylated, triacetylated and dehydrogenated bile acids CDCA, LCA, DCA and CA at positions 3 (α, β), 7α and 12α (Fig. 1) with PXR, FXR, and VDR nuclear receptors in a cellular assay as well as with recombinant nuclear receptor proteins in coactivator TR-FRET assays. Employing RT-PCR and *in silico* docking, we confirmed interactions of acetylated DCA and CA bile acids with PXR ligand binding domain (LBD) and PXR target genes regulation in HepaRG cells by DCA 3,12-diacetate. We further analyzed lipid extracts of mouse liver and human bile samples using HPLC/MS-MS.

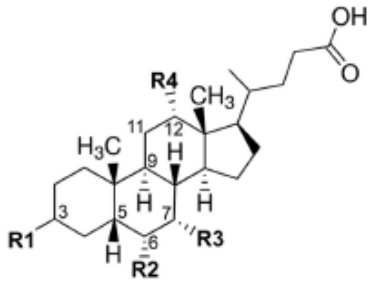
## 2. Material and methods

### 2.1. Chemicals

The bile acid derivatives (Fig. 1) were synthesized at the Institute of Organic Chemistry and Biochemistry, Czech Academy of Sciences. Synthesis procedures and NMR spectra are available upon request and will be published elsewhere.

### 2.2. Plasmids

The FXR response elements (FXRE)-driven luciferase reporter plasmid (pFXRE-luc2P) was constructed by inserting




| | Position | | | |
|---|---|---|---|---|
| | 3 (R1) | 6 (R2) | 7 (R3) | 12 (R4) |
| **LCA** | ⋯OH | | - | - |
| Iso LCA | —OH | | - | - |
| Iso LCA 5(6)-en 3-acetate | —OAc | | - | - |
| 6-hydroxy LCA | ⋯OH | ⋯OH | - | - |
| **CA** | ⋯OH | | ⋯OH | ⋯OH |
| 7-oxo CA | ⋯OH | | =O | ⋯OH |
| 3,7,12-trioxo CA | =O | | =O | =O |
| CA 3,7,12-triacetate | ⋯OAc | | ⋯OAc | ⋯OAc |
| CA 3,7-diacetate | ⋯OAc | | ⋯OAc | ⋯OH |
| **DCA** | ⋯OH | | - | ⋯OH |
| Iso DCA | —OH | | - | ⋯OH |
| DCA 3-acetate | ⋯OAc | | - | ⋯OH |
| DCA 3,12-diacetate | ⋯OAc | | - | ⋯OAc |
| 3,12-dioxo DCA | =O | | - | =O |
| 12-oxo DCA | ⋯OH | | - | =O |
| 12-oxo 9(11)-en- DCA | ⋯OH | | - | =O |
| 12-oxo DCA 3-acetate | ⋯OAc | | - | =O |
| **CDCA** | ⋯OH | | ⋯OH | - |
| CDCA 3,7-diacetate | ⋯OAc | | ⋯OAc | - |
| CDCA 3-acetate | ⋯OAc | | ⋯OH | - |
| 7-oxo CDCA | ⋯OH | | =O | - |

**Systematic Names (Lipid Maps™ database):**

LCA, 3α-Hydroxy-5β-cholan-24-oic Acid, Lithocholic acid; Iso LCA, 3β-Hydroxy-5β-cholan-24-oic Acid, Isolithocholic Acid; Iso LCA 5(6)en 3-acetate, 3β-Hydroxy-5β-cholan-5-en-24-oic Acid 3-acetate; 6-hydroxy LCA, 3α,6α-Dihydroxy-5β-cholan-24-oic Acid, Hyodeoxycholic Acid; CA, 3α,7α,12α-Trihydroxy-5β-cholan-24-oic acid, Cholic Acid; 7-oxo CA, 3α,12α-Dihydroxy-7-oxo-5β-cholan-24-oic Acid; 3,7,12-trioxo CA, 3,7,12-Trioxo-5β-cholan-24-oic Acid; CA 3,7,12-Triacetate, 3α,7α,12α-Trihydroxy-5β-cholan-24-oic Acid 3,7,12-triacetate; CA 3,7-diacetate, 3α,7α,12α-Trihydroxy-5β-cholan-24-oic Acid 3,7-diacetate; DCA, 3α,12α-Dihydroxy-5β-cholan-24-oic Acid, Deoxycholic Acid; Iso DCA, 3β,12α-Dihydroxy-5β-cholan-24-oic Acid, Isodeoxycholic Acid; DCA 3-acetate, 3α,12α-Dihydroxy-5β-cholan-24-oic Acid 3-acetate; DCA 3,12-diacetate, 3α,12α-Dihydroxy-5β-cholan-24-oic Acid 3,12-diacetate; 3,12-dioxo DCA, 3,12-Dioxo-5β-cholan-24-oic Acid; 12-oxo DCA, 3α-Hydroxy-12-oxo-5β-cholan-24-oic Acid; 12-oxo 9(11)en- DCA, 3α-Hydroxy-12-oxo-5β-chol-9(11)-en-24-oic Acid; 12-oxo DCA 3-acetate, 3α-Hydroxy-12-oxo-5β-cholan-24-oic Acid 3-acetate; CDCA, 3α,7α-Dihydroxy-5β-cholan-24-oic Acid, Chenodeoxycholic Acid; CDCA 3,7-diacetate, 3α,7α-Dihydroxy-5β-cholan-24-oic Acid 3,7-diacetate; CDCA 3-acetate, 3α,7α-Dihydroxy-5β-cholan-24-oic Acid 3-acetate; 7-oxo CDCA, 3α-Hydroxy-7-oxo-5β-cholan-24-oic Acid.

**Fig. 1.** Common names of tested bile acids.
Underlined bile acids are formed by gut microbiota (Ridlon and Bajaj, 2015; Ridlon et al., 2006, 2014).

complementary oligonucleotides containing two copies of FXRE, an inverted repeat in which consensus receptor-binding hexamers are separated by one nucleotide (IR-1) from the phospholipid transfer protein (PLTP) promoter (5′-aaactgaGGGTCAgTGACC-Caagtgaa-3′) and one FXRE (IR-1) of the SHP gene promoter (−291GAGTTAaTGACCT−279) into *KpnI-XhoI* cloning sites of pGL4.27 (Promega, Hecules, CA, USA) upstream of the minimal promoter. The experiments with pCMX-GAL-hFXR, PXR-responsive p3A4-luc and pM-GAL4-PXR LBDmut (S247W/C284W) constructs were performed as we have previously described (Hirsova et al., 2013; Krausova et al., 2011; Rulcova et al., 2010). $pDR_3$-luc plasmid containing four repeats of consensus VDR response element $(AGGTCANNNNN)_5$ was used (DR3 *cis*-Reporting System, Stratagene). The pGL5-luc, pGL4.23 and pRL-TK constructs were purchased from Promega (Madison, WI, USA). pSG5-hFXR and pSG-hPXR expression constructs were kindly provided by Dr. S.

Kliewer (University of Texas, Dallas, TX, USA). The expression plasmids pSG5-hRXRα and pSG5-hVDR were a generous gift from Dr. C. Carlberg (University of Kuopio, Kuopio, Finland).

### 2.3. Reporter gene assay and mammalian two-hybrid assays

All transient transfection reporter gene assays were performed with Lipofectamine® 2000 transfection reagent (Life Technologies, Carlsbad, CA, USA, now part of Thermo Fisher) in the HepG2 cells (between passages 15 to 25). Cells were seeded at the density of 40,000 cells/cm$^2$ onto 48-well Plates 24 h before transfection. HepG2 cells were transfected with luciferase reporter gene constructs (150 ng/well) together with NRs expression vectors (100 ng/well) and pRL-TK *Renilla* construct (30 ng/well) 24 h after seeding, according to the manufacturers protocol. For pilot screening of bile acids interactions with PXR, VDR and FXR receptors, 30 μM concentration has been used. In the concentration, tested bile acids have no effect on HepG2 viability (*data not shown*).

Mammalian two-hybrid assays were carried out with the pM-GAL4-PXRwt fusion expression plasmid for wild-type PXR and the double mutant pM-GAL4-PXRmut (S247W/C284W) expression plasmid. The double PXR mutant (S247W/C284W) has replaced the serine at position 247 with the larger tryptophan, which effectively fills the ligand binding pocket of PXR. This replacement blocks the ligand binding pocket-dependent activity of PXR, rendering the construct constitutively active independently of a ligand. HepG2 cells were transiently transfected with the pGL5-luc reporter plasmid (150 ng per well in 48-well plates) together with either the pM-GAL4-PXR LBDwt or pM-GAL4-PXR LBDmut (S247W, C248W) (100 ng per well) fusion expression plasmid, the VP16-SRC1 fusion expression plasmid or the VP16 empty vector (100 ng per well), as well as the pRL-TK control plasmid (30 ng per well) for transfection normalization. After 24 h of stabilization, the cells were treated with the DCA 3,12-diacetate and CA 3,7,12-triacetate (30 μM), rifampicin (RIF, 10 μM) or vehicle (DMSO; 0.1%, v/v) for an additional 24 h. The VP16-SRC1-RID fusion expression construct for SRC1 coactivator 1 has been described previously (Krausova et al., 2011). During the length of the experiments, cells were kept in an incubator at temperature 37 °C and 5% $CO_2$. After treatment, the cells were lysed and assayed for both firefly and *Renilla* luciferase activities with the use of the Dual-Luciferase Reporter Assay kit (Promega, Hercules, CA, USA). All test values were normalized to the mean value of the experimental control group (empty expression constructs with vehicle) and the data are presented as fold of the control group's mean value ($n = 3$ or more). More detailed protocols can be found in our previous papers (Krausova et al., 2011; Smutny et al., 2014).

### 2.4. PXR ligand binding assay

The LanthaScreen® TR-FRET PXR Competitive Binding Assay (Invitrogen/Life Technologies, Carlsbad, CA) was performed according to the protocol described in our previous paper (Smutny et al., 2014).

### 2.5. HepaRG cell cultivation

Cryopreserved HepaRG™ (GIBCO®) cells and media were obtained from Life Technologies (Carlsbad, CA, USA). The HepaRG™ cell line is an immortalized and terminally differentiated hepatic cell line that retains many liver-specific characteristics of primary human hepatocytes including high endogenous activities of nuclear receptors and biotransformation enzymes. The HepaRG cells were initially isolated from a liver tumor of a female patient suffering from hepatocarcinoma (Gripon et al., 2002). The HepaRG cell line was cultivated and differentiated for qRT-PCR expression experiment as previously described (Gripon et al., 2002; Hyrsova et al., 2016). On the contrary, HepG2 cells have been preferred to HepaRG cells in transient transfection experiments, since majority of transfection reagents display low transfection efficiency or toxicity in HepaRG cells.

### 2.6. qRT-PCR assay

Total RNA isolation, cDNAs synthesis and RT-PCR experiments were performed as described previously (Smutny et al., 2014).

### 2.7. CYP3A4 enzymatic activity assay

Human recombinant CYP3A4 (P450-Glo™ CYP3A4 Screening System with Luciferin-PPXE) was used to evaluate the interaction of the DCA 3,12-diacetate with human CYP3A4 (Smutny et al., 2014).

### 2.8. HPLC/MS-MS analyses of the DCA 3,12-acetate and CA 3,7, 12-triacetate

Three mouse liver samples and human bile samples (collected according to the Declaration of Helsinki of 1964 and approved by the local ethics committee) have been used for the analyzed. Patients with a diagnosis of biliary complications were admitted at the Endoscopy unit, Gastroenterology department of the University of Heidelberg Hospital. Samples have been prepared as described in *Supplementary data* protocol.

For HPLC–MS/MS analyses, the separation of bile acids was achieved using a Luna C18 column (Phenomenex, CA; 100 mm × 2.0 mm, 3 μm particle size) fitted on a separation module (Waters 2695, Milford, MA). Binary solvents used for the analysis were 80% $H_2O$/methanol with 8 mM ammonium acetate, pH = 8.0 (solvent A) and 95% methanol/$H_2O$ with 8 mM ammonium acetate, pH = 8.0 (solvent B). The flow rate was maintained at 0.2 mL/min, and the gradient started with 100% solvent A for 2.5 min, changed to 100% solvent B in 1 min, held for 16.5 min, and finally switched back to the initial condition within 3 min. The HPLC column was maintained at 40 °C and coupled with an electrospray ionization source of the tandem mass spectrometer (Quattro micro API, Micromass Waters, Manchester, UK). The mass spectrometer was operated with the source, with desolvation temperatures set at 130 °C and 300 °C, respectively. The bile acids in free acid form and their taurine- glycine −conjugates were detected in a negative mode. The capillary, cone, extractor, and RF voltages were used at 4100, 70, 10, and 0.6 V, respectively. The source and desolvation gases (nitrogen) were set at a flow rate of 800 and 90 L/h, respectively. Collision energies were 60, 48, and 30 eV for glyco-, tauro-, and unconjugated BAs, respectively. The DCA 3,7-diacetate and CA 3,7,12-triacetate were detected by the multiple reaction monitoring (MRM) transitions at 475.1 > 433.3 and 533.2 > 491.2, respectively. Peak assignment was achieved by direct comparison with the retention times at 9.19 min and 8.16 min of authentic standards of the DCA 3,7-diacetate and CA 3,7,12-triacetate, respectively. The concentrations of bile acids were determined by the peak area ratio between the bile acid and the internal standard.

### 2.9. Statistical and bioinformatic analyses

All data are presented as the mean ± standard deviations (SDs). A one-way analysis of variance (ANOVA) with a Dunnett's *post hoc* test was applied. $EC_{50}$ (BAs concentration required to achieve half-maximum promoter activation), $IC_{50}$ (BAs concentration required to achieve 50% inhibition in TR-FRET assay or CYP3A4 enzymatic

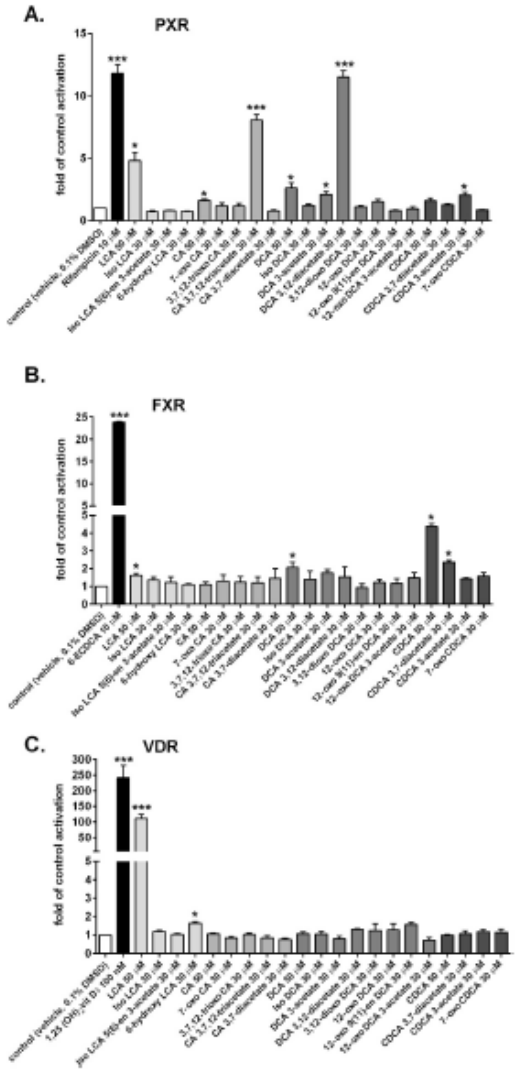



**Fig. 2.** Interaction of the bile acid derivates with PXR, FXR and VDR in luciferase reporter gene assays. Transient transfection reporter gene experiments with NRs responsive luciferase constructs (p3A4-luc, pFXRE-luc2P and pDR3-luc) were performed in HepG2 cells cotransfected with appropriate NRs expression constructs to establish the interactions of the tested compounds with PXR, FXR and VDR. The data are presented as the means ± SD from three independent experiments ($n = 3$) and are expressed as the fold-change of normalized luciferase activities relative to the vehicle-treated sample (control) group mean value (set to 1).
*$p < 0.05$, ***$p < 0.001$—statistically significant difference compared to vehicle-treated cells (ANOVA with Dunnett's post-hoc test).

assay) and $I_{max}$ (representing the overall maximal calculated induction produced by the tested compound; maximal responding capacity; maximal efficacy) values were determined according to Hill's equation by nonlinear regression analysis. All of the statistical analyses were performed using GraphPad Prism 6 Software (GraphPad Software, Inc., San Diego, CA) based on at least three independent experiments ($n = 3$). A $p$ value of $<0.05$ was considered to be statistically significant.

#### 2.9.1. Molecular modeling

3D structures of the compounds were prepared and all hydrogens were added with the Marvin 14.9.8 program (ChemAxon, http://www.chemaxon.com). The crystal structure of PXR with ethinyl estradiol and *trans*-nonachlor (PDB ID: 4 × 1G) was used as final docking template with a docking grid of 14 Å around the center of the ligand in the crystal structure (Delfosse et al., 2015), which was deleted prior to docking. Polar hydrogens were added to the receptor and all ligands with the AutoDock Tools 1.5.4 program (Morris et al., 2009) prior to docking with the AutoDock Vina program (Trott and Olson, 2010), whereas side-chains of the flexible residues identified within available PXR crystal structures (amino acids H407 and R410) were set flexible.

## 3. Results

### 3.1. Interaction of the tested bile acid derivatives with PXR, FXR and VDR in reporter gene assays

In the first set of experiments, we tested a series of interactions between bile acids derivatives with PXR, FXR and VDR by using reporter gene assays in HepG2 cells. Validated prototype ligands of the receptors such as rifampicin, 6-ECDCA and 1,25(OH)$_2$vitD$_3$, respectively, have been used as comparators (black columns) (Fig. 2A–C). We found that the DCA 3,12-diacetate and CA 3,7,12-triacetate derivatives significantly ($p < 0.001$) activated human PXR at a concentration of 30 μM (Fig. 2A). We also observed a mild but significant ($p < 0.05$) PXR activation by the DCA 3-acetate and CDCA 3-acetate, and with LCA, DCA and CA, which are known to interact with PXR.

In the experiments, known FXR ligands 6-ECDCA, CDCA, LCA, and DCA significantly activated FXR-responsive luciferase construct (Fig. 2B). We also observed a weak but statistically significant ($p < 0.05$) activation of FXR by the CDCA 3,7-diacetate (Fig. 2B).

Finally, in the experiments with the VDR-responsive luciferase reporter gene construct, we did not observe any significant activation of VDR by any of the tested compounds except for the known ligands LCA and 6-hydroxy LCA (Fig. 2C). Furthermore, the 3β epimers Iso LCA and Iso LCA 5(6)en 3-acetate did not have any significant effects on any of the nuclear receptors examined (Fig. 2).

Thus our results showed that the acetylation of CA and DCA was able to increase the activation of PXR, and the acetylation of CDCA may increase the activation of FXR.

### 3.2. The DCA 3,12-diacetate interacts with the PXR ligand binding domain (LBD) in a dose-dependent manner

Next we performed dose-response studies using the gene reporter assays in HepG2 cells with the p3A4-luc construct to measure the affinity of the DCA 3,12-diacetate to PXR LBD. We found that the DCA 3,12-diacetate had lower affinity, but higher efficacy to activate PXR (Fig. 3A). $EC_{50}$ of the DCA 3,12-diacetate and rifampicin was 32.1 ± 1.13 and 9.1 ± 1.65 μM, respectively.

In the next experiments, we aimed to determine the affinity of the DCA 3,12-diacetate to PXR-LBD in an *in vitro* TR-FRET PXR coactivation assay using recombinant PXR-LBD protein. In this assay, the affinity of the tested compounds to PXR was evaluated based on competition with a fluorescent PXR ligand. We found that the DCA 3,12-diacetate had similarly low $IC_{50}$ in comparison with prototype non-fluorescent PXR ligand SR12813 ($IC_{50}$ 3.68 ± 1.80

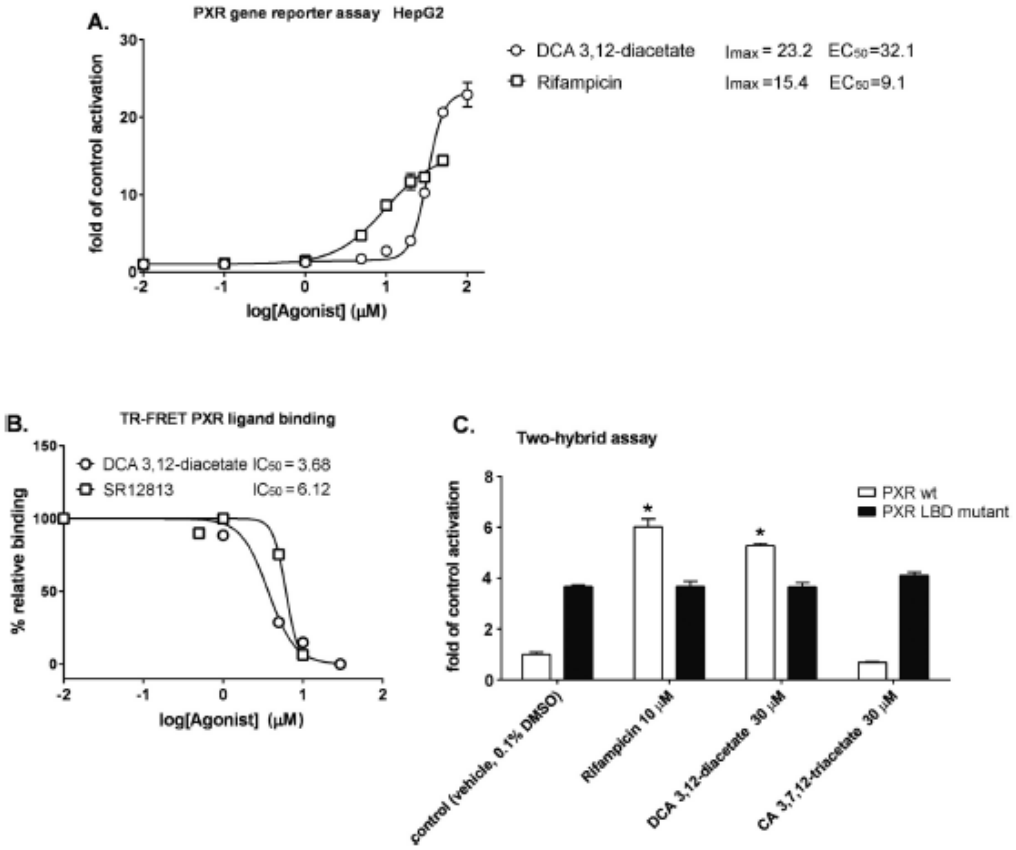



**Fig. 3.** Interaction of the DCA 3,12-diacetate and CA 3,7,12-triacetate with the PXR ligand binding domain.
(A) Dose-response activation of PXR by the DCA 3,12-diacetate in reporter gene assays. Reporter gene experiments were performed with a p3A4-luc luciferase construct in HepG2 cells treated with the indicated range of concentrations of PXR ligands for 24 h. The data are presented as the means ± SD from three independent experiments ($n = 3$) and are expressed as the fold-change in activation relative to the vehicle-treated samples (control) means. $EC_{50}$ is the concentration required to achieve half-maximum promoter activation; $I_{max}$ represents the overall maximal calculated induction (in μM).
(B) Interaction of the DCA 3,12-diacetate with PXR in a LanthaScreen® TR-FRET Pregnane X Receptor competitive binding assay. The TR-FRET assay was performed to determine the affinity of the DCA 3,12-diacetate to recombinant PXR. SR12813, a model non-fluorescent PXR agonist, was used as a positive control. Data are presented as the relative binding of fluorescent PXR substrate to PXR LBD versus the DCA 3,12-diacetate or SR12813 concentrations. The maximum value was set as 100% in the absence of a competitor. $IC_{50}$ (a tested compound concentration required to achieve 50% inhibition in PXR-SRC-1 interaction measured by TR-FRET fluorescence) was calculated. The data are presented as the mean ± SD from three independent experiments ($n = 3$) performed in triplicate measurements.
(C) The mammalian two-hybrid assay in HepG2 cells transiently transfected with the pGL5-luc reporter plasmid together with either the pM-GAL4-PXR LBD wt or pM-GAL4-PXR LBD mut (S247W, C248W) fusion expression plasmid, the VP16-SRC1 fusion expression plasmid or the VP16 empty vector, as well as the pRL-TK control plasmid. After 24 h of stabilization, the cells were treated with the DCA 3,12-diacetate and CA 3,7,12-triacetate (30 μM), rifampicin (RIF, 10 μM) or vehicle (DMSO; 0.1%, v/v) for an additional 24 h. The data are presented as the means ± SD from three independent experiments ($n = 3$).
*$p < 0.05$—statistically significant difference compared to vehicle-treated cells transfected with wild-type PXR LBD (ANOVA with Dunnett's post-hoc test).

and 6.12 ± 1.05 μM, respectively) (Fig. 3B). These data indicated a high affinity of the DCA 3,12-diacetate to PXR.

Finally, we analyzed whether the DCA 3,12-diacetylate could affect the recruitment of the SRC1 coactivator in mammalian two hybrid assay to either the wild-type (wt) PXR LBD or the constitutively active mutant of PXR (S247W/C284W) with an obstructed ligand-binding pocket. We observed that the DCA 3,12-diacetylate at 30 μM significantly ($p < 0.05$) augmented the interaction between the wt PXR LBD and SRC1, as detected by an increase of pGL5-luc reporter vector activity (Fig. 3C). On the other hand, the CA 3,7,12-triacetate did not stimulate an interaction between the wild-type PXR LBD and SRC1 coactivator. Mutated PXR was not stimulated by any of the compounds tested. These results indeed demonstrated that the DCA 3,12-diacetylate was a direct agonist in the LBD of PXR.

### 3.3. The DCA 3,12-diacetate induces PXR-target genes mRNAs in differentiated HepaRG cells

The HepaRG cells were cultivated under differentiated conditions and treated with rifampicin (10 μM) and DCA 3,12-diacetate (30 μM). As shown in Fig. 4A, the expression of CYP3A4

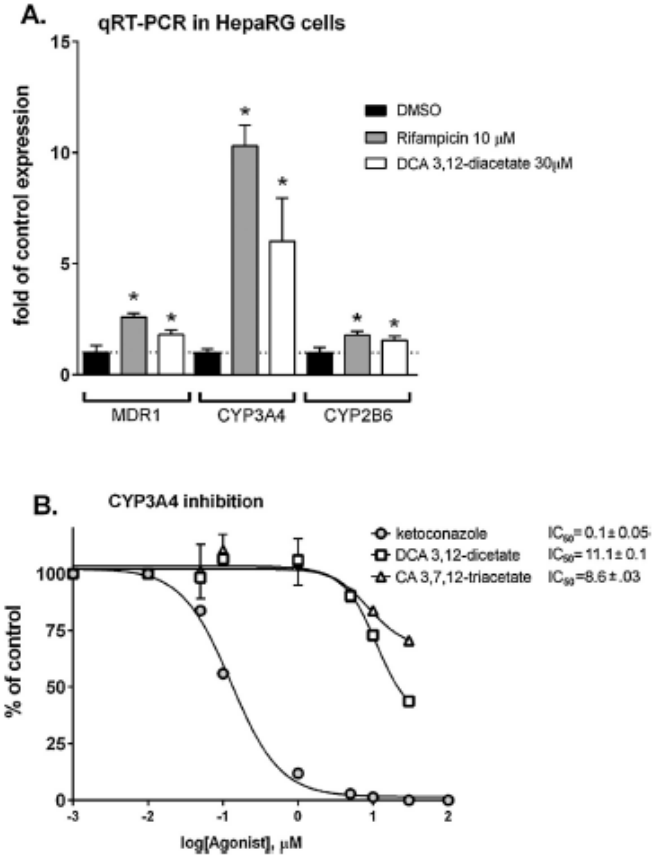



**Fig. 4.** The DCA 3,12-diacetate significantly induces PXR target genes mRNA expression and inhibits CYP3A4 catalytic activity.
(A) RT-PCR experiments were performed in differentiated HepaRG cells with TaqMan primers/probes after a 24 h treatment with rifampicin 10 μM (Rif) and the DCA 3,12-diacetate (30 μM), which significantly induced CYP3A4, CYP2B6, MDR1 mRNA expression in differentiated HepaRG cells. The data are presented as the means ± SD from three experiments ($n = 3$) and are expressed as the fold-change in induction relative to vehicle-treated controls (normalized to 1). *$p < 0.05$ represents a statistically significant difference compared to control (vehicle-treated) cells (ANOVA with a Dunnett's post hoc test).
(B) The P450-Glo™ CYP3A4 Screening System with Luciferin-PPXE was used to evaluate the interaction of the DCA 3,12-acetate with human CYP3A4 in CYP3A4-expressed microsomes. Ketoconazole was used as a prototype CYP3A4 inhibitor. CYP3A4 reactions were performed according to manufacturer's protocol in three independent experiments ($n = 3$) in triplicates. Luminescence was recorded using a plate-reader, with values displayed as relative light units related to control vehicle-treated samples. Data are presented as the means ± SD of CYP3A4 inhibition related to vehicle-treated membranes (control mean was set to be 100%). $IC_{50}$ is the tested compound concentration required to achieve 50% inhibition of CYP3A4 activity (in μM).

mRNA, a major PXR target gene, was induced by the DCA 3,12-diacetate to ~7 folds in comparison with vehicle (DMSO)-treated controls while the activation by rifampicin was ~10 folds (Fig. 5). In addition, the DCA 3,12-diacetate significantly induced other PXR target genes including CYP2B6 and ABCB1/MDR1.

### 3.4. The DCA 3,12-diacetate and CA 3,7,12-triacetate are weak inhibitors of CYP3A4 enzymatic activity

Next, we examined whether the DCA 3,12-diacetylate could inhibit the catalytic activity of CYP3A4, which is an important cytochrome P450 enzyme involved in BA metabolism and is a major target gene induced by PXR activation. We observed an inhibition with $IC_{50}$ ~10 μM for both the DCA 3,7-diacetate and CA 3,7,12-triacetate (Fig. 4B). These data indicate that the DCA 3,12-diacetate up-regulates CYP3A4 expression and at the same time inhibits CYP3A4 enzymatic activity.

#### 3.4.1. Docking of DCA derivatives to PXR structure

Recently, a PXR structure with the steroid ligand inside – ethinylestradiol – (PDB ID: 4X1G) has been released (Delfosse et al., 2015) and this was used as a docking template (Fig. 5A). Furthermore, as some side-chain conformations have been shown to differ among individual published PXR-LBD crystal structures, we set two polar residues as flexible—H407 and R410. With this approach, the molecular docking of DCA derivatives showed a common binding pose for all molecules (Fig. 5).

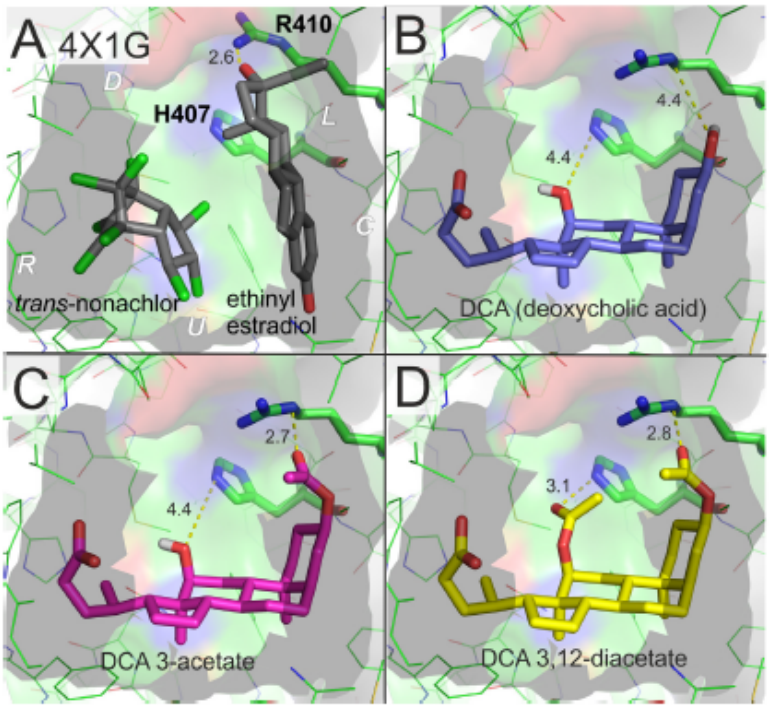



**Fig. 5.** Binding poses within PXR structure.
Best binding poses of derivatives of deoxycholic acid in comparison with crystal structure with bound ethinylestradiol and *trans*-nonachlor (PDB ID: 4 × 1G[1]) with annotated positions of hotspots (see main text for definition). Derivatives of DCA share the same binding pose, whereas acetylation affects hydrogen bonds from residues H407 and R410, which are known for their role in PXR activation.

DCA occupies several of hotspots, the regions of protein surface that are major contributors to the binding energy (Ngan et al., 2009). The hydrophobic part of the steroid tail occupies hotspot R, whereas carboxylic group is exposed towards water inside of the cavity center. The hydrophobic groove in hotspot U is filled with methyls between the A/B and C/D rings and the steroid edge interacts with hotspot C as well through hydrophobic interactions. Nevertheless, nonpolar interactions with hotspot D were too far, as interactions with hotspot L are important for its influence on the activation function helix (Fig. 5B). On the other hand, this hotspot was fulfilled with the DCA 3-acetate, which had one hydrogen bond acceptor interacting with R410, with the DCA 3,12-diacetate, which had two hydrogen bonds with both flexible residues R410, and more importantly with H407 (Fig. 5C and D). As a result of this docking, the DCA 3,12-diacetate as a PXR ligand exhibited the strongest interaction energy.

### *3.5. Analysis of the DCA 3,12-acetate and CA 3,7,12-triacetate in human bile and mouse liver samples*

We further determined the presence of the DCA 3,12-acetate and CA 3,7,12-triacetate in biological samples to provide evidence as to whether these bile acids could be endogenous ligands *in vivo*. We had previously set-up bile acid profiling by HPLC/MS-MS (Jiao et al., 2015), which was implemented for detection of the DCA 3,12-acetate and CA 3,7,12-triacetate. By using pure standards, the mass for a precursor scan (Fig. 6A and A′) and corresponding daughter scan (Supplemental material Fig. S1) was detected for the DCA 3,12-acetate and CA 3,7,12-triacetate at retention time 9.19 min and 8.16 min, respectively.

We further analyzed lipid extracts of mouse liver and several human bile samples. However, none of these two acetyl bile acids was detected in these samples (Fig. 6B–D and 6B′–6D′). We therefore concluded that these acetylated bile acids may not be endogenous bile acid PXR ligands in bile or in the liver or that their concentrations may be lower than of the detection limit of HPLC/MS-MS.

## 4. Discussion

Three nuclear receptors of the nuclear receptor superfamily have been recently established as bile acid receptors, and their role in bile acid synthesis regulation or detoxification has been clearly documented (Ishizawa et al., 2008; Makishima et al., 2002, 1999; Staudinger et al., 2001b; Wang et al., 1999; Xie et al., 2001). Bile acids have thus been established as endogenous regulatory ligands of nuclear receptors in the regulation of numerous target genes. Recently, 3-keto LCA has been found as a potent ligand for the VDR, FXR and PXR (Adachi et al., 2005; Makishima et al., 1999; Staudinger et al., 2001b), and the LCA acetate methyl ester as a highly potent VDR ligand (Adachi et al., 2005; Makishima et al., 1999; Staudinger et al., 2001b). Therefore, we hypothesized that oxidized or acetylated bile acids DCA, LCA, CA, and CDCA at position 3, 7 and 12 may have an effect on the interactions with bile

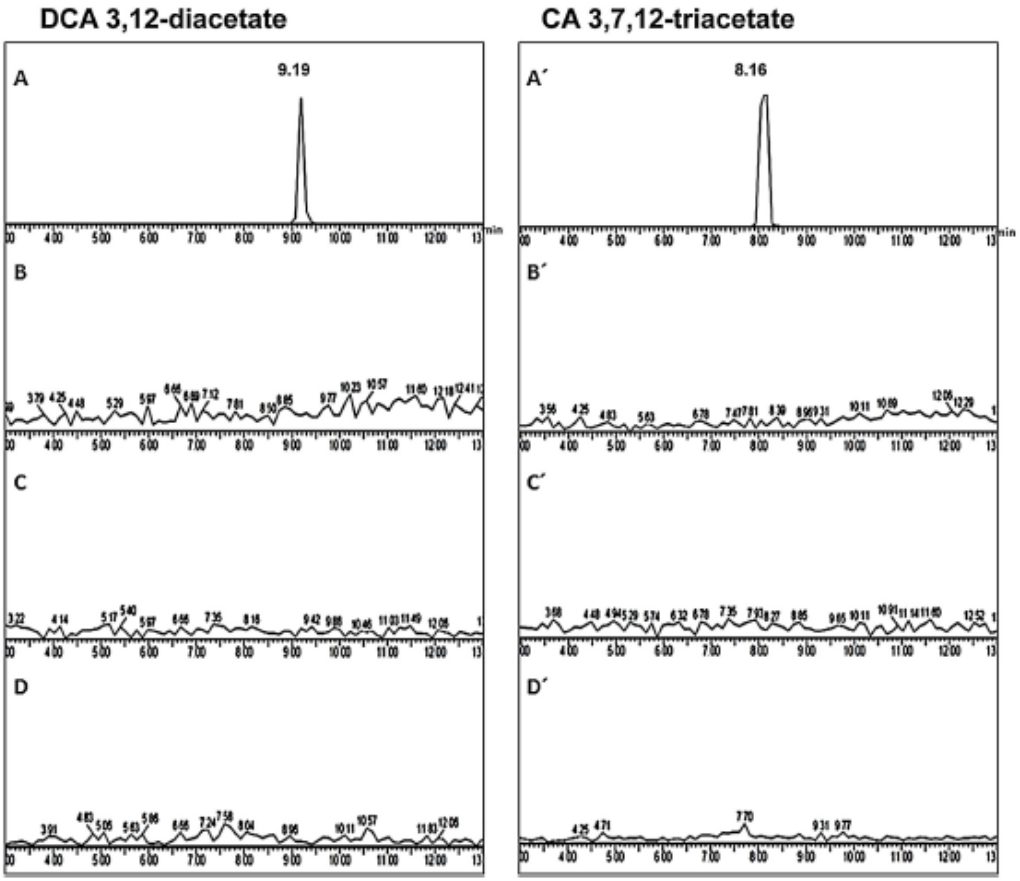



**Fig. 6.** HPLC/MS-MS analyses of acetylated bile acids.
In the left panel, HPLC–MSMS analysis of the DCA 3,7-diacetate by MRM of 475.1 > 433.2 in an authentic standard detected at 9.19 min (A) as well as in a representative lipid extract from mouse liver homogenate (B), and human bile samples collected in 2010 (C) and in 1995 (D) from a representative donor. In the right panel, HPLC–MSMS analysis of CA 3,7,12-triacetate by MRM of 533.2 > 491.2 in an authentic standard detected at 8.16 min (A′) as well as in a representative lipid extract from mouse liver homogenate (B′), and human bile samples collected in 2010 (C′) and in 1995 (D′) from a representative donor.

acid nuclear receptors. For this purpose, we synthetized a series of dehydrogenated (keto) and acetylated derivatives of DCA, LCA, CA, and CDCA, and, to our knowledge for the first time, we tested their interactions with human PXR, VDR, and FXR in cellular and *in vitro* assays.

We demonstrated that the DCA 3,12-diacetate was an efficient and high-affinity PXR ligand with greater potency than the parent secondary bile acid DCA ($EC_{50}$ = 32.1 μM versus 150.2 μM, respectively) (Krasowski et al., 2005). We showed consistently that the DCA 3,12-diacetate was able to upregulate the mRNA expression of PXR target genes CYP3A4, CYP2B6, and MDR1 in a differentiated HepaRG cells, a superior human hepatocyte model with endogenous expression of functional PXR. Dehydrogenation or acetylation of the 3, 7 and 12 hydroxyl groups of CDCA, LCA, DCA and CA did not result in any significant increase of VDR or FXR activities.

We could only observe, respectively, a weak activation of FXR with the CDCA 3,7-diacetate and of PXR with the DCA 3-acetate and CDCA 3-acetate. Oxidation of 3, 7 and 12 hydroxyl groups did not result in any augmented activity of the tested compounds to all the nuclear receptors studied. Although Iso LCA has been reported as a weak activator of VDR (Adachi et al., 2005), our data did not confirm the results. Cotransfection of ASBT bile acid transporter into HepG2 cells in a parallel experiment did not significantly improve interaction of the tested bile acids with any of the nuclear receptors examined (*A.C., P.P. unpublished observations*).

Based on docking experiments, we proposed that the acetylation of DCA may improve interactions with PXR ligand-binding domain residues, even though we should also take into account the easier entry of the acetylated bile acid into cells due to their high lipophilicity.

PXR was found to be efficiently activated by lithocholic acid, a hydrophobic bile acid formed by 7α-dehydroxylation of CDCA by intestinal anaerobic bacteria. CDCA, DCA and CA have only a mild effect to activate PXR in high micromolar concentrations (Krasowski et al., 2005; Staudinger et al., 2001b).

Activation of PXR induces genes encoding enzymes involved in the metabolism and detoxification of secondary bile acids (mainly CYP3A4 cytochrome P450 enzyme) and down-regulates CYP7A1

(cholesterol 7alpha-hydroxylase), a rate limiting enzyme of BA synthesis (Banerjee et al., 2015; Staudinger et al., 2001a; Xie et al., 2001). DCA is metabolized by CYP3A4, a main target enzyme of PXR and VDR in the liver and in the intestine, into 1β-hydroxy-DCA and 3-dehydro-DCA (3-oxo DCA) (Chen et al., 2014). By this mechanism, PXR has been documented as a key factor conferring resistance to LCA toxicity in various transgenic or knockout animal models (Staudinger et al., 2001b; Xie et al., 2001). In addition, activation of PXR has been proposed beneficial in the elevation and potential treatment of inflammatory liver and bowel diseases (Cheng et al., 2012; Wallace et al., 2010). Recently, it was shown that symbiotic bacteria regulate gastrointestinal barrier function and inflammation via the xenobiotic sensor PXR and Toll-like receptor 4 by production of indole 3-propionic acid (IPA) (Venkatesh et al., 2014). Therefore, discovery of a potent endogenous bile acid ligand of PXR receptor, produced by either gut microflora or liver metabolism, may help us to uncover additional regulatory functions of microflora or other regulatory pathways of bile acid synthesis and detoxification In addition, the potent endogenous PXR ligands could be considered as a safe therapy in inflammatory and cholesteric liver diseases (Ridlon and Bajaj, 2015).

In our study, we presented the novel finding that the acetylation of DCA and CA led to a significant increase in binding to PXR, and the DCA 3,7-diacetate was identified as a potent PXR ligand which was able to induce the PXR target genes CYP3A4 and CYP2B6 as well as the P-glycoprotein/MDR1 transporter in a HepaRG hepatocyte model. Since there have been no reports of the existence of acetylated BAs in biological samples, we analyzed the presence of these BAs in mouse liver and human bile samples. However, we observed neither the DCA 3,12-diacetate nor CA 3,7,12-triacetace in these samples. It is possible that these acetylated BAs do not exist in free form but in conjugated forms with glycine, taurine, sulfates, and glucuronide. At this time, it is unlikely that the DCA 3,12-diacetate can be considered as an important endogenous PXR ligand. Whether the conjugates of the DCA 3,12-diacetate exist *in vivo* and whether they are capable of activating PXR awaits further investigation in our laboratories. Another possibility is that intestinal microflora may produce the DCA 3,12-diacetate, which may in turn activate intestinal PXR. Further studies are needed to examine the presence of the DCA 3,12-diacetate and its conjugates within enterohepatic circulation employing HPLC/MS-MS.

## 5. Conclusions

Taken together, the majority of the newly-synthesized acetylated derivatives of LCA, CA, DCA, and CDCA did not show any improvement in their activity towards the nuclear receptors tested, the two exceptions being the DCA 3,12-diacetate and CA 3,7,12-triacetate, which were found to be powerful agonists of PXR. Dehydrogenation of position 3, 7 and 12 did not stimulate interactions with the PXR, FXR and VDR. Since we did not observe the DCA 3,12-diacetate or CA 3,7,12-triacetate in mouse liver and human bile samples, these bile acids may not be natural ligands for PXR. Our next experiments are focused on analysis of acetylated bile acid derivatives or their conjugates in the intestine and on their putative physiological function in the intestinal barrier.

## Acknowledgements

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.toxlet.2016.11.013.

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## A.4. 2-(3-Methoxyphenyl)quinazoline Derivatives: A New Class of Direct Constitutive Androstane Receptor (CAR) Agonists

Smutny T, Nova A, Drechslerova M, Carazo A, Hyrsova L, Hruskova ZR, Kunes J, Pour M, Spulak M and Pavek P (2016) 2-(3-Methoxyphenyl)quinazoline Derivatives: A New Class of Direct Constitutive Androstane Receptor (CAR) Agonists. *J Med Chem* **59**:4601-4610. $IF_{2015}$:5,589

In this research work, we tested interaction of several potential antituberculotics compounds with several nuclear receptors. Some derivates of the 2-(3-methoxyphenyl)quinazoline structure showed activator effect for VDR, AhR, PXR and importantly, CAR. Three of these compounds (3a, 7 and 7a) showed strong CAR activation, and their activities on other nuclear receptors make of them promising molecules in the study of drug metabolizing enzymes (DME) and physiological processes regulated by these transcription factors.

In gene reporter assays, compounds 3a, 7 and 7a proved to be strong CAR activators. Complementary experiments were performed to study their effect in different recombinant human CYP isoforms and their interaction with CAR-LBD. The effect of the compounds on CAR target genes expression (CYP3A4, CYP2B6, CYP1A2, CYP2C9 and CYP2D6) were studied using RT-PCR. All these genes were significantly upregulated in primary human hepatocytes treated with these compounds. In enzymatic experiments, a significant inhibitory effect for CYP2C9 was observed. For all the other tested enzymes, the compounds showed weak or no inhibition at all.

Next, in TR-FRET CAR assay, the three compounds showed direct binding into CAR-LBD with $EC_{50}$ 2.07, 0.69 and 5.12 μM for compounds 3a, 7 and 7a, respectively, while CITCO had a $EC_{50}$ of 0.02 μM.

Taken together, these data prove that these compounds are promising CAR ligands. However, these compounds did not show specificity for CAR since they also activate other receptors. These effects make these molecules promising tools for the study of different detoxification functions.

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# 2-(3-Methoxyphenyl)quinazoline Derivatives: A New Class of Direct Constitutive Androstane Receptor (CAR) Agonists

Tomas Smutny,[†] Alice Nova,[†,§] Marcela Drechslerová,[‡] Alejandro Carazo,[†] Lucie Hyrsova,[†] Zuzana Rania Hrušková,[‡] Jiří Kuneš,[‡] Milan Pour,[‡] Marcel Špulák,[*,‡] and Petr Pavek[*,†]

[†]Department of Pharmacology and Toxicology, [‡]Department of Inorganic and Organic Chemistry, Faculty of Pharmacy, Charles University, Heyrovského 1203, Hradec Kralove CZ-500 05, Czech Republic

[§]Institute of Molecular and Translation Medicine, Faculty of Medicine, Palacky University in Olomouc, Hnevotinska 5, CZ-779 00 Olomouc, Czech Republic

Supporting Information

**ABSTRACT:** Constitutive androstane receptor (CAR) is a key regulator of xenobiotic and endobiotic metabolism. Together with pregnane X (PXR) and aryl hydrocarbon (AHR) receptors, it is referred to as "xenobiotic receptor". The unique properties of human CAR, such as its high constitutive activity, both direct (ligand-binding domain-dependent) and indirect activation have hindered the discovery of direct selective human CAR ligands. Herein, we report a novel class of direct human CAR agonists in a group of 2-(3-methoxyphenyl)quinazoline derivatives. The compounds are even more potent activators of human CAR than is prototype 6-(4-chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime (CITCO). The three most potent ligands are at the same time extremely potent activators of the other xenobiotic or hormonal receptors, namely PXR, AHR, and vitamin D receptor, which regulate major xenobiotic-metabolizing enzymes and efflux transporters. Thus, the novel CAR ligands can be also considered as constituting the first class of potent pan-xenobiotic receptor ligands that can serve as potential antidotes boosting overall metabolic elimination of xenobiotic or toxic compounds.


## INTRODUCTION

Constitutive androstane receptor (CAR, NR1I3), together with pregnane X receptor (PXR, NR1I2) and aryl hydrocarbon receptor (AHR), are ligand-activated transcription factors that play pivotal roles in xenobiotic clearance. These transcription factors control gene expression across a broad spectrum of target genes that encode key phase I and phase II drug metabolizing enzymes (DMEs) and some drug transporters.[1] They are activated by a variety of exogenous ligands that include drugs, environmental toxicants, industrial chemicals, and herbal compounds. They are therefore sometimes referred to as "xenosensors" or "xenobiotic receptors."[2]

Although CAR was originally identified as a "xenosensor" of environmental, dietary, natural, and synthetic ligands,[3,4] recent findings suggest that CAR also plays important roles in energy metabolism of fatty acids, bile acids, lipids, and glucose, in thyroid hormone metabolism, in cell-cycle regulation, and in cell−cell interaction.[5,6] CAR is composed of three domains: a highly conserved DNA-binding domain, a hinge region, and a divergent ligand binding/dimerization/transcriptional activation domain.[7] In contrast to other nuclear receptors, activation of this receptor is complex. Both ligand-binding domain (LBD)-dependent and independent activation of human CAR have been shown. These release CAR from its cytoplasmic tethering complex and translocate the receptor into the nucleus, where CAR transactivates CAR-inducible genes as a heterodimer with retinoid X receptor $\alpha$ (RXR$\alpha$) nuclear receptor. Dephosphorylation-induced translocation of CAR to the nucleus is a key step for indirect activation.[8]

Numerous ligands, agonists, antagonists, as well as inverse agonists have been reported for human CAR. There is discrepancy in assigning a CAR-interacting compound because validated methods are not widely used for direct human CAR ligand identification or distinguishing between indirect activators (such as acetaminophen, bilirubin, and phenobarbital) and ligands interacting within its ligand-binding domain.[3,4] Moreover, CAR is constitutively active and it is localized in the nucleus in model tumor cell lines as opposed to its cytoplasmic localization in normal hepatocytes. CAR activation by ligands is determined by their effect on the position of the C-terminal helix12 that binds coactivators of corepressors. In addition, there are significant species differences in the ligand specificity of CAR ligands.[4] Currently, there is *no* specific high-affinity agonist for human CAR that would help decipher the diverse physiological functions of CAR. Known to date is 6-(4-chlorophenyl)imidazo[2,1-*b*]thiazole-5-carbaldehyde-*O*-(3,4-dichlorobenzyl)oxime (CITCO, **1**), which is a potent human, but not mouse, CAR agonist.[9] However, this unstable compound also activates PXR.[9] The potent mouse CAR ligand 1,4-bis[(3,5-dichloropyridine-2-yl)oxy]benzene (TCPOBOP, **2**) does not activate human or rat CAR.[10] Reported activators

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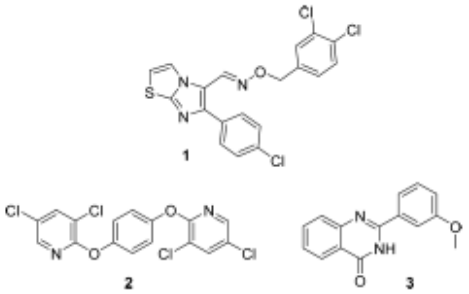
of human CAR identified to date include pesticides such as pyrethroids (permethrin, cypermethrin), the carbamate benfuracarb, and organochlorines (e.g., methoxychlor), synthetic compounds such as CITCO, FLB1, octicizer, thiazolidin-4-ones, 6-arylpyrrolo[2,1-*d*][1,5]benzothiazepine derivatives, and sulfonamides, and natural flavonoids such as chrysin and drugs (e.g., artemisinin derivatives, nevirapine, nicardipine, efavirenz, carbamazepine).[4,11−17]

In this work, a library of compounds previously prepared as potential antituberculotics was subjected to random screening. The screening revealed that 2-(3-methoxyphenyl)-3,4-dihydroquinazolin-4-one (**3**) displayed promising activation of the CAR receptor comparable to that of CITCO in reporter gene assay. We therefore synthesized other derivatives of 2-(3-methoxyphenyl)quinazoline and analyzed their interaction with the human CAR receptor. Interaction of the compounds with the additional "xenoreceptors" PXR and AHR as well as with other nuclear receptors controlling expression of certain drug metabolism genes, such as vitamin D receptor (VDR) and glucocorticoid receptors (GR),[18] was analyzed simultaneously.

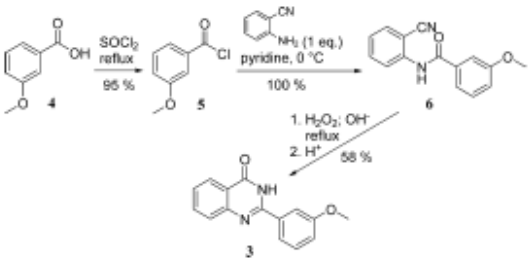
Herein we report that three 2-(3-methoxyphenyl)quinazoline derivatives are robust ligands of CAR as well as of the other "xenobiotic receptors" PXR and AHR and even exceed their prototype ligands in their potencies to activate the receptors in cell-based reporter gene assays.




## RESULTS

In a random screening of a library previously prepared as potential antituberculotics, we identified a hit compound 2-(3-methoxyphenyl)-3,4-dihydroquinazolin-4-one (**3**) that displayed promising activation of the CAR receptor comparable to that of CITCO. Quinazolinone **3** (Scheme 1) was therefore prepared[19] in order to confirm its activity in relationship to the CAR receptor. Thus, 3-methoxybenzoic acid (**4**) was converted to its chloride **5** which was subsequently treated with 2-aminobenzonitrile to yield amide **6**. Compound **6** was then cyclized to afford the title quinazoline **3** in 55% overall yield. The repeated evaluation of 2-(3-methoxyphenyl)-3,4-dihydroquinazolin-4-one (**3**) in reporter gene assays fully confirmed the ability to activate the CAR receptor.

**Scheme 1. Synthesis of 2-(3-Methoxyphenyl)-3,4-dihydroquinazolin-4-one**




A library of six 2-(3-methoxyphenyl)quinazolines was subsequently prepared via simple alkylation conditions using RX/NaI/$K_2CO_3$ at reflux in acetone (Scheme 2). In contrast to the alkylation of 3,4-dihydroquinazolin-4-one,[20] we observed no *N*3-alkylated products. The reactions afforded only *O*-alkylated species, the structures of which were clearly confirmed by $^{13}$C NMR shifts of −O−$CH_n$− carbon atom (Table 1).[20]

**Scheme 2. Alkylation of Quinazoline 3 with Various Primary Halides**

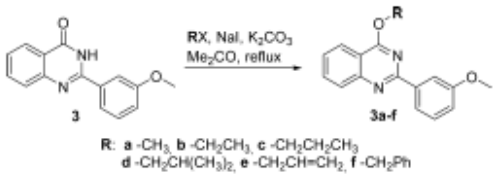
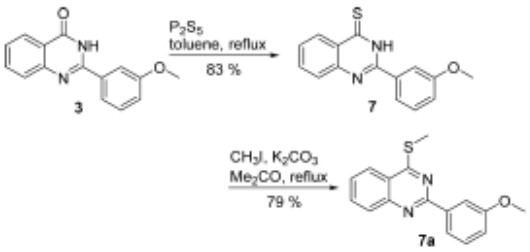



**Table 1. Isolated Yields and $^{13}$C NMR Chemical Shifts in $CDCl_3$ of C(1′) Group of Compounds 3a−f**

| compd | yield (%) | δC (ppm) |
|---|---|---|
| 3a | 85 | 54.0 |
| 3b | 91 | 62.7 |
| 3c | 88 | 68.3 |
| 3d | 78 | 72.8 |
| 3e | 58 | 67.3 |
| 3f | 78 | 68.2 |

Quinazoline derivative **7a** bearing methylsulfanyl moiety in position 4 was further synthesized. The starting 2-(3-methoxyphenyl)-3,4-dihydroquinazolin-4-one **3** was first converted into its sulfur analogue **7**, followed by the same alkylation protocol (Scheme 3).

**Scheme 3. Preparation of 4-Methylsulfanyl Derivative 7a**




**Library Screening and Ligands Identification.** We randomly screened a library of compounds previously prepared as potential antituberculotics for activation of the human CAR receptor using a reporter gene assay with p(ER6)$_3$-luc construct transfected together with human CAR expression vector into HepG2 cells. This assay showed itself to be a sensitive test for selecting several potential activators of CAR. 2-(3-Methox-



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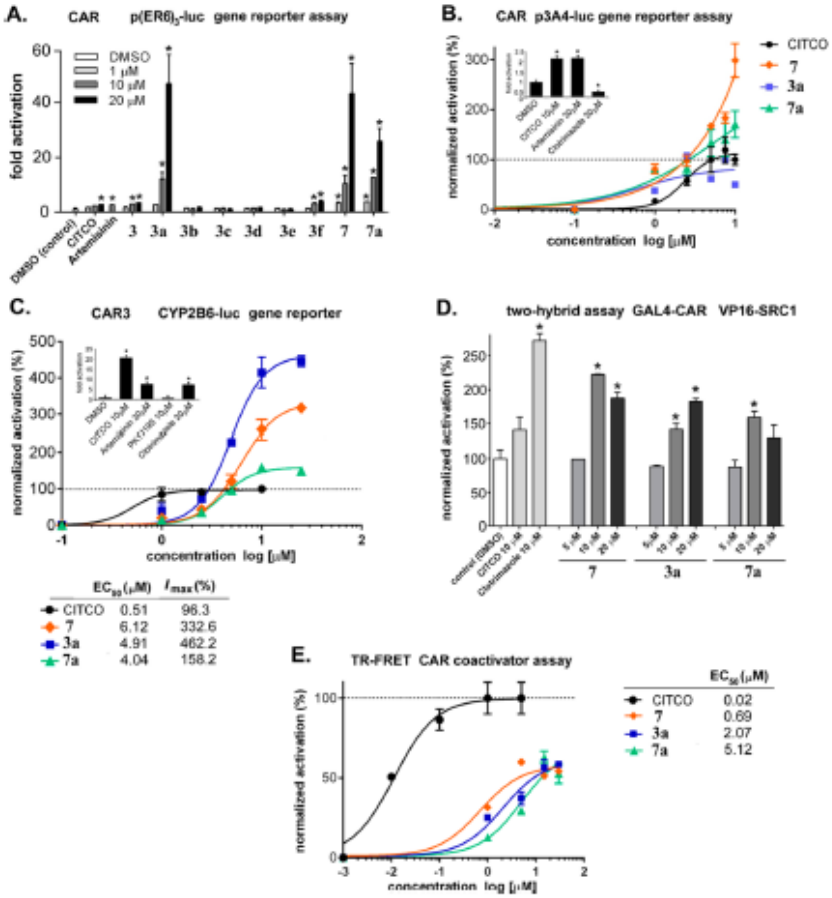


**Figure 1.** Compounds **7**, **3a**, and **7a** activate human wild-type CAR and its CAR variant **3** in transient transfection reporter gene assays and in TR-FRET CAR coactivation assays.

yphenyl)-3,4-dihydroquinazolin-4-one (**3**) displayed the most promising activation of CAR in the assay and was used as the "lead" compound.

In the next series of experiments, we tested the interactions of the newly synthesized compounds **3a**–**f**, **7**, and **7a** with CAR using the same assay (Figure 1A). Interestingly, we found even more robust activation of the CAR-responsive assay with derivatives **3a**, **7**, and **7a**. The activation was even more potent than that produced by the prototype CAR activator/ligand CITCO in equimolar concentrations (Figure 1A).

Next, the compounds **3**, **3a**, **7**, and **7a** were tested using luciferase gene reporter vectors with promoter responsive sequences of CAR target genes CYP3A4 and CYP2B6. We found that **7**, **3a**, and **7a** compounds significantly activated CYP3A4-luc and CYP2B6-luc reporter constructs in a dose-dependent manner through both wild-type CAR (CAR) characterized by high constitutive activity as well as its variant **3** (CAR3) with low constitutive activity[21] (Figure 1B,C). All tested compounds appeared as lower affinity but high potency activators of human CAR and CAR3 receptors in the assays, as indicated by the $EC_{50}$ and $I_{max}$ parameters (Figure 1C). No effects of compounds **7**, **3a**, and **7a** on HepG2 cell viability have been observed (Figure 1, Supporting Information).

In next set of experiments, we employed mammalian two hybrid assay with GAL4-CAR LBD and VP16-SRC1 fusion constructs. All tested compounds **7**, **3a**, and **7a** significantly stimulated interaction of CAR ligand binding domain with steroid receptor coactivator 1 (SRC1)-VP16 fusion protein (Figure 1D).

Finally, we used the in vitro LanthaScreen time-resolved (TR)-FRET constitutive androstane receptor (CAR) coactivator assay that monitors ligand-dependent, but not indirect phenobarbital activator-dependent, interaction with CAR ligand binding domain (LBD) as we have shown in our previous report.[22] We observed that the tested compounds **7**, **3a**, and **7a** all activated the assay, thus suggesting direct interaction with human CAR LBD (Figure 1E). These activations were, however, weaker than that of CITCO, therefore indicating potential indirect cellular signaling-dependent effects of the quinazoline compounds on CAR activation or a ligand specific interactions of CAR with its coactivators SRC-1 (Figure 1D) and PGC1$\alpha$ (Figure 1E) or corepressors based on position of helix 12 determined by the ligands.[23,24]



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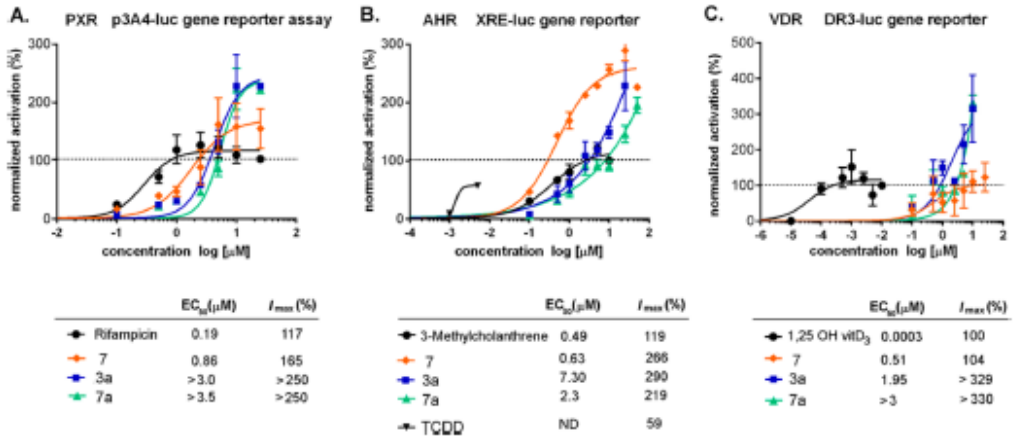


Figure 2. Compounds 7, 3a, and 7a activate PXR, AHR, and VDR receptors in transient transfection reporter gene assays.

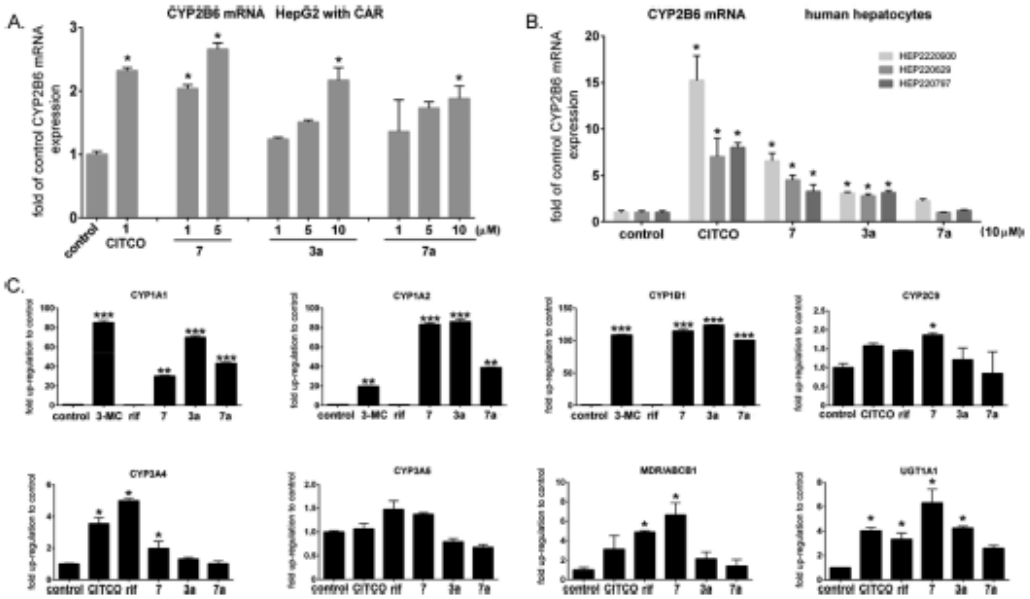


Figure 3. Compounds 7 and 3a induce target genes expression in CAR-expressing HepG2, HepaRG cells, or in primary human hepatocytes.

**Compounds 7, 3a, and 7a Activate PXR, AHR, and VDR Receptors.** In the next set of experiments, we analyzed whether 7, 3a, and 7a compounds activate other xenobiotic receptors, such as PXR, AHR, and VDR, which control expression of many xenobiotic metabolizing enzymes in the liver and intestine.[25] We found that compounds 7, and, to a lesser extent 3a and 7a, significantly activated both PXR and AHR receptors, as indicated by low micromolar $EC_{50}$ and high $I_{max}$ (Figure 2A,B). In addition, compound 7 significantly activated the vitamin D receptor (VDR) in transient transfection reporter gene assay in low micromolar concentrations ($EC_{50}$ = 0.51 $\mu$M) (Figure 2C). Glucocorticoid receptor was not significantly activated by the compounds (data not shown).

**Compounds 7, 3a, and 7a Induce Prototype CAR Target Gene CYP2B6 mRNA in HepG2 Cells and in Primary Human Hepatocytes.** We analyzed whether compounds 7, 3a, and 7a induce CYP2B6 mRNA expression in HepG2 cells expressing exogenous human CAR or in three primary human hepatocyte preparations. CYP2B6 is the most responsive target gene of the CAR receptor. We found statistically significant induction of CYP2B6 mRNA expression both in CAR-expressing HepG2 cells and in human hepatocytes after treatment with compounds 7, 3a, and CITCO (Figure 3A,B). Compound 7a most likely undergoes fast metabolism in metabolically competent primary human hepatocytes because we did not observe CYP2B6 mRNA induction (Figure 3B).

**Compounds 7 and 3a Upregulate other Major Inducible Xenobiotic-Metabolizing Enzymes and P-Glycoprotein Transporter mRNAs.** Compounds 7, 3a, and



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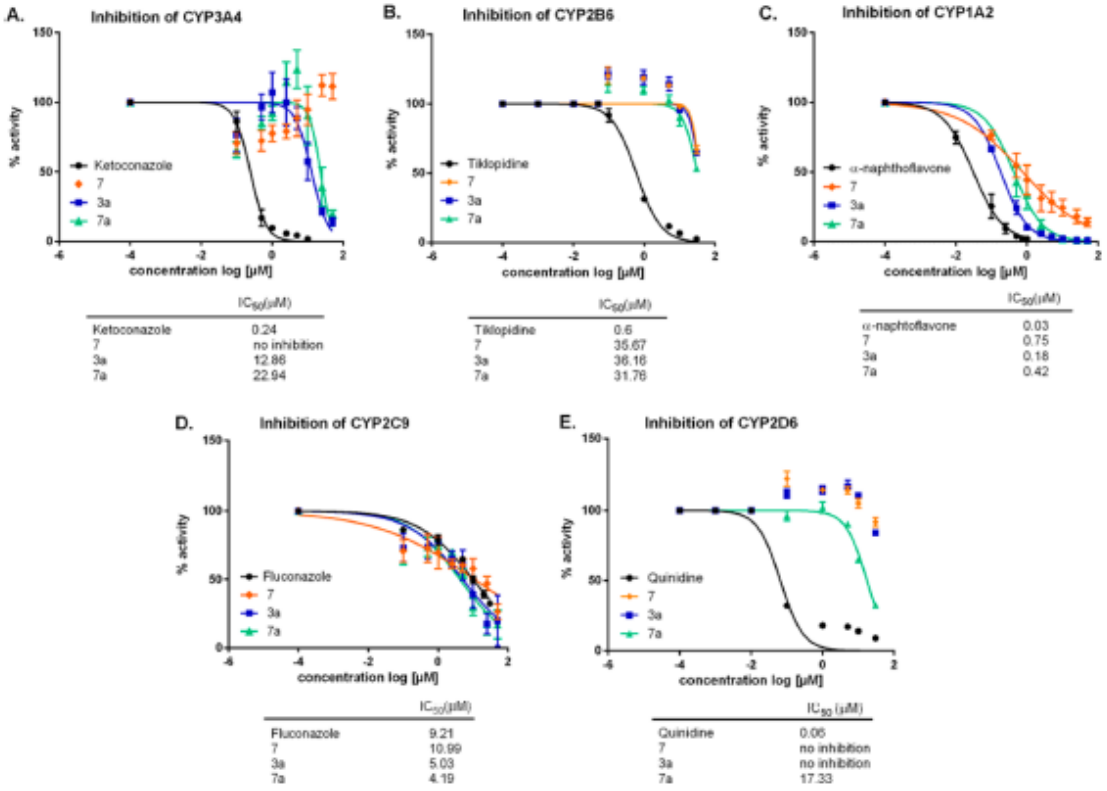


**Figure 4.** Inhibition of recombinant human CYP3A4, CYP2B6, CYP1A2, CYP2C9, and CYP2D6 enzymes with compounds 7, 3a, and 7a.

7a also significantly induced CYP3A4, CYP3A5, CYP2C9, MDR1/P-glycoprotein genes controlled by CAR, PXR, and VDR as well as AHR target genes CYP1A1, CYP1A2 (also regulated by CAR), CYP1B1, and UGT1A1 (also regulated by PXR) (Figure 3C).

**Compounds 7, 3a, and 7a Do Not Significantly Inhibit Major Cytochrome P450 Enzymes CYP3A4 and CYP2C9.** Finally, we analyzed if compounds 7, 3a, and 7a interfere enzymatic activities of the most abundant liver CYP enzymes induced by CAR, PXR, VDR, and AHR receptors. We found that tested compound do not significantly affect CYP3A4 and CYP2B6 enzyme activities (Figure 4A,B). On the other hand, compounds 7, 3a, and 7a inhibit enzymatic activity of CYP1A2 enzyme, which activates numerous procarcinogens to active carcinogens in the liver (Figure 4C). In the case of CYP2C9, the $IC_{50}$ were at concentrations leading to CAR, PXR, or VDR nuclear receptors activation (Figure 4D). We observed no significant interaction of tested compounds with CYP2D6 enzyme (Figure 4E).

These data thus indicate that enzymatic activities of the major induced CYP liver enzymes via CAR, PXR, and VDR are not significantly affected with the compounds and that compound 7 least interferes with the enzymes.

## ■ DISCUSSION

CAR is one of the key regulators of xenobiotic and endobiotic metabolism. Recently, the therapeutic potential for treatment of such metabolic disorders as obesity, type 2 diabetes mellitus, insulin resistance, dyslipidemia, and atherosclerosis has been indicated in animal models using a ligand of rodent CAR.[26] However, there exists no potent, specific, and nontoxic agonist of *human* CAR receptor that can serve as a chemical tool to address various biological functions of CAR or to consider CAR as a therapeutic target. The unique properties of human CAR, such as its high constitutive activity and the complexity of the related signaling, make discovery of specific ligands difficult. In addition, the lack of robust and validated cell-based assays to study direct interactions of compounds with the CAR ligand-binding pocket (LBP) hinders the discovery of selective human CAR ligands. Therefore, the ligand specificity of human CAR as well as of high-affinity endogenous ligands remains obscure.[11]

Our aim was to discover novel compounds that would interact with the human CAR receptor as a direct agonist with high potency. We randomly screened a library comprising about 400 compounds for the activation of human CAR receptor using a reporter gene assay with pER6-luc construct transfected together with human CAR expression vector into HepG2 cells. This assay provided to be a sensitive test for selecting the lead compound based on 2-(3-methoxyphenyl)-3,4-dihydroquinazolin-4-one (3) structure (Figure 1A). The synthesized compounds 7, 3a, and 7a displayed even more potent activation of CAR in transient transfection reporter gene assays (Figure 1A,B), in two hybrid CAR LBD/SRC-1 interaction assay (Figure 1D), and in induction experiments in HepG2 cells transfected with exogenous CAR (Figure 3A).



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At the same time, we observed activation of variant 3 of the human CAR gene with compounds 7, 3a, and 7a (Figure 1C), which was found to be a ligand-activated low-constitutive activity receptor variant. CAR3 differs from CAR1 by a 5 amino acid insertion outside of the ligand binding pocket.[21,27] Interestingly, in TR-FRET-based coactivator LBD competitive assay, CITCO activated the system more significantly at equimolar concentrations than did compounds 7, 3a, and 7a (Figure 1E). This phenomenon might reflect additional cellular or epigenetic effects of compounds 7, 3a, and 7a that stimulate CAR transactivation[23] or indicate ligand dependent coactivation with PGC1α.

Unlike other NRs that have evolved as receptors for specific high-affinity endogenous ligands, CAR and PXR are highly promiscuous. Only two human CAR crystal structures with agonist bound have been reported to date.[28] The CAR LBD sequence creates a ligand-binding pocket with volume ranging from 525 to 675 Å.[5,28] The LBD cavity has flexible and hydrophobic character, which correlates with the structural promiscuity of CAR ligands.[4,7] The apo-PXR binding cavity volume is even double (approximately1150 Å$^3$) and can extend in the presence of ligands to 1290−1540 Å$^3$. Cavity of VDR is larger but narrower than that of CAR (870 Å$^3$) (Figure 2, Supporting Information). A single residue (T350M) difference in the C-terminal region of the mouse versus human CAR might account for the extensive species selectivity for some agonists.[7] The coactivator recruitment appears to play a central role in fixing ligands in the correct arrangement in large CAR and PXR cavities. In addition, it was clearly shown that ligands determine helix12 position, which is critical for coactivator or corepressor binding and subsequent CAR activation.[24]

The ligand pockets of CAR, PXR, as well as VDR are lined by mostly hydrophobic residues. The 27 residues of the murine CAR pocket create a highly hydrophobic environment, with only a quarter of them being polar. Similarly, the cavity of PXR is lined by 28 amino acids, of which eight have polar or charged side chains.[5,29] Therefore, van der Waals forces play crucial roles in the interactions of the ligand with CAR and hydrogen bonding is rare. For example, CITCO form no hydrogen bonds and makes weak electrostatic interactions with His203, Asn165, and Tyr326. Another CAR agonist 5β-pregnanedione forms a single hydrogen bond with His-203[5,30] but display hydrophobic interactions with Phe161, Ile164, Leu206, Phe217, Tyr224, Phe234, and Leu242 in human CAR cavity.[28] In our docking study, we found that compounds 7 and 3a form hydrophobic bonding with three amino acids, Phe161, Phe234, and Leu242, that also interact with CITCO and 5β-pregnanedione (Figure 2, Supporting Information).

CAR appears to cross-talk with other two members of subfamily NR1I PXR (NR1I2) and VDR (NR1I1) because these receptors recognize similar response elements, coactivators, and corepressors and share a number of similar target genes involved in xenobiotic detoxification.[31] CAR, PXR, and VDR mainly control inducible expression of such major xenobiotic-metabolizing enzymes of cytochrome P450 as CYP3A4, CYP2C9, CYP2B6, or P-glycoprotein efflux transporter in the hepatocytes or in enterocytes.[32]

Aryl hydrocarbon receptor (AHR) is another key ligand-activated "xenosensor", although it belongs to the basic helix−loop−helix/Per-Sim-ARNT (bHLH/PAS) family of transcription factors. It controls another set of genes involved in the metabolism and transport of xenobiotics including CYP1A1, CYP1A2, CYP1B1, GST1, UGT1A1, UGT1A6, NQO1, ALDH3A1, and BCRP transporter.[33,34] The AHR ligands comprise a wide variety of known toxic and highly hydrophobic environmental contaminants such as halogenated aromatic hydrocarbons including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), polychlorinated biphenyls (PCBs), and the polycyclic aromatic hydrocarbons (PAHs) benzopyrene and 3-methylcholanthrene (MC).[35] Many AHR agonists represent planar aromatic chemicals; however, the SAR analysis showed that absolute planarity is not necessary for ligand binding of polychlorinated biphenyls. It was estimated that an AhR ligand are hydrophobic, electronegative compounds with hydrogenbonding properties between 12−14 Å in length, less than 12 Å in width, and no more than 5 Å deep.[35]

Because of enormous substrate ligand variability of xenosensors PXR, CAR, and AHR, the receptors are sometimes supposed not to strictly discriminate between molecules on the basis of size or chemical structure. The only one 3D QSAR model has been published for human CAR.[24] Considering our tested compounds structural features, we can see that methylation of oxygen or sulfur in position 4 of the quinazoline ring significantly increases activation of CAR (Figure 1A). On the contrary, alkylation of quinazoline 3 employing longer-chain alkyl halides (**b**−**f**) abolish activity toward CAR activation. This correlates with our docking experiment showing reasonable positioning of compounds 7 and 3a in CAR LBD (Figure 2, Supporting Information).

## ■ CONCLUSION

In summary, we have shown that compounds 7, 3a, and 7a are potent ligands of human CAR receptor. We also observed in the study that compounds 7, 3a, and 7a are at the same time highly potent ligands of the xenobiotic receptors PXR and AHR. We can therefore suppose that the compounds boost overall xenobiotic metabolism via induction of their target genes. To our knowledge, this is the first group of such xenobiotic metabolism "boosters" and pan-xenobiotic receptor agonists with potential application as antidotes to toxic compounds dominantly cleared from the body through metabolism. We assume that such antidotes could be seriously considered for treatment of intoxication by some natural compounds (such as mycotoxins) or synthetic toxic compounds, or, in cases of drug-induced liver injury (DILI),[36] under the assumption that the toxicants are mainly eliminated by biotransformation and are not significantly bioactivated to reactive intermediates by the xenobiotic detoxification system. Second, the new class of metabolism activators might be considered in some cases for controlled and stimulated activation of prodrugs.

Additional experiments should further characterize the toxicological properties of the compounds and consider their applications as antidotes in several intoxications or as prodrug activators. In addition, the compound could help us to more precisely characterize the limits of xenobiotic metabolism in different tissues or in the organism as a whole. We are currently synthesizing further set of quinazoline derivatives with a view to identify either more potent human CAR ligands or more selective human CAR agonists.

## ■ EXPERIMENTAL SECTION

**Biology.** *Activation of Human CAR in Transient Transfection Reporter Gene Assays (Figure 1).* HepG2 (A,B) or COS-1 (C) cells were transiently transfected with either p(ER6)$_3$-luc (A), CYP3A4-luc (B), or CYP2B6-luc (C) luciferase gene reporter constructs together



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with wild-type CAR expression vector (A,B) or its low-constitutive activity variant CAR3 (C) and pRL-TK control plasmid for transfection normalization. After 24 h of stabilization, the cells were treated with the tested compounds 7, 3a, and 7a, CITCO, and artemisinin (CAR agonists), clotrimazole (an inverse agonist) in indicated concentrations or with vehicle (DMSO; 0.1%, v/v) for an additional 48 h (A) or 24 h (B,C). HepG2 cells transfected with CYP3A4-luc and wild-type CAR have been in parallel treated with androstenol (5 $\mu$M) to suppress high basal activity (Figure 1B). In experiments with CAR3, RXR$\alpha$ has been cotransfected into COS-1 cells (100 ng/well). After treatment, the cells were lysed and assayed for both firefly and *Renilla* luciferase activities. The data are presented as the means ± SD from triplicate measurements of a representative experiment (A) or as means of three independent experiments ($n$ = 3). The results are expressed as the fold-change in inducing firefly luciferase activities relative to vehicle-treated cells (normalized to 1) *$p$ < 0.05, statistically significant. (D) Compounds 7, 3a, and 7a activate interaction of CAR ligand binding domain with SRC-1 coactivator in a two-hybrid assay. HepG2 cells have been transfected with GAL4-CAR LBD construct together with the VP16-SRC1 construct and the pGL5-luc reporter vector. After 24 h of stabilization, cells were treated with tested compounds, CITCO, and clotrimazole (known ligands of CAR, 10 $\mu$M) or vehicle (0.1%, control) for 24 h. Cells were lysed and analyzed for firefly and *Renilla* luciferase activities. The results are expressed as the fold-change in inducing firefly luciferase activities relative to vehicle-treated cells (normalized to 1). (E) Effect of compounds 7, 3a, and 7a on CAR activation in the TR-FRET LanthaScreen CAR coactivator assay. Compounds were tested in a serial dilution together with the prototype CAR agonist CITCO. Data are presented as the relative activation to background activity (no CAR LBD in the reaction mixture, set to 0%) and to the effect of CITCO (1 $\mu$M) set as 100% activation. The dotted line represents the constitutive activity of CAR LBD (vehicle-treated samples). Data are presented as the means and SD from three independent experiments ($n$ = 3). $EC_{50}$ indicates the xenobiotic concentration required to achieve half-maximum activation and relative $I_{max}$ represents the overall maximal calculated activation produced by the tested compound (i.e., maximal efficacy).

*Activation of PXR, AHR, and VDR Receptors in Transient Transfection Reporter Gene Assays (Figure 2).* HepG2 (A,B) or Huh-7 (C) cells were transiently transfected with either p3A4-luc (A), pXRE-luc (B), or $pDR_3$-luc (C) luciferase gene reporter constructs together with appropriate expression vectors pSG5-PXR (A) or pSG5-VDR (C) and pRL-TK control plasmid for transfection normalization. After 24 h of stabilization, the cells were treated with the tested compounds 7, 3a, and 7a, 3-methylcholantrene (an AHR ligand), rifampicin (a PXR ligand), and 1$\alpha$,25-dihydroxyvitamin $D_3$ (1,25OHvit D3, a VDR ligand) at the concentration range indicated for an additional 24 h. After treatment, the cells were lysed and assayed for both firefly and *Renilla* luciferase activities. The results are expressed as the relative change in induction of firefly luciferase activities versus vehicle-treated cells (normalized to 1) when activation with maximal tested concentration of 3-methylcholantrene (10 $\mu$M), rifampicin (25 $\mu$M), and 1,25OHvitD3 (100 nM) was set to 100%. The data are presented as the relative means ± SD from triplicate measurements of three independent experiments ($n$ = 3). $EC_{50}$ (xenobiotic concentration required to achieve half-maximum promoter activation) and relative $I_{max}$ (representing the overall maximal calculated induction produced by the tested compound, i.e., maximal efficacy) values were determined using GraphPad Prism Software.

*Induction of Target Genes mRNA Expression in CAR-Expressing HepG2 Cells, HepaRG Cells, or in Primary Human Hepatocytes (Figure 3).* (A) HepG2 cells were transfected with CAR expression plasmid and 24 h later treated with the tested compounds 7, 3a, and 7a (1, 5, or 10 $\mu$M) or CITCO (1 $\mu$M) or vehicle (DMSO; 0.1%, v/v) for 24 h. (B) Three primary human hepatocyte preparation have been treated with tested compounds 7, 3a, and 7a (10 $\mu$M) or CITCO (10 $\mu$M) or vehicle (DMSO; 0.1%, v/v) for 24 h. (C) Primary human hepatocytes (batch no. 2220900, Biopredic) or HepaRG cells (for CYP1A1 and CYP1B1) have been treated with 3-methylcholanthrene (3-MC, an AHR prototype ligand, 10 $\mu$M), rifampicin (rif, a prototype PXR ligand, 10 $\mu$M), CITCO (CAR ligand, 10 $\mu$M), and tested compounds 7, 3a, and 7a (10 $\mu$M). Total RNA was isolated and CYP2B6 (the gene dominantly controlled by CAR) or CYP1A1, CYP1A2, CYP1B1, CYP2C9, CYP3A4, CYP3A5, MDR1/ABCB1, and UGT1A1 genes mRNAs were assessed via qRT-PCR. The data in Figure 3A are presented as the means ± SD from three experiments ($n$ = 3) performed in triplicates and are expressed as the fold-change in CYP2B6 mRNA induction relative to vehicle-treated cells (normalized to 1). In Figure 3B,C, experiments were performed with three samples for each treatment. The values were normalized to HPRT mRNA as a reference gene. *$p$ < 0.05 indicates statistically significant difference from vehicle-treated cells transfected with appropriate reporter construct (ANOVA with Dunnett's post hoc test).

*Inhibition of Recombinant Human CYP3A4, CYP2B6, CYP1A2, CYP2C9, and CYP2D6 Enzymes with Compounds 7, 3a, and 7a (Figure 4).* Assays with recombinant human enzymes CYP3A4, CYP2B6, CYP1A2, CYP2C9, and CYP2D6 have been performed according to manufacturer's protocols (Promega). $IC_{50}$ (xenobiotic concentration required to achieve half-maximum enzyme inhibition) was determined using GraphPad Prism Software fitting for each compound.

**Transient Transfection and Luciferase Reporter Gene Assays.** All transient transfection assays were carried out using TransFectin transfection reagent purchased from Bio-Rad (Hercules, CA, USA) in HepG2, HuH7, or COS-1 cells, as described previously.[37] DNA constructs are described in the Supporting Information. Briefly, cells were seeded into 48-well plates and transfected with a luciferase reporter construct (150 ng/well), an expression plasmid (100 ng/well), and the *Renilla reniformis* luciferase transfection control plasmid (pRL-TK) (30 ng/well) 24 h later. In the case of the mammalian two-hybrid assay, HepG2 cells have been transfected with GAL4-CAR LBD (100 ng/well), VP-16-SRC-1 (100 ng/well), and pGL5-luc (150 ng/well) constructs in 48-well plate format. Cells were maintained in a phenol-red-free medium (220 $\mu$L) supplemented with 10% charcoal/dextran-stripped FBS and treated with the tested compounds (at indicated concentrations in a range from 0.1 up to 30 $\mu$M) or reference compounds CITCO, artemisinin,[38] and clotrimazole (CAR ligands and agonists, 10 $\mu$M), 1,25OHvit$D_3$ (10 nM, a VDR receptor ligand), rifampicin (10 $\mu$M, PXR ligand), and 3-methylcholantrene (10 $\mu$M, an AHR ligand) for 24 h. Reference compound have been purchased from Sigma-Aldrich. Luminescence activity in the cell lysate was measured using a commercially available luciferase detection system (Dual Luciferase Reporter Assay Kit, Promega). The data are expressed as the fold-change of firefly luciferase activity normalized to *Renilla* luciferase activity in each sample and relative to the vehicle (DMSO 0.1%)-treated controls, which were set equal to 1. Activity of a reference agonist at the maximum tested concentration has been set equal to 100% activation.

**Primary Cultures of Human Hepatocytes and HepaRG Cells.** Preparation of primary human hepatocytes and information about donors is described in the Supporting Information. HepaRG cells were cultivated as we described recently.[39]

**TR-FRET Constitutive Androstane Receptor (CAR) Coactivator Assay.** LanthaScreen time-resolved (TR)-FRET constitutive androstane receptor (CAR) coactivator assay was performed according to the recently published protocol.[72] Data are presented as means and SD from three independent experiments, and the curve fitting was performed using a sigmoidal dose response (variable slope) algorithm in GraphPad software version 6. Activity of CITCO at the maximum tested concentration has been set equal to 100% activation.

**qRT-PCR Analysis.** Total RNA was isolated from HepG2 or HepaRG cells, and primary human hepatocytes were treated with the tested compounds or with CITCO (1 $\mu$M), rifampicin (10 $\mu$M), and 3-MC (10 $\mu$M) for 24 h. qRT-PCR expression analyses were performed as previously described.[37] In expression experiments with CAR-expressing HepG2 cells, $2 \times 10^5$ cells were seeded into 12-well plates and transiently transfected with the pCR3-hCAR expression construct. After 24 h of stabilization, the cells were treated with the



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tested compounds for 24 h in the indicated concentrations. The data are presented as the fold-changes in gene expression relative to the vehicle-treated control (DMSO 0.1%) samples (set equal to 1). Statistical analyses were performed using the means of at least three independent experiments ($n = 3$) performed in triplicate.

**Chemistry.** *General.* The structural identities of the prepared compounds were confirmed by $^1$H NMR and $^{13}$C NMR spectroscopy (Varian Mercury-Vx BB 300 and Varian VNMR S500 spectrometers). Each of the reported compounds had ≥95% purity, as determined by combustion analysis (CHNS-OCE FISONS EA1110CE automatic microanalyzer instrument).

*Experimental Procedure for the Preparation of Compound 3.* A mixture of 3-methoxybenzoic acid **4** (9.700 g, 63.8 mmol) and thionyl chloride (30 mL) was heated under reflux for 3 h. The resultant carboxylic acid chloride **5** was purified by distillation with 95% yield and immediately used for the next reaction step.

Chloride **5** (7.165 g, 42.0 mmol) was slowly added to a cooled (0 °C) solution of 2-aminobenzonitrile (4.962 g, 42.0 mmol) and dry pyridine (17 mL). The reaction mixture was stirred at 0 °C for 4 h, then pyridine was removed under reduced pressure and crude product was recrystallized from an ethanol−water (2:1) mixture with quantitative yield.

A mixture of the resultant amide **6** (7.291 g, 28.9 mmol), sodium hydroxide (32 g), and aqueous hydrogen peroxide solution (30%, 48 mL) in water (200 mL) was heated at 100 °C for 1 h. After cooling to room temperature, the reaction mixture was acidified with concentrated aqueous hydrogen chloride solution (36%) to pH ≅ 4. The product crystallized out of the solution, after which it was filtered, washed with water, and dried to afford 2-(3-methoxyphenyl)-3,4-dihydroquinazolin-4-one (**3**) in 58% yield. White crystalline compound, mp 214 °C, mp lit.[40] 209−210 °C. $^1$H NMR: (300 MHz, DMSO-$d_6$) $\delta$ 12.52 (1H, bs, OH), 8.14−8.18 (1H, m, Ar), 7.85−7.71 (4H, m, Ar), 7.54−7.21 (2H, m, Ar), 7.15−7.11 (1H, m, Ar), 3.83 (3H, s, $OCH_3$). $^{13}$C NMR: (75 MHz, DMSO-$d_6$) $\delta$ 162.5, 159.5, 152.3, 148.8, 134.8, 134.2, 129.9, 127.7, 126.8, 126.1, 121.2, 120.3, 117.8, 112.7, 55.6. IR: 1047, 1148, 1223, 1251, 1267, 1286, 1310, 1445, 1471, 1482, 1586, 1612, 1670, 3038 cm$^{-1}$. LRMS (APCI): $m/z$ (relative intensity) 253.6 $[M + H]^+$ (100), 130.6 (3), 88.6 (1.5), 87.6 (6), 73.6 (8). Anal. Calcd for $C_{15}H_{12}N_2O_2$: C, 71.42; H, 4.79; N, 11.10. Found: C, 71.38; H, 5.00; N, 11.21.

*General Experimental Procedure for the Preparation of Compounds 3a−f.* A mixture of 2-(3-methoxyphenyl)-3,4-dihydroquinazolin-4-one (**3**, 0.5 mmol), sodium iodide (0.05 mmol), potassium carbonate (2.5 mmol), and an appropriate alkylating agent (2.5 mmol) in acetone (5 mL) was heated under reflux for 24 h. The resultant mixture was diluted with ethyl acetate (10 mL), washed with brine (10 mL), and the organic phase dried with sodium sulfate. Crude products were purified by column chromatography (hexane−ethyl acetate 9:1).

*4-Methoxy-2-(3-methoxyphenyl)quinazoline (3a).* Yield: 85%. Yellowish crystalline compound, mp 92−94 °C. $^1$H NMR: (500 MHz, $CDCl_3$) $\delta$ 8.22−8.15 (3H, m, Ar), 8.03−7.99 (1H, m, Ar), 7.86−7.8 (1H, m, Ar), 7.55−7.41 (2H, m, Ar), 7.08−7.03 (1H, m, Ar), 4.30 (3H, s, $OCH_3$), 3.96 (3H, s, $OCH_3$). $^{13}$C NMR: (125 MHz, $CDCl_3$) $\delta$ 167.0, 159.8, 159.8, 151.8, 139.7, 133.4, 129.4, 128.0, 126.4, 123.4, 121.1, 116.6, 115.3, 113.4, 55.4, 54.0. IR: 1047, 1108, 1184, 1195, 1282, 1327, 1355, 1380, 1443, 1453, 1509, 1561, 1574, 1620, 2966 cm$^{-1}$. LRMS (APCI): $m/z$ (relative intensity) 267.6 $[M + H]^+$ (100), 252.6 (75), 224.6 (7), 223.6 (25), 148.6 (7). Anal. Calcd for $C_{16}H_{14}N_2O_2$: C, 72.17; H, 5.30; N, 10.52. Found: C, 72.26; H, 5.37; N, 10.43.

*4-Ethoxy-2-(3-methoxyphenyl)quinazoline (3b).* Yield: 91%. Yellowish crystalline compound, mp 93 °C, mp lit.[41] not mentioned. $^1$H NMR: (300 MHz, $CDCl_3$) $\delta$ 8.24−8.14 (3H, m, Ar), 8.02−7.97 (1H, m, Ar), 7.84−7.77 (1H, m, Ar), 7.5−7.41 (2H, m, Ar), 7.08−7.03 (1H, m, Ar), 4.8−4.72 (2H, q, $J$ = 7.2 Hz, $OCH_2$), 3.94 (3H, s, $OCH_3$), 1.56−1.51 (3H, t, $J$ = 7.2 Hz, CH). $^{13}$C NMR: (75 MHz, $CDCl_3$) $\delta$ 166.5, 159.7, 159.7, 151.6, 139.6, 133.2, 129.3, 127.8, 126.2, 123.4, 120.9, 116.4, 115.3, 113.3, 62.7, 55.3, 14.3. IR: 1023, 1050, 1105, 1161, 1282, 1322, 1346, 1356, 1380, 1426, 1458, 1507, 1561, 1575, 2989 cm$^{-1}$. LRMS (APCI): $m/z$ (relative intensity) 281.5 $[M + H]^+$ (24), 255.5 (2), 254.4 (13), 253.6 (100), 238.5 (4), 210.5 (6), 91.5 (5). Anal. Calcd for $C_{17}H_{16}N_2O_2$: C, 72.84; H, 5.75; N, 9.99. Found: C, 72.81; H, 5.64; N, 10.08.

*2-(3-Methoxyphenyl)-4-propoxyquinazoline (3c).* Yield: 88%. Yellowish amorphous compound. $^1$H NMR: (300 MHz, $CDCl_3$) $\delta$ 8.24−8.17 (3H, m, Ar), 8.02−7.98 (1H, m, Ar), 7.83−7.78 (1H, m, Ar), 7.53−7.41 (2H, m, Ar), 7.07−7.03 (1H, m, Ar), 4.65 (2H, t, $J$ = 7.0 Hz, $OCH_2$), 3.93 (3H, s, $OCH_3$), 2.06−1.93 (2H, m, $CH_2$), 1.15 (3H, t, $J$ = 7.0 Hz, $CH_3$). $^{13}$C NMR: (75 MHz, $CDCl_3$) $\delta$ 166.7, 159.7, 151.7, 139.6, 133.3, 129.3, 127.8, 126.2, 123.4, 121.0, 116.4, 115.3, 113.3, 68.3, 55.3, 22.1, 10.6. IR: 1046, 1105, 1162, 1252, 1279, 1346, 1363, 1421, 1499, 1561, 1575, 1965 cm$^{-1}$. LMRS (APCI): $m/z$ (relative intensity) 295.6 $[M + H]^+$ (16), 254.4 (12.5), 253.6 (100), 238.6 (3), 210.6 (3). Anal. Calcd for $C_{18}H_{18}N_2O_2$: C, 73.45; H, 6.16; N, 9.52. Found: C, 73.12; H, 6.00; N, 9.31.

*4-Isobutyloxy-2-(3-methoxyphenyl)quinazoline (3d).* Yield: 78%. Yellowish amorphous compound. $^1$H NMR: (300 MHz, $CDCl_3$) $\delta$ 8.23−8.15 (3H, m, Ar), 8.01−7.97 (1H, m, Ar), 7.82−7.77 (1H, m, Ar), 7.52−7.41 (2H, m, Ar), 7.07−7.03 (1H, m, Ar), 4.49 (2H, d, $J$ = 6.8 Hz, $OCH_2$), 3.95 (3H, s, $OCH_3$), 2.35−2.22 (1H, m, CH), 1.14 (6H, d, $J$ = 6.8 Hz, $CH_3$). $^{13}$C NMR: (75 MHz, $CDCl_3$) $\delta$ 166.7, 159.8, 159.7, 151.7, 139.7, 133.3, 129.3, 127.9, 126.2, 123.4, 121.0, 116.4, 115.4, 113.3, 72.8, 55.3, 27.9, 19.3. IR: 1047, 1107, 1162, 1253, 1280, 1344, 1385, 1421, 1461, 1499, 1561, 1575, 1620, 2959 cm$^{-1}$. LRMS (APCI): $m/z$ (relative intensity) 309.6 $[M + H]^+$ (7.5), 255.5 (2), 254.4 (16), 253.6 (100), 238.6 (2.5), 210.6 (3). Anal. Calcd for $C_{19}H_{20}N_2O_2$: C, 74.00; H, 6.54; N, 9.08. Found: C, 73.87; H, 6.44; N, 9.21.

*4-Allyloxy-2-(3-methoxyphenyl)quinazoline (3e).* Yield: 58%. Yellowish crystalline compound, mp 50−52 °C. $^1$H NMR: (300 MHz, $CDCl_3$) $\delta$ 8.22 (3H, m, Ar), 8.02−7.98 (1H, m, Ar), 7.82−7.77 (1H, m, Ar), 7.52−7.4 (2H, m, Ar), 7.07−7.03 (1H, m, Ar), 6.31−6.18 (1H, m, CH), 5.56 (1H, d, $J$ = 17.2 Hz, $=CH_2$ A), 5.37 (1H, d, $J$ = 10.4 Hz, $=CH_2$ B), 5.21 (2H, d, $J$ = 5.5 Hz, $OCH_2$), 3.95 (3H, s, $OCH_3$). $^{13}$C NMR: (75 MHz, $CDCl_3$) $\delta$ 166.1, 159.7, 159.5, 151.7, 139.5, 133.4, 132.6, 129.3, 127.9, 126.3, 123.4, 120.9, 118.3, 116.5, 115.2, 113.3, 67.3, 55.3. IR: 1001, 1052, 1109, 1217, 1280, 1317, 1339, 1355, 1408, 1457, 1500, 1561, 1574, 1619, 2939 cm$^{-1}$. LRMS (APCI): $m/z$ (relative intensity) 293.6 $[M + H]^+$ (100), 291.7 (2.5), 265.6 (3), 264,6 (11), 253.6 (50), 250.7 (9), 210.6 (2). Anal. Calcd for $C_{18}H_{16}N_2O_2$: C, 73.95; H, 5.52; N, 9.58. Found: C, 74.16; H, 5.65; N, 9.42.

*4-Benzyloxy-2-(3-methoxyphenyl)quinazoline (3f).* Yield: 78%. White crystalline compound, mp 109−111 °C. $^1$H NMR: (300 MHz, $CDCl_3$) $\delta$ 8.32−8.17 (3H, m, Ar), 8.07−8.03 (1H, m, Ar), 7.84−7.78 (1H, m, Ar), 7.64−7.6 (2H, m, Ar), 7.52−7.37 (5H, m, Ar), 7.13−7.08 (1H, m, Ar), 5.76 (2H, s, $OCH_2$), 3.97 (3H, s, $OCH_3$). $^{13}$C NMR: (75 MHz, $CDCl_3$) $\delta$ 166.2, 159.7, 159.5, 151.7, 139.4, 136.4, 133.3, 129.3, 128.4, 128.1, 128.0, 127.8, 126.3, 123.3, 120.9, 116.5, 115.1, 113.2, 68.2, 55.2. IR: 1041, 1091, 1222, 1255, 1280, 1331, 1342, 1356, 1416, 1459, 1500, 1563, 1575, 1600, 1619, 2940 cm$^{-1}$. LRMS (APCI): $m/z$ (relative intensity) 343.6 $[M + H]^+$ (65), 253.5 (1), 92.5 (7.5), 91.5 (100). Anal. Calcd for $C_{22}H_{18}N_2O_2$: C, 77.17; H, 5.30; N, 8.18. Found: C, 77.01; H, 5.32; N, 8.34.

*Experimental Procedure for the Preparation of Compound 7.* A mixture of 2-(3-methoxyphenyl)-3,4-dihydroquinazolin-4-one (**3**, 1.000 g, 4.0 mmol) and phosphorus pentasulfide (0.880 g, 4.0 mmol) in toluene (10 mL) was heated under reflux for 4 h. The reaction mixture was poured onto ice and washed with chloroform (3 × 20 mL). Collected organic layers were dried with sodium sulfate. Crude product was purified by column chromatography (hexane−ethyl acetate 1:1) to afford thiol **7** in 83% yield. Yellow crystalline compound, mp 222−225 °C, mp lit.[42] not mentioned. $^1$H NMR: (500 MHz, DMSO-$d_6$) $\delta$ 13.84 (1H, bs, SH), 8.64−8.57 (1H, m, Ar), 7.92−7.86 (1H, m, Ar), 7.8−7.63 (3H, m, Ar), 7.6−7.55 (1H, m, Ar), 7.48−7.43 (1H, m, Ar), 7.16−7.12 (1H, m, Ar), 3.87 (3H, s, $OCH_3$). $^{13}$C NMR: (125 MHz, DMSO-$d_6$) $\delta$ 187.9, 159.4, 151.4, 144.5, 135.6, 133.5, 129.8, 129.5, 128.5, 128.2, 127.8, 121.0, 118.0, 113.3, 55.6. IR: 1029, 1040, 1154, 1189, 1222, 1252, 1263, 1339, 1492, 1508, 1570,



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J. Med. Chem. 2016, 59, 4601−4610

2985 cm$^{-1}$. LRMS (APCI): $m/z$ (relative intensity) 269.9 [M + H]$^+$ (100), 253.6 (9), 235.5 (19), 226.5 (6), 210.6 (3); Anal. Calcd for $C_{15}H_{12}N_2OS$: C, 67.14; H, 4.51; N, 10.44; S, 11.95. Found: C, 67.01; H, 4.42; N, 10.28; S, 11.90.

*Experimental Procedure for the Preparation of Compound 7a.* A mixture of 2-(3-methoxyphenyl)-3,4-dihydroquinazolin-4-thione (7, 0.5 mmol), potassium carbonate (2.5 mmol), and an methyl iodide (2.5 mmol) in acetone (5 mL) was heated under reflux for 24 h. The resultant mixture was diluted with ethyl acetate (10 mL), washed with brine (10 mL), and the organic phase dried with sodium sulfate. The crude product was purified by column chromatography (hexane−ethyl acetate 9:1) to afford 2-(3-methoxyphenyl)-4-methylsulfanylquinazoline (7a) in 79% yield. Yellowish crystalline compound, mp 89−91 °C. $^1$H NMR: (300 MHz, $CDCl_3$) $\delta$ 8.28−8.21 (2H, m, Ar), 8.11−8.01 (2H, m, Ar), 7.86−7.8 (1H, m, Ar), 7.56−7.41 (2H, m, Ar), 7.09−7.4 (1H, m, Ar), 3.95 (3H, s, $OCH_3$), 2.83 (3H, s, $SCH_3$). $^{13}$C NMR: (75 MHz, $CDCl_3$) $\delta$ 171.4, 159.8, 158.6, 148.6, 139.5, 133.6, 129.5, 129.0, 126.7, 123.7, 122.6, 121.1, 116.7, 113.4, 55.4, 12.6. IR: 1039, 1217, 1249, 1328, 1348, 1429, 1483, 1494, 1559, 1600, 2924 cm$^{-1}$. LMRS (APCI): $m/z$ (relative intensity) 283.6 [M + H]$^+$ (100), 268.5 (7), 250.6 (3.5), 236.5 (5.5), 235.6 (38), 192.6 (1). Anal. Calcd for $C_{16}H_{14}N_2OS$: C, 68.06; H, 5.00; N, 9.92; S, 11.35. Found: C, 68.31; H, 4.89; N, 10.11; S, 11.17.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.5b01891.

General experimental procedures, supplementary data, and copies of $^1$H and $^{13}$C NMR spectra of compounds 3, 3a−f, 7, 7a (PDF)

Molecular formula strings (CSV)

## ■ AUTHOR INFORMATION


### Corresponding Authors

*For M.Š.: phone, +420 495 067 348; fax, +420 495 067 166; E-mail, spulak@faf.cuni.cz.

*For P.P.: phone, +420 495 067 334; fax, +420 495 067 170; E-mail, petr.pavek@faf.cuni.cz.


### Notes

The authors declare no competing financial interest.

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## ■ ABBREVIATIONS USED

AhR, aryl hydrocarbon receptor; CAR, constitutive androstane receptor; CYP450, cytochrome P450; GR, glucocorticoid receptor; LBD, ligand binding domain; MRP2, multidrug resistance-associated protein; NR, nuclear receptor; PGC1$\alpha$, peroxisome proliferator-activated receptor gamma, coactivator 1$\alpha$ (PPARGC1A); PXR, pregnane X receptor; SLC, solute carrier family; SRC-1, steroid receptor coactivator 1 (NCOA1); TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; XME, xenobiotic-metabolizing enzyme

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## A.5. Unpublished observations: Leflunomide and its major metabolite teriflunomide are activators of human CAR (hCAR)

In this project, I discovered that DMARD leflunomide and its metabolite teriflunomide induce CYP2B6 mRNA expression, among other CAR target genes, in HepaRG cells and in primary human hepatocytes. CYP2B6 is the key CYP enzyme regulated by CAR (Figure 8a).

Therefore, I studied if leflunomide and teriflunomide are direct or indirect activators of human CAR. I found that the drugs inhibit EGFR, which leads to inhibition ofits downstream transcription factor Elk1. In addition, leflunomide and teriflunomide significantly did not interact with CAR ligand binding domain as I have shown in CAR TR-FRET assay (Figure 8b).These data and other data I am not presenting here suggest that leflunomide and teriflunomide are indirect activators of CAR and inducers of CAR target genes via unique indirect mechanism.

Thus, I found and describe the first indirect CAR activator after phenobarbital, currently used in pharmacotherapy. The discovery can help us understand the clinical consequences of CAR indirect activation in humans.

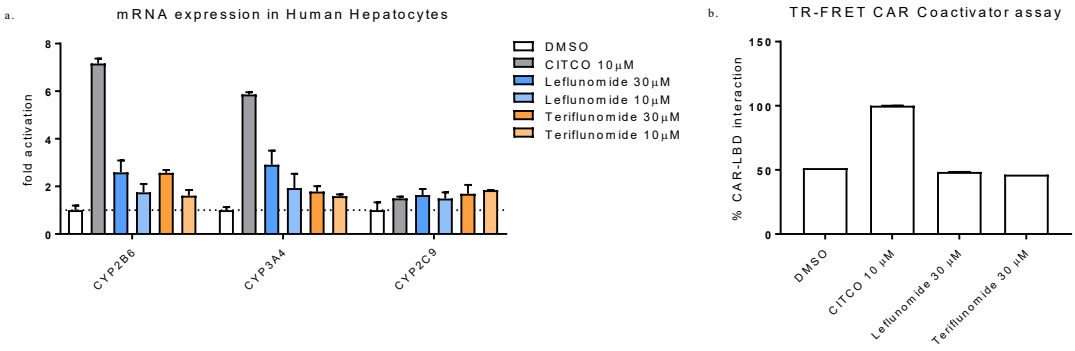


Figure 8. Leflunomide and teriflunomide activity on CAR target genes and CAR-LBD. Both DMARDs enhance the expression on CAR target genes (a), and do not directly bind to the CAR-LBD (b) pointing to an indirect activation mechanism of the receptor.

# 8. DISCUSSION

Important discoveries have been done and many outstanding studies have been reported in the last years in the field of nuclear receptors. Consequently, we have learned a lot about NRs since their first discovery (Hollenberg et al 1985). This superfamily of transcription factors have revealed as key components of the proper physiologic processes and this has raised them as attractive therapeutic targets.

A growing number of patients are being diagnosed with metabolic diseases, especially in the developed occidental world, due to changing ways of life (alimentation habits, smoking, alcohol, and decrease in exercise) (World Health Organization - www.who.int). This is intriguing due to the growing information and knowledge of the society with regards to correct dietary habits and the benefits of regular sport practice. NRs and namely CAR and PXR have been revealed as relevant factors, which can regulate and modify the homeostasis and metabolism of glucose, lipids, bile acids, hormones, etc. (Bitter et al 2015, Dong et al 2009b, Fiorucci et al 2010, Gao et al 2009, Hakkola et al 2016, Ma & Liu 2012).

In my thesis, I focused on improving the knowledge about CAR and PXR, and eventually, find new high-affinity ligands for these receptors. The case of CAR is quite special, since no safe, non-toxic ligand has been reported to date to activate the human orthologue of the receptor. For this aim, I employed well-established methods in molecular biology and introduced new methods such as CAR assembly assay or TR-FRET CAR coactivator assay, which improve sensibility and specificity in the study of CAR ligands. All evidence seems to point to a wider function of CAR than was originally believed. The metabolic implication of CAR is a fact, although its effect is not as extensive as for PXR. However, accumulating data from several research groups shows evidence in characterizing CAR as a key player in glucose and lipid metabolism. In the case of PXR, many drugs and xenobiotics have been identified as ligands. However, similarly to CAR, an endogenous high-affinity PXR ligand with clear physiological function is still enigma.

Great relevance has the cross-talk and overlapped functions of these nuclear factors (Konno et al 2008, Staudinger et al 2003, Willson & Kliewer 2002). Together, they form a complex network that keeps the correct functioning of physiological

processes and adaptation to the environment. As the activity of these receptors can be modified by drugs, the therapeutic potential of these targets is great and yet little exploited.

Animal models are a useful tool for the evaluation and testing of potential drugs targeting these receptors. However, species specificity is another barrier for the study of the physiological consequences of the regulation by these receptors. In both CAR and PXR receptors, rodent orthologues have different prototype ligands than their human variants. Therefore, only human cellular models and humanized mice models can be used for the study of cellular and *in vivo* effects of the human nuclear receptors. Thus, the development of animal models with incorporated humanized receptor helped to predict to some extent the *in vivo* outcomes after ligand treatment, but it should be kept in mind the species specificity differences.

In our first paper, we aimed to determine the mechanism of action of several flavonoids with human CAR receptor. Flavonoids are found in vegetables that are used as dietary complements and its use is increasing in the late decades. These flavonoids (galangin, baicalein, baicalin and chrysin), and several others, were reported to activate CAR (Yao et al 2010). At the same time, evidence showed that some of them interfere with EGFR receptor signaling (Gao et al 2013). However, in our laboratory, we found that these flavonoids did not activate CAR receptor directly. Therefore, we raised the question whether the reported activation of CAR could be exerted in a PB-like way through inhibition of EGFR receptor signaling (Mutoh et al 2013). Employing innovative CAR assembly assay, cell-free TR-FRET CAR assay and RT-PCR among other assays, we confirmed our initial suggestion that these flavonoids were indirect CAR activators (Carazo Fernandez et al 2015). Thus, we described for the first time a PB-like indirect CAR activation for other compounds than PB itself. These results show the complexity of the study of CAR and open the question of the exact mechanism of reported CAR activators. Due to the fact that CAR possesses extremely restricted LBD, it makes sense to think that many recently described CAR activators are in fact able to activate the receptor indirectly. In my paper, however, I indicate a clear methodology how to distinguish direct and indirect human CAR activators employing TR-FRET and CAR assembly assays.

In our next paper, we wanted to study in depth the TR-FRET CAR coactivator method. The TR-FRET assay was first used in 2001 (Navon et al 2001). In the study of

CAR, this method is extremely important since it eliminates confounding effects of cellular signaling on CAR activation. Thus, this is an ideal methodology to study direct CAR activation by ligands in nanomolar concentrations. In addition, we clearly describe in our paper that this method can be also used for identification of CAR agonists, partial agonists, antagonist or inverse agonist (Carazo Fernandez et al 2015). Finally, we showed for the first time that employing the method we can study interaction and competition of two ligands in CAR LBD.

It is well known that microflora from the intestine metabolizes some compounds and modifies them in order to facilitate absorption or their detoxification (Ridlon et al 2014). Bile acids secreted to the intestine aim to digest the fatty acids ingested with the diet and process them for absorption. Several nuclear receptors, are activated by endogenous bile acids and are nowadays believed to function as intestinal bile acid receptors, influencing their homeostasis and metabolism.

Our search for new endogenous ligands of nuclear receptors was not only focused on CAR. Knowing that PXR binds with high affinity bile acids (Staudinger et al 2001b), we aimed to study the activation of the receptor by potential derivates of parent bile acids. Bile acids are mainly metabolized through oxidation and acetylation processes in the liver or by bacterial enzymes of gut microflora (Staley et al 2017). Thus, we rationally developed several derivates or synthetized known products of intestinal microflora and studied their effects not only on PXR but also on other bile acid-binding nuclear receptors such as FXR and VDR. We reported that derivates 3,12-diacetyl DCA and 3,7,12-triacetate CA strongly activate PXR, but lack activity on the other two receptors. Strong PXR activation was showed in particular for 3,12-diacetate DCA, being able to trans-activate PXR target genes in primary human hepatocytes and showing great affinity for the PXR LBD. However, using HPLC/MS-MS techniques we were unable to report the presence of any of the derivates in human bile samples. Whether these two derivates exist in the human organism should be further studied, since their physiological concentration can be under the sensitivity of the method we used.

In my ongoing project, I discovered that leflunomide and its metabolite teriflunomide, compounds used for the treatment of rheumatoid arthritis, upregulate significantly CAR target gene CYP2B6 and induce CAR mRNA in HepaRG differentiated hepatic cells. Therefore, I investigated if the drugs are direct or indirect

activators of CAR. Employing the introduced methods from previous projects, I found that both leflunomide and teriflunomide do not bind directly to CAR LBD, but they interfere with EGFR signaling. Further experiments confirmed that these molecules may downregulate key enzymes in gluconeogenesis including PEPCK1 and G6Pase. Although these experiments are in progress, I can postulate that I discovered and described in detail first moderate affinity indirect CAR ligand used in clinical therapy.

In the light of the reports published in expert literature and taking into account our results and observations, we believe that CAR is a “second line” transcription factor that remains almost unnoticed under healthy circumstances. However, upon stress, imbalance or compromised physiological processes, CAR takes a step forward and gets involved in trying to reestablish the equilibrium and level the physiological state.

This field of study has greatly grown in the last years and decades. For instance, in the decade between 1980 and 1990, 14 000 papers were published regarding this research topic, whereas this number has grown decade to decade and from the year 2010 to this year, already 56 000 works have focused on this field. These data highlights the importance of these receptors, revealing nuclear receptors as important, yet quite unknown players in many physiological processes. Our results show solidly that nuclear receptors are an interesting growing field of study with potential therapeutic applications and I believe that my results will help to better understand the way the nuclear receptors function.

# 9. FUTURE STEPS

Nuclear receptors are a still growing field of research with promising applications. Recent works increased the interest in these receptors, revealing them as interesting therapeutic targets. These transcription factors play a role in multiple physiological processes and their modulation can influence the evolution of highly prevalent illnesses such as DM II, obesity, hyperlipidemia, cancer and other pathologies. For this purpose is key to clarify and precisely describe the mechanisms of action by which these receptors are activated or repressed and their posterior modulation of target genes.

Adopted orphan nuclear receptors, such as CAR and PXR, were initially described as xenobiotic sensors and metabolism regulators upregulating many cytochrome P450 enzymes, some phase I and phase II enzymes and several drug transporters. However, increasing evidence from the last decade points to wider functions and a complex mechanism of action, especially for the complicated receptor CAR. The close relationship between these receptors leads to overlapping regulatory functions and cross-talk with other nuclear receptors such as ER, FXR, GR, etc. This field of study has a vast potential of growth, since it is still unclear how these receptors specifically work in humans, in different tissues, under some pathological conditions or in different populations. This is mainly due to the lack of non-toxic ligands of human CAR or antagonists of PXR. In addition, discovery of true endogenous ligands of the receptors would help us to consider their physiological functions with therapeutic consequences.

Thanks to the great amount of research that has been done, our knowledge of the nuclear receptors has improved in the late years, but a great deal of work is yet ahead.

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# 11. CONFERENCES ATTENDED

## 11. 1. Oral presentations

Alejandro Carazo, Petr Pávek.
*„Leflunomide and its metabolite teriflunomide are human constitutive androstane receptor (CAR) that may influence glucose metabolism“*
**7. Postgradual & 5. Postdoctoral scientific conference, Pharmaceutical Faculty, Charles University.** February 7 – 8, 2017, Hradec Králové, Czech Republic.

Alejandro Carazo, Lucie Hyršová, Eva Kudová, Petr Pávek.
*„Interaction of keto and acetylated bile acids with PXR, FXR and VDR“.*
**6. Postgradual & 4. Postdoctoral scientific conference, Pharmaceutical Faculty, Charles University.** February 9 – 10, 2017, Hradec Králové, Czech Republic.

Alejandro Carazo, Tomáš Smutný, Lucie Hyrsová, Karel Berka, Petr Pávek.
„Chrysin, balcalein and galangin are indirect activators of the human constitutive androstane receptor (CAR).”
**5. Postgraduální & 3. Postdoctoral scientific conference, Pharmaceutical Faculty, Charles University.** February 3 – 4, 2017, Hradec Králové, Czech Republic.

## 11. 2. Poster presentations

Alejandro Carazo, Lucie Hyršová, Hana Chodounská, Karel Berka, Waleé Chamulitrat, Eva Kudová, Petr Pávek.
*„Interaction of keto and acetylated bile acids with PXR, FXR and VDR“.*
**66. Czechoslovak Pharmacological Days.** September 13 – 15, 2016, Brno, Czech Republic.
Alejandro Carazo, Tomáš Smutný, Lucie Hyršová Karel Berka, Petr Pávek.

„New methodological approaches for distinguishing direct and indirect Constitutive Androstane Receptor (CAR) activators".
**65. Czechoslovak Pharmacological Days . September** 16 – 18, 2015, Praha, Czech Republic.

Alejandro Carazo, Lucie Navratilová, Tomas Smutný, Karel Berka, Petr Pávek. "New methodological approaches for distinguishing direct and indirect Constitutive Androstane Receptor (CAR) activators".
**13th European Meeting of the International Society for the study of Xenobiotics**. June 22 – 25, 2015, Glasgow, United Kingdom.

Alejandro Carazo, Tomáš Soukup, Martin Doseděl, Jana Nekvindová, Petr Bradna, Aleš Antonín Kuběna, Tomas Veleta, Jiří Vlček, Petr Pávek. „Úloha polymorfismů C677T a A1298C methylentetrahydrofolát reduktázy (MTHFR) na účinnosti léčby methotrexátem u pacientů s revmatoidní artritidou - lze na základě farmakogenetiky predikovat účinnost *methotrexátu?"*
**XVI. Symposium of Clinical Pharmacy "René Macha".** November 21 – 22, 2014 Mikulov, Czech Republic.

### 11. 3. Conferences attended without presentation

Alejandro Carazo.
**XXI. Biological days.** September 4 – 5 2014, Brno, Czech Republic.

Alejandro Carazo.
**20th International Symposium on Microsomes and Drug Oxidations.** May 18 – 22 2014, Stuttgart, Germany.

# 12. ABBREVIATIONS

| | |
|---|---|
| Å | Ångström (unit of length) |
| ABCC2/MRP2 | Multidrug Resistance-Associated Protein 2 |
| AF-1 | Activation function 1 |
| AF-2 | Activation function 2 |
| AhR | Aryl hydrocarbon Receptor |
| AR | Androgen Receptor |
| ASC | Nuclear Receptor Coactivator-6 |
| BCRP/ ABCG2 | ATP-Binding Cassette G2 |
| CA | Cholic acid |
| CAR/ Car | Constitutive Androstane Receptor (human/ mice) |
| CCRP | Cytoplasmic CAR Retention Protein |
| CDCA | Chenodeoxycholic acid |
| CITCO | 6-(4-chlorophenyl)imidazo(2,1-b)(1,3)thiazole-5-carbaldehyde O-(3,4- dichlorobenzyl)oxime |
| CYP | Cytochrome P450 |
| DBD | DNA Binding Domain |
| DCA | Deoxycholic acid |
| DM II | Diabetes Mellitus type II |
| DM II | Diabetes Mellitus type II |
| DMARDs | Drug-Modifying Anti-rheumatic Drugs |
| DME | Drug Metabolizing Enzymes |
| DNA | Desoxyribobucleic acid |

| | |
|---|---|
| E2 | Estradiol |
| $EC_{50}$ | Effective Concentration 50 |
| EGF | Epidermal Growth Factor |
| EGFR | Epithelial growth factor receptor |
| ELISA | Enzyme-Linked ImmunoSorbent Assay |
| Elk1 | ETS domain-containing protein 1 |
| ERK | Extracellular Signal-Regulated Kinase |
| ET-743 | Ecteinascidin-743/ Trabectedin |
| FDA | Food and Drug Administration |
| FXR | Farnesoid X Receptor |
| G6P | Glucose 6 phosphatase |
| GR | Glucocorticoid Receptor |
| GRIP1 | Glutamate Receptor-Interacting Protein 1 |
| HDAC | Hystone Deacetylases |
| HFD | High Fat Diet |
| HNF4α | Hepatocyte Nuclear Factor 4 alpha |
| HPLC/MS-MS | High Performance Liquid Chromatography / Tandem Mass Spectrometry |
| Hsp90 | Heat Shock Protein 90 |
| IBD | Inflammatory Bowel Disease |
| Kbp | Kilo base pairs |
| LBD | Ligand Binding Domain |
| LCA | Litocholic acid |
| LXR | Liver X Receptor |
| MDR1/ P-gp | Multidrug Resistance Protein |

| | |
|---|---|
| miRNA | micro RNA |
| mRNA | messenger RNA |
| NCoR | Nuclear Receptor Co-repressor 1 |
| NF-κB | Nuclear Factor - κB |
| NR | Nuclear Receptor |
| NRNC | Nuclear Receptor Nomenclature Committee |
| NURR1 | Nuclear Receptor Related-1 Protein |
| NURSA | Nuclear Receptor Signaling Atlas |
| OATP | Organic Anion-Transporting Protein |
| OCA | Obeticholic acid |
| PB | Phenobarbital |
| PCN | Pregnenolone 16α-carbonitrile |
| PEPCK1 | Phosphoenolpyruvate carboxykinase 1 |
| PGC-1α | Peroxysome Proliferator-Activated Receptor Gamma Coactivator1α |
| PPAR | Peroxisome proliferator-activated receptor |
| PR | Progesterone Receptor |
| PXR/ Pxr | Pregnane X Receptor (human/ mice) |
| RAR | Retinoid Acid Receptor |
| RNA | Ribonucleic acid |
| RXR | Retinoid X Receptor |
| SHP | Small Heterodimer Partner |
| SMC-1 | Structural Maintenance Chromosome Protein 1 |
| SMRT | Nuclear Receptor Co-repressor 2 |
| SNP | Single Nucleotide Polymorphism |

| | |
|---|---|
| SRC-1 | Steroid Receptor Coactivator |
| SREBP-1 | Sterol Regulatory Element Binding Protein 1 |
| SULT | Sulfotransferase |
| SXR | Steroid X Receptor |
| T4 | Thyroxine |
| TCM | Traditional Chinese Medicine |
| TCPOBOP | 1,4-Bis-[2-(3,5-dichloropyridyloxy)]benzene,3,3′,5,5′-Tetrachloro-1,4-bis(pyridyloxy)benzene |
| TF | Transcription Factor |
| TIF | Transcriptional Intermediary Factor |
| TR | Thyroid hormone Receptor |
| TR-FRET | Time Resolved – Fluorescence Energy Transfer |
| UDCA | Ursodeoxycholic acid |
| UGT | UDP-Glucuronosyltransferase |
| VDR | Vitamin D Receptor |
| WHO | World Health Organization |

# NOTES

All figures and tables are of my authorship, except for figures 2. and 3., that were taken from https://en.wikipedia.org/wiki/Nuclear_receptor