PALACKÝ UNIVERSITY OLOMOUC

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ENANTIOSPECIFIC INTERACTIONS BETWEEN CLINICALLY USED CHIRAL DRUGS AND REGULATORY PATHWAYS OF HUMAN CYTOCHROME P450s

Ph.D. THESIS

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I hereby declare that the Ph.D. thesis is based on my own research carried out in the Department of Cell Biology and Genetics, Faculty of Science, Palacký University, Olomouc, from September 2013 to June 2017. The co-authors agree with the inclusion of published results. All literary sources cited in this work are listed in the 'References' section.

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Mgr. Martina Štěpánková

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Abstract

The pharmaceutical industry produces many chiral drugs with an emphasis on the development and introduction of more enantiopure drugs for pharmacotherapy. Numerous studies have revealed that individual enantiomers of a chiral drug can qualitatively and quantitatively differ in their biological activities, including their pharmacokinetics, pharmacodynamics, toxicokinetics, and toxicodynamics. For this reason, elimination of the inactive isomer in some cases can be advantageous, because it can reduce adverse effects of the drug. In other cases, however, the racemate is more beneficial. Therefore, research on enantiospecific interactions between individual optical isomers of chiral drugs and drug-metabolizing enzymes is crucial for an effective and safe treatment. I have studied single isomers of clinically used drugs prescribed for hypertension (benidipine [BENI], felodipine [FELO], and isradipine [ISRA]) and hypercholesterolaemia (atorvastatin [AT], fluvastatin [FLU], and rosuvastatin [ROS]) and their influence on the activity of cytochrome P450 (CYP) transcriptional regulators, including pregnane X receptor (PXR), aryl hydrocarbon receptor (AhR) and glucocorticoid receptor (GR) by the means of gene reporter assays and an electrophoretic mobility shift assay (EMSA). Moreover, effects of the tested stereoisomers on the expression of drug-metabolizing P450s were evaluated using Simple western blotting and quantitative PCR (qPCR) for quantification of proteins and mRNAs, respectively. Overall, I showed that AT, FLU and ROS enantiospecifically activate PXR and induce genes *CYP2A6, CYP2B6* and *CYP3A4*. The calcium channel blockers BENI, FELO and ISRA enantiospecifically activated AhR and induced genes *CYP1A1* and *CYP1A2*, while they non-enantiospecifically activated PXR and induced genes *CYP2A6, CYP2B6,* and *CYP3A4*. The data obtained may have toxicological and clinical implications.

Keywords	enantiopure drugs, cytochrome P450, xenoreceptors, statins, dihydropyridines
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Abstrakt

Farmaceutický průmysl produkuje velké množství chirálních léčiv s důrazem na rozvoj a využití enantio-čistých léčiv. Četné studie prokázaly, že se účinky jednotlivých enantiomerů kvalitativně i kvantitativně liší na farmakokinetické, farmakodynamické, toxikokinetické a toxikodynamické úrovni. Z tohoto důvodu bývá eliminace neaktivního isomeru v některých případech výhodná, jelikož se zmírní vedlejší účinky léčiva. V jiných případech je však pro požadovaný terapeutický účinek vhodnější racemát. Z toho důvodu hraje studium enantiospecifických interakcí mezi jednotlivými optickými izomery chirálních léčiv a enzymy metabolizující léčiva důležitou roli pro zajištění efektivní a bezpečné léčby. Z tohoto důvodu jsem studovala jednotlivé izomery klinicky používaných léčiv často předepisovaných pro léčbu hypertenze (benidipin [BENI], felodipin [FELO], and isradipin [ISRA]) a hypercholesterolémie (atorvastatin [AT], fluvastatin [FLU], and rosuvastatin [ROS]) a jejich vliv na aktivitu transkripčních regulátorů cytochromů P450 (CYP), konkrétně pregnanového X receptoru (PXR), aryl uhlovodíkového receptoru (AhR) a glukokortikoidního receptoru (GR), pomocí reportérových esejí a gelové retardační analýzy (EMSA). Dále byly hodnoceny účinky testovaných stereoizomerů na expresi CYP450 enzymů metabolizujících léčiva za použití Simple western blottingu a kvantitativní PCR (qPCR) na úrovni proteinů a mRNA. Celkově jsem došla k zajímavému zjištění, že AT, FLU and ROS enantiospecificky aktivují PXR a indukují geny *CYP2A6, CYP2B6* a *CYP3A4*. Dále BENI, FELO and ISRA enantiospecificky aktivují AhR a indukují geny *CYP1A1* a *CYP1A2* a zároveň ne-enantiospecificky aktivují PXR a indukují geny *CYP2A6, CYP2B6* a *CYP3A4*. Získaná data mohou mít toxikologický a klinický význam.

Klíčová slova	enantio-čistá léčiva, cytochrom P450, xenoreceptory, statiny, dihydropyridiny
Počet stran	109
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Jazyk	Angličtina

Contents

Abbreviations

AhR	Aryl hydrocarbon receptor
AhRR	Aryl hydrocarbon receptor repressor
AF	Activation function
AIP	Aryl hydrocarbon receptor-interacting protein
AP	Activation protein
ARNT	Aryl hydrocarbon receptor nuclear translocator
AT	Atorvastatin
AUC	Area under the curve
BCRP	Breast cancer resistance protein
BENI	Benidipine
bHLH-PAS	Basic helix-loop-helix-PER-ARNT-SIM
CAR	Constitutive androstane receptor
CCB	Calcium channel blocker
CITCO	6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehydeO-(3,4-dichlorobenzyl)oxime
COMT	Catechol O-methyl transferase
CYP	Cytochrome P450
DBD	DNA-binding domain
DDI	Drug-drug interaction
DEX	Dexamethasone
DMEM	Dulbecco’s modified Eagle’s medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DR	Direct repeat
DRE	Dioxin response element
EC_{50}	Half maximal effective concentration
ECACC	the European Collection of Authenticated Cell Cultures
EMSA	Electrophoretic mobility shift assay
ER	Everted repeat
FELO	Felodipine
FLU	Fluvastatin
FXR	Farnesoid X receptor

GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC	Glucocorticoid
GR	Glucocorticoid receptor
GRE	Glucocorticoid-responsive element
GST	Glutathione S-transferase
GTP	Guanosine triphosphate
HMG-CoA	3-Hydroxy-3-methylglutaryl coenzyme A
HNF	Hepatocyte nuclear factor
hsp	Heat-shock protein
IC_{50}	Half maximal inhibitory concentration
IR	Inverted repeat
ISRA	Isradipine
IUPAC	International Union of Pure and Applied Chemistry
LBD	Ligand-binding domain
LCA	Lithocholic acid
LXR	Liver X receptor
MDR	Multidrug resistance
M-MuLV	Moloney Murine Leukemia Virus
MRP	Multidrug resistance protein
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NADPH	Nicotinamide adenine dinucleotide phosphate
NAT	N-acetyltransferase
NR	Nuclear receptor
OATP	Organic anion transporting polypeptide
OCT	Organic cation transporter
OeR	Oestrogen receptor
PBREM	Phenobarbital-responsive enhancer module
PBS	Phosphate buffered saline
P-gp	P-glycoprotein
PP	Pyrophosphate
PPAR	Peroxisome proliferator-activated receptor
PXR	Pregnane X receptor
qPCR	Quantitative polymerase chain reaction
RAR	Retinoic acid receptor

RE	Response element
RIF	Rifampicin
RNA	Ribonucleic acid
ROS	Rosuvastatin
RXR	Retinoid X receptor
SULT	Sulphotransferase
TAT	Tyrosine aminotransferase
TCDD	2,3,7,8-Tetrachlorodibenzo-[p]-dioxin
TCPOBOP	1,4-bis[2-(3,5-dichloropyridyloxy)]benzene
TPMT	Thiopurine S-methyl transferase
TR	Thyroid hormone receptor
UDP	Uridine diphosphate
UGT	UDP-glucuronosyltransferase
UPL	Universal Probe Library
USF	Upstream stimulatory factor
VDR	Vitamin D receptor
XREM	Xenobiotic Responsive Enhancer Module
XME	Xenobiotic-metabolizing enzyme

1 INTRODUCTION

A specific spatial arrangement is required for many biochemical processes including binding of a ligand to its receptor and a substrate–enzyme interaction. Chirality is abundant in living systems; thus, proteins, amino acids, carbohydrates, nucleosides and other natural molecular entities are chiral (asymmetric). Metabolism of drugs and other xenobiotics could also be stereoselective because it is facilitated by many enzymes with potential three-dimensional (3D) substrate specificity including cytochrome P450s, flavin-containing monooxygenases, alcohol dehydrogenases, sulphotransferases, glutathione S-transferases, UDP-glucuronosyltransferases, and many others (Jakoby and Ziegler, 1990).

Cytochrome P450s (CYPs) play a crucial role in phase I of xenobiotic biotransformation. These iron-containing proteins catalyse the monooxygenase reaction involving various endogenous and exogenous substrates. CYP1, CYP2, and CYP3 family members metabolise mainly xenobiotics, and these genes are inducible by endogenous and foreign substances through activation of steroid receptors, nuclear receptors, and xenoreceptors. These receptors are ligand-activated transcription factors and include aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), pregnane X receptor (PXR), glucocorticoid receptor (GR), oestrogen receptor (OeR), vitamin D receptor (VDR), and retinoid X receptor (RXR) (Aranda and Pascual, 2001).

Many currently used drugs are chiral, being marketed as racemic mixtures of stereoisomers. They are enantiomers (non-superimposable mirror images) or diastereomers (not mirror images). In either case, stereoisomers can differ markedly from each other in properties such as bioactivity, pharmacokinetics, and toxicity. Therefore, the United States Food and Drug Administration requires the identification and characterisation of each individual component of racemic drugs (Brocks, 2006). There are numerous reports about chiral drugs with one being a more potent stereoisomer than the other. The examples are S-atenolol (Stoschitzky *et al.*, 1993), S-propranolol (Stoschitzky *et al.*, 1989), S-carvedilol (Bartsch *et al.*, 1990), S-metoprolol (Wahlund *et al.*, 1990), S-warfarin (Hewick and McEwen, 1973), S-omeprazole (Andersson *et al.*, 2001), R-methadone (Olsen *et al.*, 1977), R-lansoprazole (Katsuki *et al.*, 1996). As a result, pharmaceutical manufacturers tend to produce single-isomer (enantiopure)

drugs to achieve higher therapeutic efficacy and diminish adverse effects and toxicity of the drug. This concept led to the introduction of enantiopure drugs, which until then had been used as racemates. The examples are omeprazole–esomeprazole, citalopram–escitalopram, modafinil–armodafinil, and many other pairs. However, in some cases, the use of a racemate is more suitable than the use of a pure enantiomer because of the complementary effects of stereoisomers (Smith, 2009). Thus, it is worthwhile to explore enantiospecific interactions between individual optical isomers of chiral drugs and drug-metabolizing enzymes, to ensure effective and safe pharmacotherapy.

Monitoring of drug–drug interactions (DDIs) is essential to guarantee drug safety. DDIs are caused by multiple mechanisms, including activation or inhibition of major transcriptional regulators of xenobiotic-metabolizing CYPs, in particular, AhR and PXR. Consequently, induction or down-regulation of CYPs may occur. Indeed, it was recently demonstrated that several clinically used chiral drugs, including ketoconazole (Novotna *et al.*, 2014a; Novotna *et al.*, 2014b), warfarin (Rulcova *et al.*, 2010), omeprazole and lansoprazole (Novotna and Dvorak, 2014; Novotna *et al.*, 2014c) have enantiospecific effects on these xenoreceptors.

2 AIMS

The main aim of this work was to study the effects of individual optical isomers of chiral drugs on regulatory pathways of human CYPs. Well-known cholesterol-lowering drugs (statins) and calcium channel blockers (CCBs) of the dihydropyridine class were selected for this study. The specific goals were to investigate the effects of pure stereoisomers of CCBs and statins on:

a) Function and transcriptional activities of AhR, GR and PXR in human cancer cell lines and in primary human hepatocytes.

b) The expression and catalytic activities of major drug-metabolizing CYPs in human cancer cell lines and in primary cultures of human hepatocytes.

3 THEORETICAL BACKGROUND

3.1 Metabolism of xenobiotics

Xenobiotics such as drugs, environmental pollutants, and agrochemicals undergo biotransformation catalysed by phase I and phase II xenobiotic metabolizing enzymes (XMEs) and are subject to transport by phase III transporters (Xu *et al.*, 2005). The oxidation, conjugation, and transport of xenobiotics and eubiotics by XMEs are complicatedly regulated by multiple mechanisms, including the regulation of gene expression by ligand-activated transcription factors such as nuclear receptors, steroid receptors, and xenoreceptors (Omiecinski *et al.*, 2011).

3.1.1 Phase I XMEs

Phase I reactions facilitate transformation of hydrophobic xenobiotics into more polar metabolites by unmasking or *de novo* formation of functional groups, such as -OH, -NH_2, or -SH. These reactions include mainly N- and O-dealkylation, aliphatic and aromatic hydroxylation, N- and S-oxidation, and deamination.

The predominant phase I XMEs are heme-containing monooxygenases called cytochrome P450s (CYPs). They are anchored in the endoplasmic reticulum together with NADPH-cytochrome P450 reductase mainly in the liver and to a lesser extent in the kidneys, lungs, skin, brain, intestines, placenta, and other tissues (Nebert and Russell, 2002). CYPs belong to a large superfamily and are classified according to similarity of their amino acid sequences into 18 mammalian families (40% congruency; e.g. CYP1) and other subfamilies (55% congruency; e.g. CYP1A, CYP1B). Individual members of a family or a subfamily are labelled by Arabic numerals (e.g. CYP1A1, CYP1A2). To represent an individual member, a new P450 sequence should differ by more than 3% (Nelson *et al.*, 2004). CYP1, CYP2, and CYP3 families are responsible for the biotransformation of most foreign substances including 70–80% of all the drugs in clinical use, while other families are involved more in the biotransformation of endobiotic compounds, such as fatty acids, cholesterol, bile acids, prostaglandins, steroid hormones, and retinoids (Anzenbacher and Anzenbacherova, 2001; Monostory and Pascussi, 2008). Isoforms CYP1A1/2, CYP1B1, CYP2A6, CYP2B6, CYP2C8/9, CYP2C19

and CYP3A4/5 are inducible by xenobiotics (e.g. polycyclic aromatic hydrocarbons, aromatic amines, polychlorinated biphenyls, rifampicin, phenobarbital, or midazolam) and endogenous compounds (e.g. steroid hormones, retinoic acid, or vitamin D). Induction proceeds via activation of xenosensors (AhR, CAR, and PXR), steroid receptors (GR and OeR), and nuclear receptors (VDR and RXR). These receptors are ligand-activated transcription factors that are responsible for initiation of the *de novo* synthesis of CYPs (Aranda and Pascual, 2001; Monostory and Pascussi, 2008; Zanger and Schwab, 2013).

Expression of CYPs is also influenced by other factors including genetic polymorphisms, regulation by cytokines, hormones, and disease states as well as by sex, age, and other factors. Multi-allelic genetic polymorphisms, which strongly depend on ethnicity, play a key role in the function of some CYPs and lead to distinct pharmacogenetic phenotypes termed as *poor*, *intermediate*, *extensive*, and *ultrarapid metabolisers* (Zanger and Schwab, 2013). Clinically significant instances of this pharmacogenetic variation have been reported for CYP2C9 [warfarin (Takeuchi *et al.*, 2009)], CYP2C19 [clopidogrel (Hou *et al.*, 2014)], CYP2D6 [tamoxifen and codeine (Gryn *et al.*, 2014; Madadi *et al.*, 2013)], and CYP3A5 [tacrolimus (Rojas *et al.*, 2015)].

Other XMEs participating in phase I metabolism include flavin-containing monooxygenases (Krueger and Williams, 2005), amine oxidases (Benedetti, 2001), epoxide hydrolases (Fretland and Omiecinski, 2000), and alcohol and aldehyde dehydrogenases (Vasiliou *et al.*, 2000).

3.1.2 Phase II XMEs

Phase II enzymes have a significant function in the biotransformation of endogenous compounds and xenobiotics into more easily excretable compounds. Phase II XMEs are mostly transferases catalysing conjugation reactions. As a result, the conjugates are more hydrophilic than the parent compounds. The reactions include mostly glucuronidation by UDP-glucuronosyltransferases (UGTs), sulphation by sulphotransferases (SULTs), methylation mainly by thiopurine S-methyl transferase (TPMT) and catechol O-methyl transferase (COMT), acetylation by N-acetyltransferases (NATs), and glutathione conjugation by glutathione S-transferases (GSTs). Because drug

interactions relating to phase II enzymes are relatively rare, these enzymes have attracted much less attention in clinical pharmacology than CYPs have (Jancova *et al.*, 2010). However, gene polymorphisms or a lack of the genes encoding these enzymes may be involved in the development of severe disorders. For instance, deficiency of the UGT1A1 isoform can cause Crigler-Najjar syndrome or Gilbert syndrome, both associated with hyperbilirubinaemia (Kadakol *et al.*, 2000). Several studies have also described participation of a SULT1A1 polymorphism in the development of cancers, such as lung cancer (Arslan *et al.*, 2009), urothelial carcinoma (Huang *et al.*, 2009) and meningiomal brain tumours (Bardakci *et al.*, 2008).

3.1.3 Phase III transporters

Xenobiotic transporters influence plasma and tissue concentrations of a broad variety of xenobiotics. They can be classified into transporters mediating the uptake of xenobiotics into cells, such as organic-anion-transporting polypeptides (OATPs), and organic-cation transporters (OCTs), or transporters mediating the export of xenobiotics out of cells, such as breast cancer resistance protein (BCRP), P-glycoprotein (P-gp; also known as multidrug resistance protein 1: MDR1), and other multidrug resistance proteins (MRPs). Transporters are located in the small intestine, liver, and kidneys and are important determinants of drug disposition and DDIs (Muller and Fromm, 2011; Zhang *et al.*, 2011). Moreover, transporters expressed in blood–tissue barriers, such as the blood–brain barrier or placental barrier, protect sensitive tissues from potentially toxic compounds (Ghosh *et al.*, 2011; Vahakangas and Myllynen, 2009).

3.2 Cellular signalling by nuclear receptors (NRs)

NRs are ligand-activated transcription factors. The NR superfamily is thought to be evolutionarily derived from a common ancestor. Evolutionary analysis of the receptors has given rise to subdivision into six subfamilies containing nuclear hormone receptors with a known hormonal ligand and numerous orphan receptors without an identified endogenous ligand (Laudet, 1997). In humans, 48 NRs have been recognised. The first cloned human nuclear receptors were GR, together with OeR and thyroid hormone receptor (TR) (Hollenberg *et al.*, 1985; Walter *et al.*, 1985; Weinberger *et al.*, 1986).

Just as other transcriptional regulators, NRs share a characteristic protein architecture that consists of five to six domains of homology (A–F), in the N terminus to C terminus direction (Figure 1). The A/B region contains a ligand-independent transactivation domain called activation function 1 (AF-1). It is the most variable in both size and sequence; this property may cause differences in binding affinity for DNA or other members of the transcription initiation complex (Briancon and Weiss, 2006; Hollenberg *et al.*, 1996; Mascrez *et al.*, 2001). The DNA-binding domain (DBD) or C domain is the most conserved region of NRs. It can recognise and bind specific regulatory sites (in DNA) termed response elements (REs). This compact domain contains the P-box, a short motif responsible for direct interaction with DNA and DNA-binding specificity (Danielsen *et al.*, 1989; Smit-McBride and Privalsky, 1994). The D domain serves as a hinge between the DBD and ligand-binding domain (LBD), allowing for rotation of the DBD. Moreover, nuclear localisation sequences were found in this region that are responsible for regulation of the subcellular distribution of NRs (Shaffer *et al.*, 2005; Tanner *et al.*, 2010). The LBD or E domain is multifunctional. Besides the binding of a ligand, it mediates homodimerization or heterodimerization (see below) and the interaction with co-activators or co-repressors. LBD contains two well-conserved regions (a 'signature motif' and C-terminal AF-2 motif) responsible for ligand-dependent transcriptional activation (Wurtz *et al.*, 1996). The F domain is located at the C terminus of NR. Given the high variability in sequence (0–80 amino acid residues long), little is known about its functional role and structure (Kumar *et al.*, 1987).

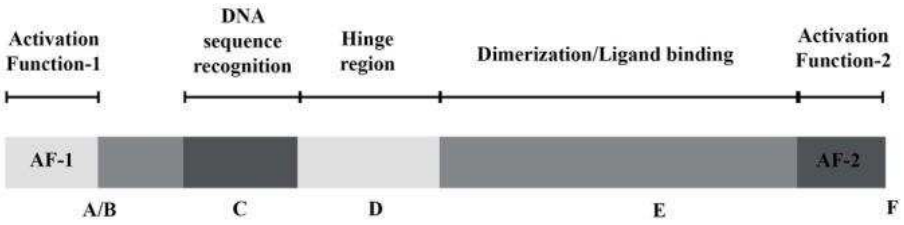

Figure 1: General structural organisation of nuclear receptors. Letters from A to F represent nuclear receptor domains from the N terminus to C terminus of a nuclear receptor (Pawlak *et al.*, 2012).

NRs regulate transcription by binding to the REs that consist of one or two hexameric half-site motifs located upstream of the transcription initiation site of the target gene. Although some monomeric NRs can bind to a single hexamer, most

NRs bind as homodimers or heterodimers to two hexameric half-site motifs. REs may form palindromes organised as direct (DR), everted (ER), or inverted repeats (IR), separated by a spacer of varied length and sequence (Aranda and Pascual, 2001). Receptors for steroid hormones bind to DNA as homodimers, i.e. two monomeric receptors bind cooperatively to a RE. Formation of stable homodimers depends on dimerization interfaces located in both the DBD and LBD. As an example of a homodimer, OeR recognises two consensus half-sites arranged as IRs separated by 3 bp (Klinge, 2001; Schwabe *et al.*, 1993). Other NRs such as PXR, retinoic acid receptor (RAR), VDR, or TR, form heterodimers with RXR and recognise REs arranged as DR, IR, or ER with the core consensus sequence 5′-AGGTCA-3′ (Kliewer *et al.*, 1992; Umesono *et al.*, 1991). Depending on the RXR partner, heterodimers can be permissive, non-permissive, and conditional (Figure 2). Permissive RXR heterodimers can be activated by ligands of either RXR or the partner. In the presence of both ligands, a synergistic response is achieved. Such heterodimers form RXR with peroxisome proliferator-activated receptor (PPAR), liver X receptor (LXR), or farnesoid X receptor (FXR). Non-permissive RXR heterodimers can be activated by the ligand of the partner only (e.g. TR). Conditional heterodimers are unresponsive to RXR agonists; however, these ligands overactivate transcription by synergizing with partner ligands (e.g. RAR, VDR) (Brtko and Dvorak, 2015; Pawlak *et al.*, 2012).

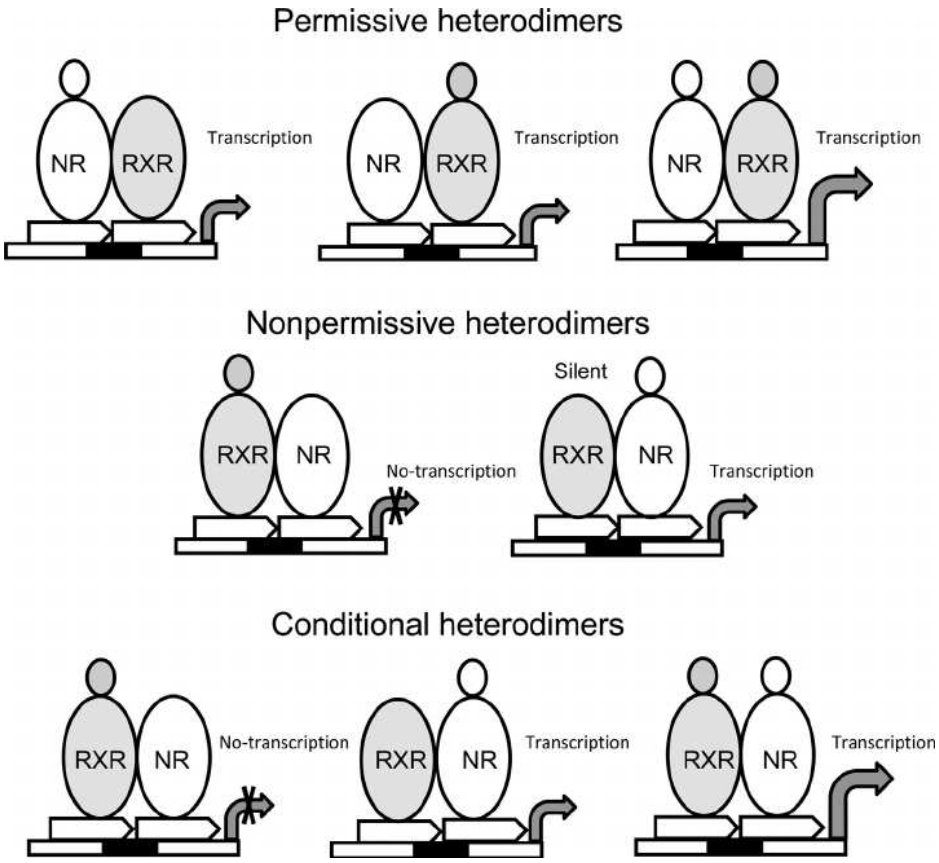

***Figure 2: Types of RXR heterodimers*.** Permissive heterodimers are activated by a ligand of either NR (nuclear receptor) or of RXR (retinoid X receptor). Non-permissive heterodimers are activated by the ligand of the NR only. Conditional heterodimers are not activated by an RXR ligand exclusively; however, the RXR ligand overactivates transcription together with a ligand of NR (Brtko and Dvorak, 2015).

3.2.1 Aryl hydrocarbon receptor (AhR)

AhR, also known as dioxin receptor, is not a true nuclear receptor. It is a member of the basic helix-loop-helix-PER-ARNT-SIM (bHLH-PAS) family of heterodimeric transcriptional regulators. bHLH-PAS proteins are involved in the control of diverse physiological processes including circadian rhythms, organ development, metabolism, and neurogenesis (Kewley *et al.*, 2004). AhR is constitutively expressed in many cell types and tissues. Basal AhR expression has been observed in human and mouse lungs, liver, kidneys, spleen, and placenta (Dohr *et al.*, 1996; Yamamoto *et al.*, 2004).

In the absence of a ligand, AhR is located in the cytosolic compartment and is bound to a molecular chaperone complex that stabilises AhR and prevents it from uncontrolled nuclear translocation. This complex is composed of two molecules of a heat-shock protein (hsp90), co-chaperone p23, and immunophilin-like AhR-interacting protein (AIP; also termed XAP2 or ARA9) (Denis *et al.*, 1988;

Kazlauskas *et al.*, 1999; Ma and Whitlock, 1997). After ligand binding, AhR undergoes conformational changes and the co-factors dissociate (Figure 3). AhR then translocates into the nucleus, where it dimerizes with its partner molecule called aryl hydrocarbon receptor nuclear translocator (ARNT) via interaction of HLH and PAS domains of the two proteins (Probst *et al.*, 1993). The AhR–ARNT heterodimer is the transcriptionally active form that can bind to dioxin-responsive element (DRE) motifs, containing the core sequence 5′-GCGTG-3′ in the promoter and enhancer regions of target genes (Shen and Whitlock, 1992; Swanson *et al.*, 1995). Binding of the AhR–ARNT heterodimer to the DRE motif leads to the recruitment of several co-activators that relax local chromatin structure so that nucleosomes change their positions, followed by recruitment of the general transcription machinery. These co-activators are SRC-1 (Beischlag *et al.*, 2002), Brg-1 (Wang and Hankinson, 2002), p300, and CBP (Kobayashi *et al.*, 1997). AhR signalling needs to be quickly terminated. Proteolytic degradation of AhR is induced by the nuclear export signal of AhR. The receptor is removed from the nucleus and is rapidly degraded by the ubiquitin-proteasome-dependent mechanism (Davarinos and Pollenz, 1999). Moreover, AhR repressor (AhRR) was identified as a negative regulator of AhR activity. AhRR is a competitor of AhR and can form a heterodimer with ARNT and consequently bind to DRE. This process leads to recruitment of other co-repressors, resulting in termination of active transcription (Haarmann-Stemmann *et al.*, 2007).

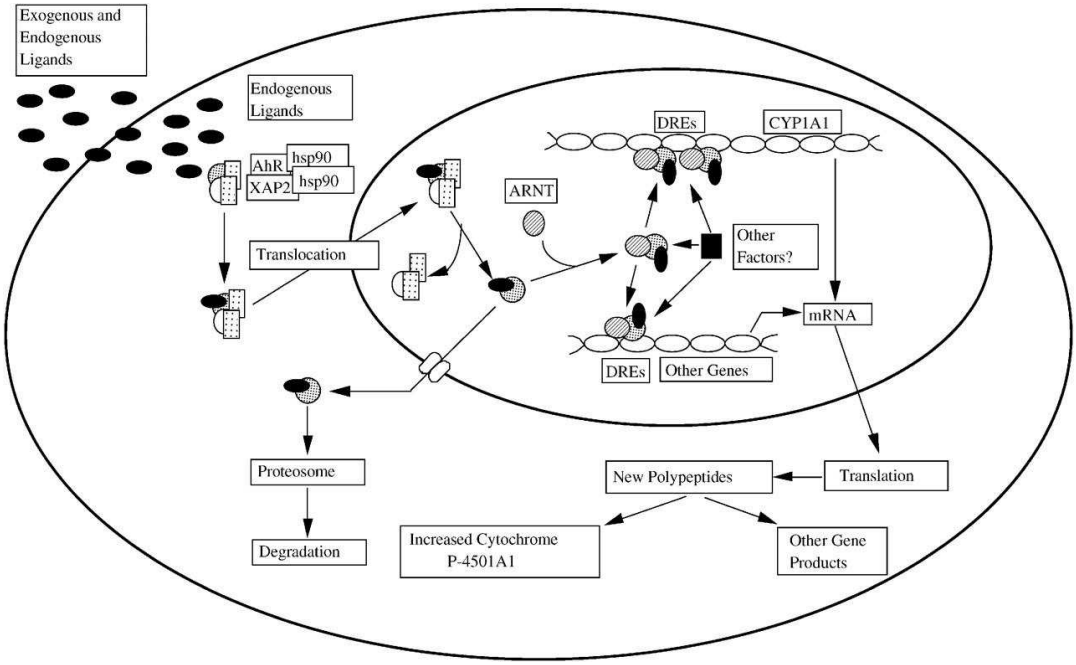

Figure 3: Key components and events in activation of the AhR signalling pathway. The ligands enter a responsive cell and bind with high affinity to the cytosolic AhR, which exists as a multiprotein complex, containing two molecules of chaperone protein hsp90 (a heat shock protein of 90 kDa) and X-associated protein 2 (XAP2). After ligand binding, AhR undergoes a conformational change resulting in translocation of the complex into the nucleus, where it can dimerize with its partner molecule aryl hydrocarbon receptor nuclear translocator (ARNT). This heterodimer can bind to its specific DNA recognition site, DRE, upstream of *CYP1A1* and other AhR-responsive genes in order to stimulate transcription of these genes. A nuclear export sequence present in AhR is responsible for the cytoplasmic shuttling of nuclear AhR complexes that fail to dimerize with ARNT and/or bind to DNA and leads to its ubiquitination and degradation (Denison and Nagy, 2003).

A wide range of environmental pollutants such as persistent planar halogenated polycyclic hydrocarbons (many dioxins, dibenzofurans, and biphenyls) and polycyclic aromatic hydrocarbons (benzo[a]pyrene and benzanthracene) activate AhR. 2,3,7,8-Tetrachlorodibenzo-[p]-dioxin (TCDD) is considered the most potent man-made AhR ligand that can cause teratogenesis, endocrine disruption, and immune and liver toxicity as well as skin disorders (Murray *et al.*, 2014). AhR also binds drugs such as the proton pump inhibitor omeprazole (Quattrochi and Tukey, 1993); or the non-steroidal anti-inflammatory drug sulindac (Ciolino *et al.*, 2006) or diclofenac (Bass *et al.*, 2009). Furthermore, the large group of naturally occurring substances that bind to AhR comprises food ingredients of plant origin, in particular flavonoids and related polyphenols, alkaloids, and indole derivatives. Most of them are known as chemopreventive agents such as resveratrol (Ciolino and Yeh, 1999), luteolin (Bothe *et al.*, 2010), quercetin and kaempferol (Ciolino *et al.*, 1999).

The existence of endogenous physiological AhR ligands has been reported in several studies when the AhR signalling pathway was found to be active in the absence of exogenous ligands (Denison and Nagy, 2003). The most important endogenous ligands are tryptophan metabolites, such as indirubin, indigo, indoleacetic acid, L-kynurenine, and 6-formylindolo[3,2-b]carbazole (Adachi *et al.*, 2001; Heath-Pagliuso *et al.*, 1998; Smirnova *et al.*, 2016; Wei *et al.*, 2000). Other physiological compounds binding to AhR are bilirubin, and biliverdin (Phelan *et al.*, 1998; Sinal and Bend, 1997; Togawa *et al.*, 2008). Tissue-specific roles for AhR endogenous ligands have been postulated. Besides metabolic production of these ligands by human cells, formation of photo-reactive metabolites of tryptophan in the skin exposed to ultraviolet light or production of AhR ligands by the intestinal microbiota has been reported (Esser *et al.*, 2009; Zelante *et al.*, 2013).

Exposure to AhR ligands triggers the expression of family 1 of cytochrome P450 genes (*CYP1A1*, *CYP1A2*, and *CYP1B1*) (Whitlock, 1999). Additional target genes of AhR include *UGT1A1* (Yueh *et al.*, 2003) and *UGT1A6* (Munzel *et al.*, 1998), *GSTA2* (Rushmore *et al.*, 1990) or NADPH:quinone oxidoreductase (Jaiswal, 1991). In addition to drug-metabolizing genes, AhR controls the expression of cell cycle and apoptosis regulators, including transforming growth factor β (Gramatzki *et al.*, 2009), p27 (Kip1) (Kolluri *et al.*, 1999), and Bax (Matikainen *et al.*, 2001). Agonists of AhR also induce the expression of AHRR providing a negative feedback loop that prevents over-expression of AhR target genes (Harper *et al.*, 2006; Mimura *et al.*, 1999).

3.2.2 Glucocorticoid receptor (GR)

GR (NR3C1) is a nuclear hormone receptor activated by natural and synthetic glucocorticoids (GCs) such as cortisone, hydrocortisone, and dexamethasone. GCs are synthesised in the adrenal cortex and have important functions in glucose homeostasis, development, metabolism, neurobiology, apoptosis, and many other processes (Sapolsky *et al.*, 2000). Furthermore, GCs are widely used in pharmacology owing to their anti-inflammatory and immunosuppressive effects as well as pro-apoptotic properties suitable for chemotherapy (Barnes, 1998; Kirkham *et al.*, 1991).

Prior to binding of a ligand, GR stays in the cytoplasm attached to two molecules of hsp90 and several immunophilins such as FKB51 (Gallo *et al.*,

2011). Upon ligand binding, cytoplasmic chaperones dissociate, and GR is translocated into the nucleus, where it forms a homodimer that binds to a glucocorticoid-responsive element (GRE) found in promoters of GR's target genes (Gross and Cidlowski, 2008; Reichardt *et al.*, 1998). These genes include human immunodeficiency virus type 1 genes (Soudeyns *et al.*, 1993), human corticotrophin-releasing hormone (Malkoski and Dorin, 1999) and neuronal serotonin receptor (Ou *et al.*, 2001).

GR also regulates the expression of several CYP genes. This regulation can take place either via direct or indirect binding of GR to a promoter (Dvorak and Pavek, 2010). Direct binding of GR was observed in the promoter of *CYP2C8* (Ferguson *et al.*, 2005), *CYP2C9* (Gerbal-Chaloin *et al.*, 2002), *CYP2C19* (Chen *et al.*, 2003), and *CYP3A5* (Schuetz *et al.*, 1996). However, indirect binding of GR involves an interaction with co-activators (NCOA1/2), co-repressors (NCOR1/2), chromatin-remodelling enzymes (PRMT1, CREB-binding protein), and other proteins (Lu and Cidlowski, 2006). This '*trans*-regulation' has been reported for genes *CYP2A6* and *CYP3A4* possessing no functional GRE in their promoter sequences. The process is enabled by the interaction of HNF4α with a HNF4-RE (Onica *et al.*, 2008). Similarly, *CYP3A4* is *trans*-activated indirectly by GCs via HNF-3/CCAAT-enhancer protein α in the promoter (El-Sankary *et al.*, 2002).

3.2.3 Pregnane X receptor (PXR)

PXR (NR1I2) is a member of the nuclear hormone receptor family of ligand-activated transcription factors and is intimately involved in the mechanism of defence against xenobiotics (Kliewer *et al.*, 1998). PXR is highly expressed in the liver, intestines, and kidneys, and small amounts of PXR have also been found in the lungs, stomach, uterus, ovaries, placenta, breasts, heart, and adrenal glands, as well as in bone marrow, peripheral blood monocytes, and osteoclasts (Kliewer *et al.*, 1998; Masuyama *et al.*, 2001; Miki *et al.*, 2005; Zhang *et al.*, 1999). The PXR-binding site is highly hydrophobic and flexible, permitting binding of lipophilic molecules of varied size (Watkins *et al.*, 2001; Watkins *et al.*, 2003). Indeed, PXR is activated by a variety of compounds, including natural and synthetic GCs (dexamethasone), anti-GCs (mifepristone), steroids, pregnane derivatives, herbal extracts, and many drugs currently in use, such as clotrimazole, lovastatin,

nifedipine, rifampicin (RIF), ritonavir, and tamoxifen (Bertilsson *et al.*, 1998; Desai *et al.*, 2002; Dussault *et al.*, 2001; Kliewer *et al.*, 1998).

Upon ligand binding, PXR is translocated from the cytoplasm into the nucleus (Squires *et al.*, 2004). Subsequently, PXR binds as a heterodimer with RXR to a variety of REs containing two copies of the half-site consensus sequence AG(G/T)TCA with various spacers. PXR/RXR was found to bind efficiently to DR-3 located in the proximal promoters of genes *CYP3A23* and *CYP3A2* and in the *CYP3A4* enhancer as well as to the ER-6 element in the proximal promoter of *CYP3A4* (Goodwin *et al.*, 1999; Kliewer *et al.*, 1998). DR-3 and ER-6 in another xenobiotic-inducible gene, *CYP3A7*, are also target REs for PXR (Pascussi *et al.*, 1999). Additionally, the PXR–RXR heterodimer can bind to DR-4 and DR-5 located for instance in promoters of *CYP2B* family members or the *MDR1* gene (Geick *et al.*, 2001; Goodwin *et al.*, 2001). Recently, the ER-8 element present in the *MRP2* gene was shown to bind PXR as well (Kast *et al.*, 2002). The PXR–RXR complex is non-permissive; therefore, it should not be activated by RXR agonists. Nevertheless, 9-*cis* retinoic acid and LG100268 (typical RXR ligands) can activate the heterodimer by binding directly to PXR, but only at micromolar concentrations (Jones *et al.*, 2000). PXR can also recruit several co-activators such as steroid receptor coactivators 1 and 2, hepatocyte nuclear factors HNF1α and HNF4α, upstream stimulatory factor USF1, activating protein AP-1, and PPARγ coactivator 1α (Matsumura *et al.*, 2004; McKenna *et al.*, 1999; Tirona *et al.*, 2003).

In addition to *CYP3A*, phase I genes regulated by PXR include *CYP2B6*, *CYP2B9*, *CYP2C8*, *CYP2C9*, and *CYP2C19* (Gerbal-Chaloin *et al.*, 2001; Goodwin *et al.*, 2001). PXR can regulate also phase II genes, including *UGT*, *SULT*, *GST*, and carboxylesterase families (Xu *et al.*, 2005). Among the drug transporters regulated by PXR are MDR1, MRP, BCRP, OATP, and the OCT family (Assem *et al.*, 2004; Hagenbuch and Meier, 2004; Jigorel *et al.*, 2006; Mills *et al.*, 2004; Schrenk *et al.*, 2001).

Although PXR was originally characterised as a xenosensor, it is also an important member of the family of endobiotic receptors. Many studies revealed its importance in glucose and lipid metabolism, steroid hormone homeostasis, bile acid and bilirubin detoxification, vitamin metabolism, and inflammation. Therefore, activation of PXR may lead to many pathological conditions (Ihunnah *et al.*, 2011).

A substantial group of endogenous PXR ligands is bile acids. For example, lithocholic acid (LCA), a secondary bile acid, has been found to cause cholestasis resulting in accumulation of toxic by-products in the liver (Li and Apte, 2015). PXR acts as an LCA sensor and participates in the detoxification of this cholestatic bile acid, thus protecting against severe liver damage induced by LCA (Staudinger *et al.*, 2001; Xie *et al.*, 2001). Recently, acetylated deoxycholic and cholic acids were identified as potent activators and ligands of human PXR (Carazo *et al.*, 2017). Detoxification of bilirubin is crucial, while its accumulation in blood is potentially neurotoxic. Under-expression of UGT1A1, a key enzyme for the conjugation of bilirubin, or deficiency of MRP2, a drug transporter responsible for the hepatic excretion of conjugated bilirubin, leads to severe disorders, such as Crigler–Najjar, Gilbert's, or Dubin–Johnson syndrome (Kaplan *et al.*, 2002; Keppler, 2014). PXR has been shown to induce the expression of key enzymes in the clearance pathway of bilirubin, including UGT1A1, OATP2, GSTA1 and GSTA2, and MRP2 (Jigorel *et al.*, 2006; Wagner *et al.*, 2005; Xie *et al.*, 2003). PXR also plays an important role in lipid homeostasis (Zhou *et al.*, 2006). As an example, activation of PXR by RIF results in an increased level of total cholesterol in HepG2 cells as a consequence of induced expression of free fatty acid transporter CD36 and ABCG1, as well as several lipogenic enzymes, including stearoyl-CoA desaturase 1, long-chain free fatty acid elongase, and lecithin-cholesterol acyltransferase (Zhang *et al.*, 2013).

3.2.4 Constitutive androstane receptor (CAR)

CAR (NR1I3) is a member of the NR superfamily actively involved in xenobiotic metabolism facilitated by phenobarbital-mediated induction of the hepatic *CYP2B* gene family (Sueyoshi *et al.*, 1999; Tolson and Wang, 2010). CAR is predominantly expressed in the liver and, to a lesser extent, in the intestine (Baes *et al.*, 1994). CAR forms a heterodimer with RXR as well, and together they bind to two functional enhancer modules, namely the phenobarbital-responsive enhancer module (PBREM) and the xenobiotic-responsive enhancer module (XREM) upstream of the *CYP2B6* gene in humans (Honkakoski *et al.*, 1998; Wang *et al.*, 2003). Moreover, CAR is involved in the control of other CYPs, such as CYP1A1 and CYP1A2 (Yoshinari *et al.*, 2010), CYP2A6 (Wortham *et al.*, 2007), CYP2C8 (Ferguson *et al.*, 2005), CYP2C9 (Ferguson *et al.*, 2002; Gerbal-Chaloin

et al., 2002), and CYP3A4 (Goodwin *et al.*, 2002). In addition, CAR regulates phase II enzymes, including several UGTs (Buckley and Klaassen, 2009; Osabe *et al.*, 2008; Sugatani *et al.*, 2005), GSTs, and SULTs (Yanagiba *et al.*, 2009) as well as drug transporters, such as MDR1 (Burk *et al.*, 2005) and OATPs (Svoboda *et al.*, 2011). CAR also modulates the metabolism and excretion of many endobiotics, such as bilirubin, bile acids, and steroid hormones (Huang *et al.*, 2003; Timsit and Negishi, 2007) and participates in insulin signalling (Gao *et al.*, 2009; Masuyama and Hiramatsu, 2012).

Despite the intensive cross-talk between CAR and PXR, CAR is constitutively activated (unlike PXR) in nearly all immortalised cells and is spontaneously accumulated in the nucleus of these cells prior to chemically stimulated activation (Kawamoto *et al.*, 1999). Moreover, CAR can be activated either directly by reported ligands 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehydeO-(3,4-dichlorobenzyl)oxime (CITCO) (Maglich *et al.*, 2003) and 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) (Baskin-Bey *et al.*, 2006) or indirectly by phenobarbital (Kawamoto *et al.*, 1999; Maglich *et al.*, 2003). CAR activation is therefore a multi-step process, and most identified CAR activators may not directly bind to the receptor. For this reason, the effects on CAR were not included in the study on enantiospecific effects of chiral drugs in this thesis project.

3.3 Drug-drug interactions (DDIs)

DDIs occur when one drug influences the metabolism of another drug, leading to possible adverse effects such as altered bioavailability, hepatic clearance, or increased formation of reactive and toxic metabolites (Fuhr, 2000). CYP3A subfamily members are most abundant in the liver and metabolise over 50% of pharmaceuticals available on the market (Kliewer *et al.*, 2002). Given that PXR is a major transcriptional regulator of *CYP3A*s, it became evident that the PXR-mediated regulation of XMEs could participate in clinical DDIs (Figure 4). Indeed, a hPXR agonist, RIF (used as an antituberculosis drug) affects the pharmacokinetics of other simultaneously administered drugs (via alteration of the CYP3A4 metabolic pathway), including anti-cancer drugs, anti-hypertensives, anti-microbials, cardiac drugs, and HIV antivirals (Bolton *et al.*, 2004; Finch *et al.*, 2002; Kyrklund *et al.*, 2000; Niemi *et al.*, 2003). Herb–drug interactions involving

PXR have been observed as well. The herbal remedy St. John's wort used for the treatment of depression and inflammation was found to induce CYP3A4 in the intestine (Durr *et al.*, 2000) and to increase the metabolism of oral contraceptives, the anticoagulant warfarin, immunosuppressant cyclosporine, cardiotonic digoxin, and other pharmaceuticals (Breidenbach *et al.*, 2000; Ernst, 1999; Johne *et al.*, 1999).

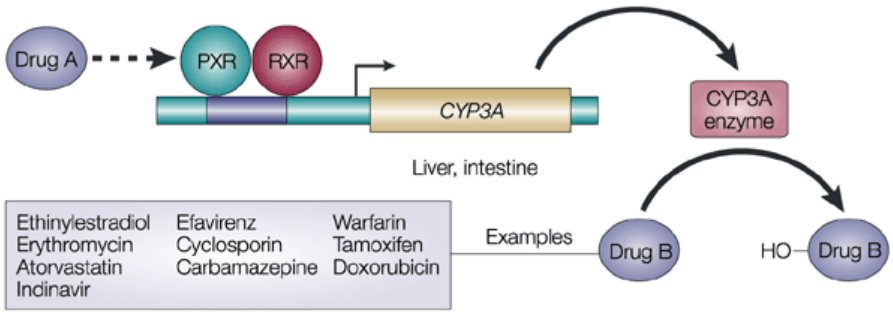

Figure 4**: **The molecular basis of a drug–drug interaction. Orphan nuclear receptor PXR forms a heterodimer with nuclear receptor RXR and acts as a transcriptional regulator of the expression of the *CYP3A* gene (yellow) in the liver and intestines. Drug A binds to PXR and induces expression of the CYP3A enzyme (pink), accelerating the metabolism of drug B, which is a substrate for CYP3A (Willson and Kliewer, 2002).

DDIs can be mediated by other CYPs as well. There is a database called SuperCYP (http://bioinformatics.charite.de/supercyp), which is a comprehensive resource focused on CYPs, their interactions with drugs, drug metabolism, and DDIs. The SuperCYP database contains 1170 drugs with more than 3800 DDIs including references. Therefore, SuperCYP is often a starting point for scientists and health professionals in their research (Preissner *et al.*, 2010).

3.4 The phenomenon of chirality

3.4.1 Chirality and enantiomers

Chirality is a property of an object that is non-superimposable onto its mirror image. It is like the left and right hand. Chiral compounds are optical isomers. Although they have identical molecular formulas, their arrangement in 3D space is different, and moreover, they are optically active, i.e. they can rotate the plane of polarised light. Chirality is present only in substances possessing at least one asymmetric (chiral) centre. It is mostly formed by carbon or sulphur bound to four different substituents, thus forming a tetrahedron.

Chiral chemistry was founded in the 19th century by Louis Pasteur, who recrystallized the racemic sodium ammonium salt of tartaric acid. Two different forms of hemihedral facets were formed. After he re-dissolved them, the solutions rotated polarised light in opposite directions with equal intensity (Flack, 2009).

Optical isomerism allows for the formation of two non-superimposable mirror images, so called *enantiomers* that rotate polarised light either to the left or to the right (Figure 5). Furthermore, some chiral substances possess two or more asymmetrical centres. Each chiral centre gives rise to two configurations and thus increases the number of stereoisomers by a factor of two. If the stereoisomers are not mirror images, they are called *diastereomers* or *diastereoisomers*.

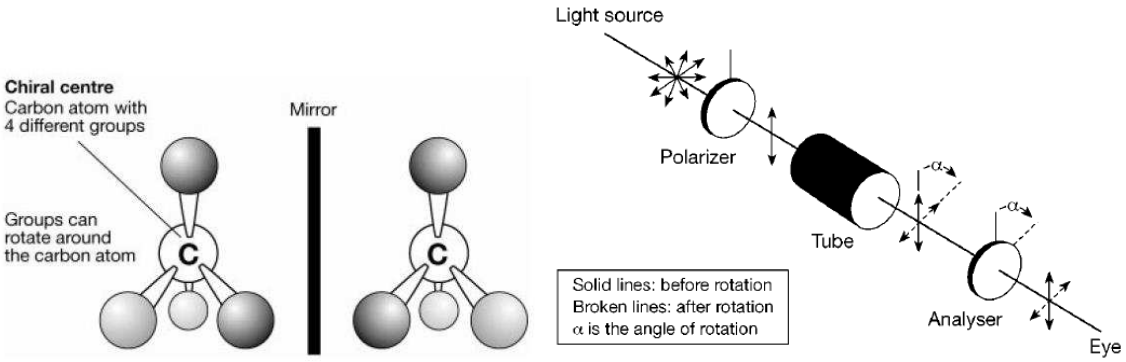

Figure 5: General structure of a chiral molecule with formation of two enantiomers and polarimeter used for measurement of rotation of polarised light by enantiomers (Burke and Henderson, 2002).

The nomenclature of individual enantiomers was established by the International Union of Pure and Applied Chemistry (IUPAC) Recommendations in 1996. The most frequent way to describe chiral molecules is based on the optical rotation of enantiomers. Relative descriptors (+) and (-) then characterise clockwise or counter clockwise rotation of polarised light. Isomers are then termed as *dextrorotatory* (D-) or *laevorotatory* (L-), respectively. Currently, enantiomers are determined by their spatial arrangement of substituents around a chiral centre according to the Cahn-Ingold-Prelog convention by assigning 'priorities' to individual substituents depending on their atomic number (Cahn *et al.*, 1956). If the sequence of the substituents in terms of size (largest to smallest) produces a clockwise direction, the enantiomer is named R- (*rectus* = right). The counter

clockwise progression is termed as S- (*sinister* = left). Thus, a chiral compound can be fully described by a combination of both absolute and relative descriptors—R(+), R(-), S(+), or S(-). A *racemate* or *racemic mixture* stands for an equimolar mixture of enantiomers. It is designated in most cases by the prefix ±; d,l; R/S; or rac- and causes no optical rotation. Finally, for description of diastereomers, the absolute configuration is stated for each chiral centre individually, e.g. 3R5S-fluvastatin.

3.4.2 Chiral pharmacology

Because the body contains numerous *homochiral* (with the same chirality), compounds (e.g. all amino acids are laevorotatory), biological structures such as receptors or enzymes then have a preference for one enantiomer over its counterpart. The enantioselective mechanism was described in the 1930s (Easson and Stedman, 1933) and is illustrated in Figure 6. If we compare affinities, the enantiomer with the highest affinity is called *eutomer* and that with the lowest affinity is termed *distomer*. The pharmacological activity of the enantiomers can be compared quantitatively by calculation of the *eudysmic ratio*.

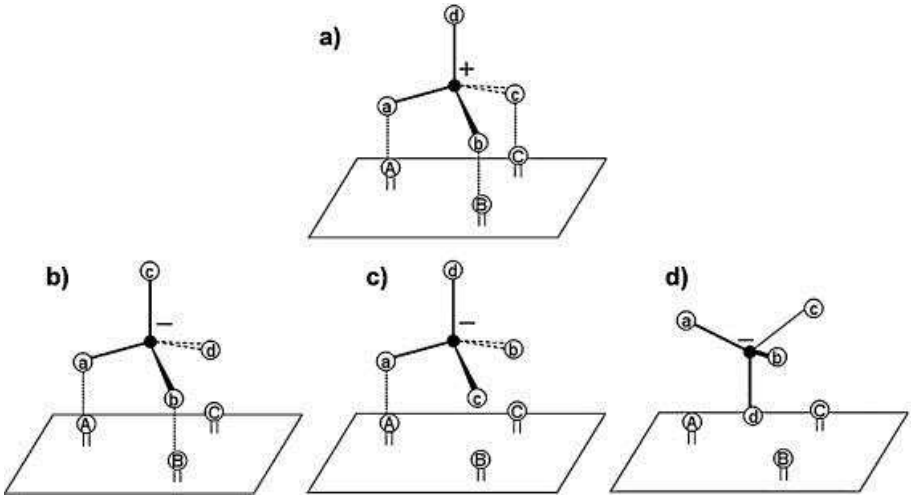

Figure 6: The Easson-Stedman model of an enantioselective interaction with a chiral biological macromolecule. The more active stereoisomer – eutomer (top) – is involved in three bonding interactions, while the less active enantiomer – distomer (bottom) – may interact at two sites at most regardless of its orientation towards the active site (Urniaz et al., 2014).

Drugs are classified into achiral, racemic, and single-enantiomer (*enantiopure*) containing one or more chiral centres in their molecule. Chiral drugs make up more than 50% of the market today. Given the different affinity of enantiomers, the majority of chiral drugs have one isomer that produces a desired therapeutic

effect, while the opposite one is inactive or is even responsible for toxic or adverse effects. Therefore, many enantiopure drugs have been developed, and their number is still growing. However, production of enantiopure drugs could be merely a marketing strategy that pharmaceutical companies have been using to ensure new patent protection (so called 'evergreening') of the products whose protection is about to expire (Agranat and Wainschtein, 2010).

Individual enantiomers are either synthesised *de novo* or derived from a racemate by a *chiral switch* (Tucker, 2000). Resolution of enantiomers is currently accomplished mainly by different variations of high-performance liquid chromatography, capillary electrophoresis, capillary electrochromatography, and gas chromatography methods (Ward and Ward, 2012).

There are numerous reports about chiral drugs with one enantiomer being more potent, including cardiovascular drugs, antibiotics, antidepressants, proton pump inhibitors, and sedatives. For instance, S-isomers of β-blockers are generally more potent at blocking β-adrenoreceptors than their R-isomers, e.g. atenolol (Stoschitzky *et al.*, 1993), propranolol (Stoschitzky *et al.*, 1989), carvedilol (Bartsch *et al.*, 1990), and metoprolol (Wahlund *et al.*, 1990). Warfarin used as an anticoagulant is more potent in its S-form (Hewick and McEwen, 1973). On the contrary, the R(-)-isomer of methadone is ~50-fold more potent than the S(+) antipode in the treatment of opiate dependence and cancer pain (Olsen *et al.*, 1977). For the treatment of gastric ulcers and lowering gastric pH, the S-enantiomer of omeprazole (Andersson *et al.*, 2001) and R-enantiomers of lansoprazole (Katsuki *et al.*, 1996) are often used.

There are only a few chiral drugs with both enantiomers having equal therapeutic potency or biological activity. This group includes anti-arrhythmic drugs (flecainide, propafenone), antimalarials (halofantrine, enpiroline), and the antidepressant fluoxetine (Nguyen *et al.*, 2006).

In some cases, racemic drugs cannot be used in pharmacotherapy because of adverse effects or toxicity of the distomer. The notorious example is thalidomide: a racemic sedative that was withdrawn from the market in the 1960s owing to selective toxicity to the embryo while being relatively safe for the mother. Experiments have revealed teratogenic effects of the S-enantiomer (Hoglund *et al.*, 1998). Because thalidomide undergoes spontaneous hydrolysis and fast chiral interconversion at physiological pH, enantiopure treatment with the R-enantiomer

was worthless (Chung *et al.*, 2003). Nevertheless, thalidomide was reintroduced as an off-label drug for the treatment of leprosy, multiple myeloma, and various types of cancer because of its immunomodulatory and anti-angiogenic properties (Matthews and McCoy, 2003; Strasser and Ludwig, 2002). Likewise, the selective serotonin reuptake inhibitor citalopram has already been used as an enantiopure drug because the activity of the S(+)-enantiomer is over 100-fold higher than that of the counterpart, and moreover, R-citalopram has more adverse effects (Hyttel *et al.*, 1992). Similarly, S-ketamine is an active anaesthetic and analgesic, whereas R-ketamine can cause hallucinations and agitation (White *et al.*, 1985). Furthermore, dextromethorphan, a synthetic analogue of codeine, is used worldwide for its antitussive effects, whereas levomethorphan, a narcotic analgesic 4- to 5-fold more potent than morphine, is a strictly controlled substance used for the treatment of severe pain (Bortolotti *et al.*, 2013).

Overall, the preference for enantiopure drugs or racemates in pharmacological use depends on therapeutic benefits, adverse effects, and costs.

3.4.3 Chiral pharmacokinetics

Unlike differences in pharmacodynamics, the differences between individual optical isomers in their pharmacokinetic parameters have been poorly studied (Hutt, 2007). Nevertheless, potential discrepancies in the processes of absorption, distribution, metabolism, and excretion are important and need to be explored as well.

Passive transport across cellular membranes is enabled by lipophilicity and the extent of ionisation at physiological pH. Because the lipid solubility and aqueous solubility are identical for individual enantiomers, there is generally only a slight if any effect of optical configuration of a drug on its passive transport. However, active transport allows for preference for one optical isomer over its antipode. For instance, the intestinal efflux of a racemic form of talinolol (β-blocker) is inhibited by the R-enantiomer of verapamil (Gramatte and Oertel, 1999). In addition, the R-form of cetirizine (an antihistamine) up-regulates P-gp expression, while S-cetirizine down-regulates it (Shen *et al.*, 2007).

Chirality may influence the basic properties of protein binding to varying degrees. Stereoselectivity of binding can have a significant effect on the concentration of a drug in blood plasma. Albumin is the most predominant plasma

protein and has stereo-specific binding preferences (Pistolozzi and Bertucci, 2008). Human albumin exhibits stereoselective binding to warfarin, where the affinity of the S(-)-enantiomer is two to five times higher than that of R(+)-warfarin (Fitos *et al.*, 2002). Furthermore, albumin has higher affinity for S(+)-chloroquine (an antimalarial) than the R(-)-form, while α-1-acid glycoprotein shows a preference for the R-form (Augustijns and Verbeke, 1993).

Due to the 3D nature of substrate recognition by enzymes, the biotransformation processes such as oxidation, reduction, hydrolysis, and conjugation may be stereospecific. For instance, S-etodolac (a non-steroidal anti-inflammatory drug) is primarily metabolised by acyl-glucuronidation, whereas R-etodolac is mainly metabolised by oxidation (Brocks and Jamali, 1992). Age, race, sex, and medical conditions may complicate this issue. Moreover, the stereoselective metabolism of S(-)-lansoprazole by CYP2C19 is influenced by polymorphism in drug metabolism—homozygous and heterozygous extensive metabolisers and poor metabolisers, such that R/S ratios for lansoprazole's area under the curve (AUC) for these polymorphisms is 12.7, 8.5, and 5.8, respectively (Miura, 2006). Interspecies variability in stereospecific metabolism is also common. For example, the metabolic oxidation of felodipine (a calcium channel blocker) in humans is greater for the S-isomer, whereas oxidative metabolism in rats and dogs shows a preference for the R-isomer (Eriksson *et al.*, 1991). Additional complications occur as a result of inversion processes, whereby one enantiomer is converted into another. This process may be spontaneous and bidirectional or may be facilitated by enzymes or other proteins and is mainly unidirectional. Thalidomide is known to undergo bidirectional inversion in human serum (Eriksson *et al.*, 1998). Unidirectional, biochemically mediated inversion is typical for 2-arylpropionic acid class of nonsteroidal anti-inflammatory drugs. The inactive R-form of ibuprofen and especially fenoprofen is known to be inverted to an active S-enantiomer in humans and laboratory animals (Berry and Jamali, 1991; Davies, 1998). The therapeutic consequence of chiral inversion of these drugs is that the administered dose of a racemate is nominally higher than needed as per the amount of the active ingredient present in the formulation.

Both biliary and urinary excretory processes may be stereoselective due to binding-site specificity of transport proteins and glomerular filtration of a drug bound to plasma proteins. For instance, the anti-arrhythmic R-disopyramide

inhibits tetraethylammonium transport by the human organic cation transporter more efficiently than its antipode (Zhang *et al.*, 1998). In addition, the anaesthetic R(-)-ketamine inhibits the elimination of S(+)-ketamine (Ihmsen *et al.*, 2001). Besides, higher renal tubular secretion was reported for dextro-cetirizine compared to its levo-form (Strolin Benedetti *et al.*, 2008).

3.5 The chiral compounds tested

3.5.1 3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors

HMG-CoA reductase inhibitors also known as statins (e.g. atorvastatin [AT], fluvastatin [FLU], lovastatin, pravastatin, rosuvastatin [ROS], and simvastatin) inhibit the synthesis of cholesterol, and therefore are widely used in the treatment of hypercholesterolaemia (Goldstein and Brown, 1990). The affinity of HMG-CoA reductase for statins is approximately three orders of magnitude greater than that for the natural substrate HMG-CoA (Moghadasian, 1999). Statins induce regression in vascular atherosclerosis and reduce cardiovascular morbidity and mortality in patients with and without coronary artery disease (Hebert *et al.*, 1997; Maron *et al.*, 2000; Pedersen, 1999). Moreover, statins have anti-inflammatory properties and participate in regulation of the immune system (Blanco-Colio *et al.*, 2003; Jain and Ridker, 2005; Kwak *et al.*, 2003; Weitz-Schmidt, 2002).

Statins, as potent inhibitors of the endogenous mevalonate pathway, consequently influence the biosynthesis of cholesterol as well as isoprenoids such as geranylgeranyl-pyrophosphate and farnesyl-pyrophosphate (Liao, 2002). These lipids are important for the post-translational modification of several proteins, including the small GTP-binding proteins Ras, Rac, and Rho (Figure 7). This post-translational modification, so-called isoprenylation, is crucial for the activation and intracellular transport of proteins responsible for a variety of cellular functions, such as cell shape maintenance, migration, differentiation, and proliferation (Mackay and Hall, 1998).

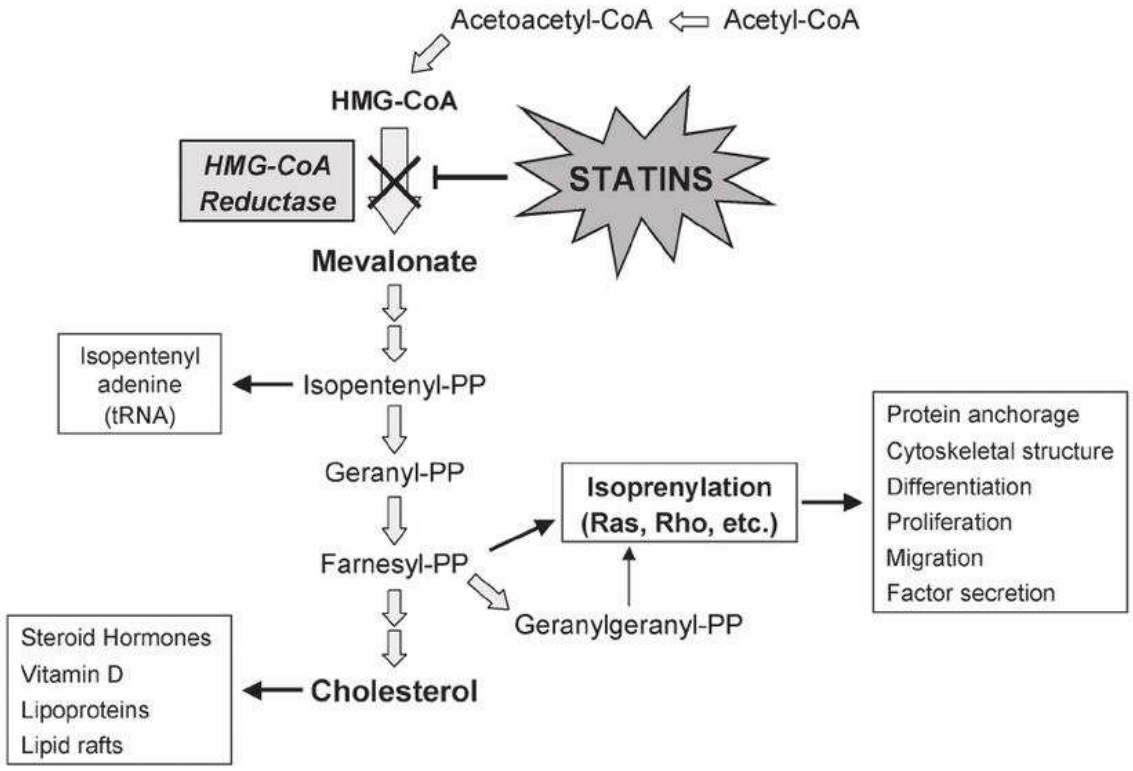

Figure 7: Inhibition of the endogenous mevalonate pathway leading to cholesterol biosynthesis. Statins inhibit the conversion of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) to mevalonate by inhibiting HMG-CoA reductase. This process leads to decreased production of cholesterol thereby causing lowered production of sterol products and impaired formation or disruption of lipid rafts. Isoprenoids, such as farnesyl-pyrophosphate (PP) and geranylgeranyl-PP are essential for isoprenylation of proteins Ras and Rho; therefore, statins also interfere with a number of cellular processes facilitated by these proteins (Kuipers and van den Elsen, 2007).

Three of the most frequently prescribed statins—AT, FLU, and ROS—were chosen for analysis of their stereospecific effects on human CYPs in this study (Figure 8). Structurally, the statins under study are chiral compounds having two asymmetrical centres in the molecule, enabling formation of four diastereomers: 3R5R-, 3R5S-, 3S5R-, and 3S5S-. The following single-isomer formulations of these drugs are used in the clinic: 3R5R-AT (Lipitor, Pfizer; generic since November 2011), 3R5S-ROS (Crestor, Astra-Zeneca; approved on 12th August 2003), and 3R5S-FLU (Lescol, Novartis; approved on 31st December 1993; generic since 2011).

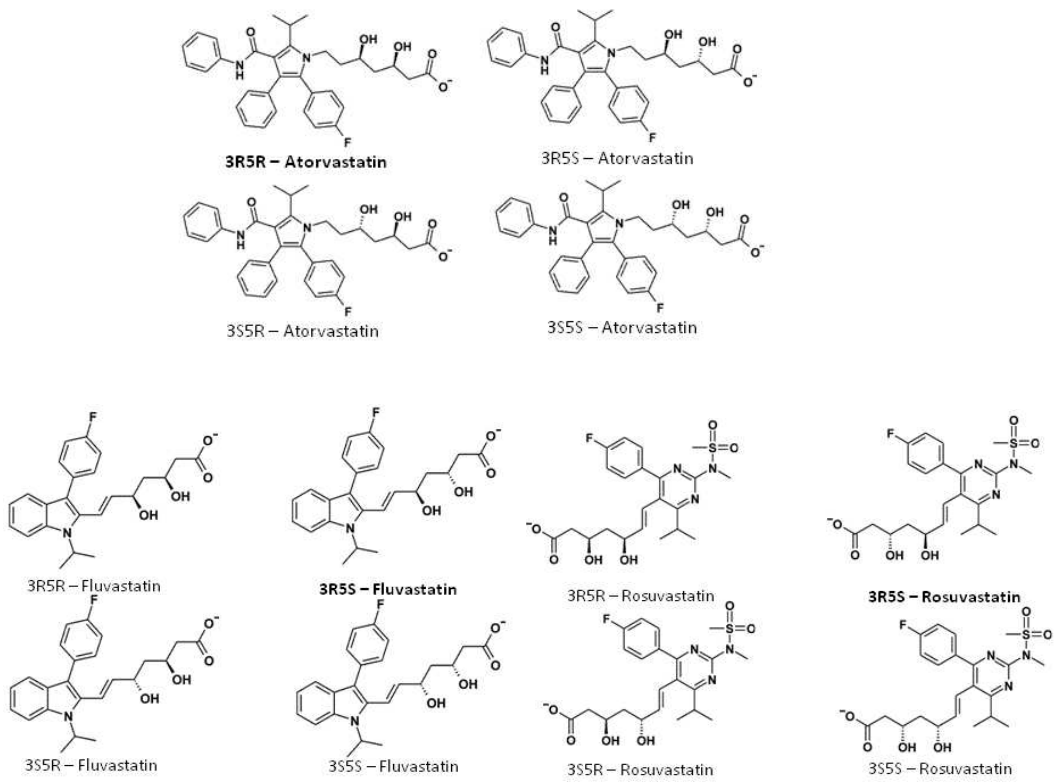

Figure 8: Chemical structures of single-isomer forms of statins. Four individual diastereomers of atorvastatin, rosuvastatin, and fluvastatin are shown. Clinically used single-isomer forms are boldfaced.

The frequent adverse effects associated with statin therapy are relatively mild: headache, rash, and gastrointestinal symptoms. However, statins may cause more serious adverse effects such as asymptomatic elevation in liver transaminases, myopathy, and increased risk of diabetes (Bellosta and Corsini, 2012). The importance of considering the safety profile of prescribed statins was further highlighted in August 2001, when cerivastatin was recalled worldwide due to its association with fatal rhabdomyolysis (Staffa *et al.*, 2002).

The risk of adverse effects increases when statins are used simultaneously with other drugs, which may cause DDIs. Pharmacokinetics of statins is complex. Statins are substrates for multiple membrane transporters, such as OATP1B1, BCRP, and MDR1 (Neuvonen *et al.*, 2006). Additionally, statins undergo substantial microsomal metabolism by CYPs. AT is metabolised primarily by CYP3A4; therefore, inhibitors of CYP3A4, including protease inhibitor nelfinavir or CCB mibefradil may influence the pharmacokinetics of AT (Hsyu *et al.*, 2001; Prueksaritanont *et al.*, 1999). On the contrary, FLU and ROS are metabolised primarily by CYP2C9 with little involvement of CYP3A4 in case of ROS (Olsson *et al.*, 2002; Scripture and Pieper, 2001). Therefore, a lower number of clinically

significant pharmacokinetic DDIs between ROS or FLU and other drugs than with AT has been reported (Cooper *et al.*, 2002). Induction or down-regulation of CYPs may occur via activation or inhibition of major xenoreceptors. There are several reports showing activation of PXR and induction of PXR-regulated genes by statins (Hoffart *et al.*, 2012; Howe *et al.*, 2011; Kocarek *et al.*, 2002; Plee-Gautier *et al.*, 2012; Yamasaki *et al.*, 2009).

3.5.2 Dihydropyridine CCBs

CCBs inhibit the flow of extracellular calcium through ion-specific channels in the cell membrane. Although several types of such channels have been identified, currently available CCBs inhibit the L-type channels in humans (Hockerman *et al.*, 1997). These channels facilitate calcium influx in response to membrane depolarisation and regulate intracellular processes such as contraction, secretion, neurotransmission, and gene expression in many different cell types. Blockade of these channels in vascular tissues results in a decrease in smooth-muscle and myocardial contractility and a reduction in blood pressure (Abernethy and Schwartz, 1999). Therefore, CCBs are used as anti-hypertensives and in the treatment of angina pectoris. There exist two subclasses of CCBs—dihydropyridine (amlodipine, benidipine [BENI], clevidipine, felodipine [FELO], isradipine [ISRA], nifedipine, nisoldipine, nitrendipine, and other '-dipines') and non-dihydropyridine compounds (diltiazem, fendiline, and verapamil). Dihydropyridine CCBs are more potent vasodilators and are often combined with β-blockers in pharmacotherapy (Handler, 2005).

Structurally, dihydropyridine CCBs are chiral compounds having at least one asymmetrical centre in the molecule, thereby existing as two or more optical isomers. Three chiral 'dipines'—BENI, FELO, and ISRA—were chosen here for analysis of stereospecific effects on human CYPs (Figure 9). BENI has two chiral atoms in the molecule thus enabling formation of four diastereomers: (+)-α-; (-)-α-; (+)-β-, and (-)-β-isomer. The approved therapeutic formulation is a racemate of α enantiomers (approved in China in 2008, sold as Coniel by Kyowa Hakko Kirin Co., Ltd.; Caritec: Stancare, Ranbaxy Laboratories Ltd.). The potency of (+)-α-BENI is 30- to 100-fold stronger than that of (-)-α-BENI in spontaneously hypertensive rats (Muto *et al.*, 1988). BENI was described as an inhibitor of

CYP3A4, CYP1A1, CYP2C9, CYP2C19, and CYP2D6 in human liver microsomes (Katoh *et al.*, 2000). In case of FELO and ISRA, there is only one chiral carbon present in the structure. Thus, they have two enantiomers: (+)(R)/(-)(S)-FELO and (+)(S)/(-)(R)-ISRA, respectively. Both drugs are clinically used as racemates (Plendil, AstraZeneca, approved 25th July 1991, generic since 2004; Dynacirc, SmithKline Beecham, approved 20th December 1990, generic since 2006). (-)(S)-FELO was found to be more potent than (+)(R)-FELO (Eltze *et al.*, 1990). Pharmacological activity of (+)(S)-ISRA was shown to be 150-fold higher as compared to (−)(R)-ISRA (Ruegg and Hof, 1990). Although FELO inhibits CYP3A4 and CYP2C9 activities in humans (Ma *et al.*, 2000), the inhibition of CYP3A4 by ISRA is rather weak (Wang *et al.*, 1999).

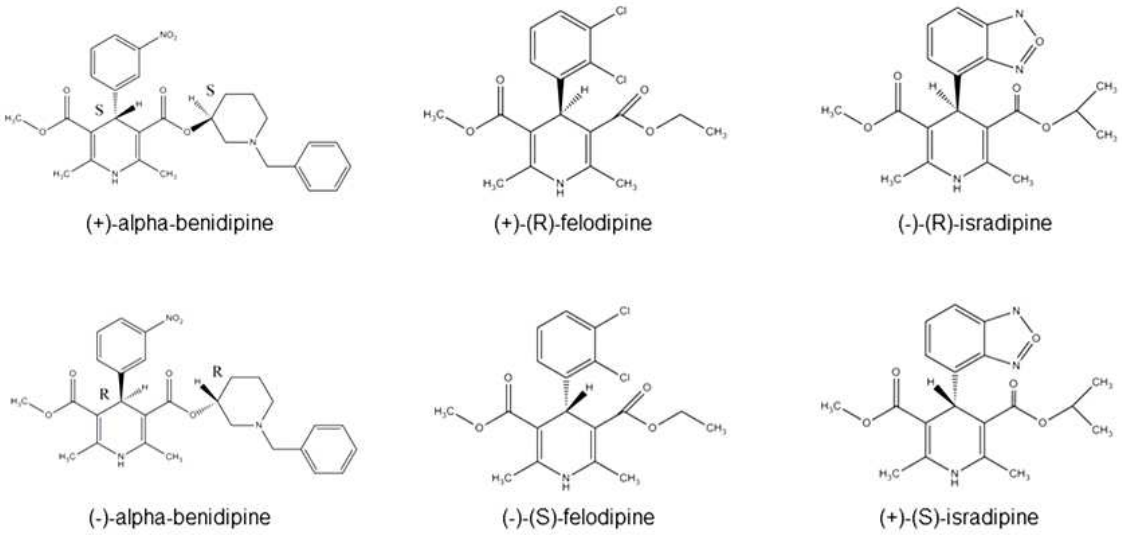

Figure 9: Chemical structures of the chiral dihydropyridines under study.

Many CCBs cause considerable adverse effects, such as tachycardia, orthostatic hypotension, fluid retention, headache, fatigue, vertigo, muscle cramps, and dizziness. Dihydropyridines were reported to be metabolised to less active metabolites mainly by CYP3A and to a lesser extent by other CYPs (Guengerich *et al.*, 1991; Yoon *et al.*, 2007). Thus, interactions with simultaneously administered CYP3A inhibitors or inducers may occur (Holtbecker *et al.*, 1996; Jalava *et al.*, 1997; Lown *et al.*, 1997). DDIs may be caused by activation of major xenoreceptors. Indeed, activation of PXR and induction of PXR-regulated genes by dihydropyridines (nifedipine, nicardipine, ISRA, or clevidipine) have been described (Drocourt *et al.*, 2001; Zhang *et al.*, 2006).

4 MATERIALS AND METHODS

4.1 Biological materials

4.1.1 Human cancer cell lines

Human Caucasian hepatocellular carcinoma cell line HepG2 (ECACC No. 85011430), human Caucasian breast adenocarcinoma cell line MCF-7 (ECACC No. 86012803), human Caucasian colon adenocarcinoma cell lines LS174T (ECACC No. 87060401) and LS180 (ECACC No. 87021202) were purchased from the European Collection of Authenticated Cell Cultures (ECACC). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% of foetal bovine serum, 100 U/mL streptomycin, 100 µg/mL penicillin, 4 mM L-glutamine, 1% of non-essential amino acids, and 1 mM sodium pyruvate. The cells were maintained at 37 °C and 5% CO_2 in a humidified incubator. Cell lines (AZ-AHR and AZ-GR) stably transfected with a gene reporter were described elsewhere (Novotna *et al.*, 2011; Novotna *et al.*, 2012).

4.1.2 Primary human hepatocytes

Hepatocytes were isolated from human liver tissue obtained from six multiorgan donors: HH59 (woman; 42 years), HH61 (man; 64 years), HH63 (man; 68 years), HH64 (man; 73 years), HH65 (man; 34 years), and HH66 (man; 65 years). The tissue acquisition protocol was in accordance with the requirements stated by the local ethics commission in the Czech Republic. The cells were plated in collagen-coated dishes in a hormonally and chemically defined medium (Isom *et al.*, 1985; Pichard-Garcia *et al.*, 2002) consisting of the mixture of William's E and Ham's F-12 media [1:1 (v/v)]. The cultures were maintained at 37 °C and 5% CO_2 in a humidified incubator.

4.2 Compounds and reagents

Dimethylsulphoxide (DMSO), rifampicin (RIF), dexamethasone (DEX), hygromycin B, 3R5R-AT, foetal bovine serum, DMEM, streptomycin, penicillin, L-glutamine, sodium pyruvate, non-essential amino acids, and TRI Reagent® were purchased from Sigma-Aldrich (Czech Republic). Racemates of α-BENI, FELO, and ISRA were also acquired from Sigma-Aldrich, and their respective enantiomers were isolated by chiral semipreparative chromatography using a

Knauer Smartline HPLC system (Germany) as described elsewhere (Stepankova *et al.*, 2016). Following chiral purity was achieved: 98.5%, and 98.5% for (+/-)-BENI, 99.5% and 97.0% for (+/-)-FELO and 99.6% and 97.5% for (+/-)-ISRA, respectively. The racemization of individual enantiomers after 48 h in aqueous solution was negative. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) was purchased from Ultra Scientific (USA), whereas 3R5S-AT, 3S5R-AT, and 3S5S-AT from Toronto Research Chemicals Inc. (Canada). 3R5R-FLU, 3R5S-FLU, 3S5R-FLU, 3S5S-FLU, 3R5R-ROS, 3S5R-ROS, and 3S5S-ROS were acquired from TLC PharmaChem Inc. (Canada). 3R5S-ROS was purchased from Santa Cruz Biotechnology Inc. (Germany), whereas luciferase lysis buffer and FuGENE® HD Transfection Reagent from Promega (USA). M-MuLV Reverse Transcriptase and random hexamers was purchased from New England Biolabs (USA). Oligonucleotide primers used in qPCR reactions were synthesised by Generi Biotech (Czech Republic). LightCycler® 480 Probes Master was purchased from Roche Diagnostic Corporation (Czech Republic). Reagents for Simple Western blotting by Sally Sue^{TM}, antibody diluent, a goat anti-rabbit IgG antibody, and goat anti-mouse IgG antibody were acquired from ProteinSimple (San Jose, CA). Antibodies against CYP1A1 (goat polyclonal, sc-9828, G-18), CYP1A2 (mouse monoclonal, sc-53614, 3B8C1), CYP2A6 (mouse monoclonal, sc-53615, F16P2D8), CYP2B6 (rabbit polyclonal, sc-67224, H-110), and CYP3A4 (mouse monoclonal; sc-53850, HL3) and a rabbit anti-goat IgG antibody (sc-2768) were purchased from Santa Cruz Biotechnology Inc. (Germany). An anti-β-actin (mouse monoclonal; 3700S, 8H10D10) antibody was acquired from Cell Signaling Technology (USA), and Light Shift Chemiluminescent EMSA Kit from Thermo Scientific (USA). All other reagents were of the highest quality commercially available.

4.3 Methods

4.3.1 Cell viability assay

AZ-AHR, AZ-GR, and LS180 cells were seeded at a density of 25 × 10^4/well (counted on CASY® Cell Counter + Analyzer System, Roche) in a 96-well plate and stabilised for 16 h prior to the treatments. To evaluate the cell damage, the cells were treated for 24 h with various concentrations of the compounds under study, Triton X-100 (2%, v/v), and/or vehicle (DMSO; 0.1%, v/v). After that,

the medium was discarded, and the cells were washed with PBS. After addition of 100 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 0.3 mg/mL), the cells were incubated at 37 °C for 30–40 min. Next, the MTT solution was removed and formazan crystals were dissolved in DMSO. Then, the absorbance was measured at 540 nm using an Infinite M200 (TECAN, Austria). The half-maximal inhibitory concentrations (IC_{50}) were calculated using the data obtained from three independent cell passages.

4.3.2 Gene reporter assay

Transcriptional activities of AhR and GR were studied in stably transfected gene reporter AZ-AHR and AZ-GR cells, respectively. For evaluation of PXR transcriptional activity, LS180 cells were transiently transfected with a chimeric p3A4-luc reporter construct by lipofection (FuGENE® HD Transfection Reagent) as described elsewhere (Pavek *et al.*, 2010). The cells were seeded at density 25×10^4/well in a 96-well plate and stabilised for 16 h prior to the treatments. After that, the cells were treated for 24 h with the tested compounds and/or vehicle (DMSO; 0.1%, v/v), in the presence (antagonist mode) or absence (agonist mode) of TCDD (5 nM), RIF (10 µM), or DEX (100 nM), respectively. After the treatments, the cells were lysed, and luciferase activity was measured on the Infinite M200 (TECAN, Austria).

4.3.3 RNA isolation, reverse transcription, and quantitative PCR (qPCR)

HepG2 cells and primary human hepatocytes were seeded in 6-well plates (1×10^6/well) and stabilised for 16 h prior to the treatments. The cells were then treated for 24 h with the tested compounds, TCDD (5 nM), RIF (10 µM), and/or vehicle (DMSO; 0.1%, v/v). Total RNA was isolated by means of the TRI Reagent®. The concentration of RNA was quantified by spectrometry at 260 nm, and purity was assessed from the ratio of absorbance values A_{260}/A_{280}. cDNA was synthesised from 1000 ng of total RNA using M-MuLV Reverse Transcriptase at 42 °C for 60 min in the presence of random hexamers. qPCR was carried out using LightCycler® 480 Probes Master on a Light Cycler® 480 II machine (Roche Diagnostic Corporation). The levels of all mRNAs were determined using primers and Universal Probes Library (UPL; Roche Diagnostic Corporation) probes described in Table 1. For quantification of mRNA of genes *CYP1A1*, *CYP1A2*, *CYP2A6*, *CYP2B6*, *CYP2C9*, *CYP3A4*, *TAT*, *PXR*, and

GAPDH, the following program was used: an activation step at 95 °C for 10 min was followed by 45 cycles of PCR (denaturation at 95 °C for 10 s and annealing with elongation at 60 °C for 30 s). The measurements were performed in triplicate. Gene expression was normalised to *GAPDH* as a housekeeping gene. The data were processed according to the delta-delta C_t method.

Table 1: Primer sequences with appropriate Universal Probes Library (UPL) numbers.

Gene symbol	Forward primer sequence	Reverse primer sequence	UPL number
CYP1A1	CCAGGCTCCAAGAGTCCA	GATCTTGGAGGTGGCTGCT	33
CYP1A2	ACAACCCTGCCAATCTCAAG	GGGAACAGACTGGGACAATG	34
CYP2A6	CCACGGGACTTCATTGACTC	CCCAATGAAGAGGTTCAACG	63
CYP2B6	TTCACTCATCAGCTCTGTATTCG	GCCCCAGGAAAGTATTTCAA	106
CYP2C9	GAAGCACCCAGAGGTCACAG	CTCGTGCACCACAGCATC	92
CYP3A4	TGTGTTGGTGAGAAATCTGAGG	CTGTAGGCCCCAAAGACG	38
GAPDH	CTCTGCTCCTCCTGTTCGAC	ACGACCAAATCCGTTGACTC	60
PXR	GCCAAAGTCATCTCCTACTTCAG	TCTCAGTTGACACAGCTCGAA	93
TAT	GCACCCCTAGAAGCTAAGGAC	CAGGTCTTGGAACCAGGATG	37

4.3.4 Simple Western blotting by Sally Sue™

Total protein extracts were prepared from HepG2 cells and primary human hepatocytes cultured in 6-well plates (1 x 10^6 cells/well) and stabilised for 16 h prior to the treatments. After the stabilisation, the cells were incubated for 48 h with the test compounds, TCDD (5 nM), RIF (10 µM), and/or vehicle (DMSO; 0.1%, v/v). Next, the cells were washed twice with ice-cold PBS and scraped into 1 mL of phosphate buffered saline (PBS). The suspension was centrifuged (4500 x g/5 min/4 °C), and the pellet was resuspended in ice-cold lysis buffer [150 mM NaCl, 10 mM Tris-HCl pH 7.2, 0.1% (w/v) SDS, anti-protease cocktail, 1% (v/v) Triton X-100, anti-phosphatase cocktail, 1% (v/v) sodium deoxycholate, 5 mM EDTA]. The mixture was vortexed and incubated for 10 min on ice and then centrifuged (15000 x g/13 min/4 °C). The supernatant was collected, and the protein content was determined by means of the Bradford reagent. For assessment of the expression level of CYP proteins, Simple Western blotting was used according to the ProteinSimple manual (Sally Sue, San Jose,

California). Primary antibodies were diluted differently according to the cell type: CYP1A1 (dilutions 1:200 for cell lines and 1:25 for human hepatocytes), CYP1A2 (dilution 1:250), CYP3A4 (dilution 1:10000), β-actin (dilutions 1:1000 for cell lines and 1:100 for human hepatocytes). Target proteins were identified using primary antibodies, a horseradish peroxidase-conjugated secondary antibody, and chemiluminescent substrate. The resulting chemiluminescent signal was detected and quantified in the Compass Software version 2.6.5.0 (ProteinSimple). For quantitative data analysis, the CYP signals were normalised to β-actin as a loading control.

4.3.5 Electrophoretic mobility shift assay (EMSA)

4.3.5.1 Binding of AhR to DNA

MCF-7 cells were seeded in 6-well plates (1 x 10^6 cells/well) and stabilised for 16 h prior to the treatments. The cells were then incubated for 2 h with the compounds under study (10 µM), TCDD (5 nM), and vehicle (DMSO; 0.1%, v/v). Nuclear fractions were isolated using the Nuclear Extract Kit (Active Motif). The following double-stranded 5′-biotinylated oligonucleotides containing a specific DNA-binding sequence for AhR corresponding to the 27-bp protein-binding site of DRE 3 were used:

sense 5′- GATCCGGCTCTTCTCACGCAACTCCGAGCTCA-3′

antisense 5′- GATCTGAGCTCGGAGTTGCGTGAGAAGAGCCG-3′

The EMSA was carried out as follows: nuclear protein (15 µg) from each sample was pre-incubated in binding buffer (final concentrations: 10 mM Tris-HCl, 50 mM KCl, 1 mM DTT; pH 7.5) along with final concentrations of 2.5% glycerol, 0.05% NP-40, double-distilled water (ddH_2O), and nonspecific competitor Poly (dI.dC) before addition of the biotin-labelled probe (10 fmol/µL). An unlabelled probe (2 pmol/µL) was added to the reaction mixture for a competitive experiment. The complete binding reaction was incubated at room temperature for 20 min. Finally, 5 µL of 5× loading buffer with bromophenol blue was added before loading onto a non-denaturing 5% polyacrylamide gel for electrophoretic separation. The protein–DNA complexes were electro-blotted to a positively charged nylon membrane. After that, transferred DNA was cross-linked using a UV-light cross-

linker instrument. Biotin-labelled DNA was detected using a streptavidin-horseradish peroxidase conjugate and a chemiluminescent substrate from the Light-Shift Chemiluminescent EMSA Kit (Thermo Scientific, USA).

4.3.5.2 Binding of PXR to DNA

LS174T cells were seeded in 10-mm Petri dishes (1 x 10^7/dish) and stabilised for 16 h. Nuclear fractions were isolated using the Nuclear Extract Kit. Then, nuclear fractions were incubated for 2 h at 30 °C with the tested compounds (10 µM), RIF (10 µM), and vehicle (DMSO; 0.1%, v/v). The following double-stranded 5′-biotinylated oligonucleotides containing the DR3 motif from the XREM sequence of the *CYP3A4* gene promoter were used:

sense 5′-GAATGAACTTGCTGACCCTCT-3′

antisense 5′-AGAGGGTCAGCAAGTTCATTC-3′

The EMSA was performed using the LightShift Chemiluminescent EMSA Kit as described above.

4.3.6 Statistical analyses

Data were expressed as mean ± SD. One-way ANOVA followed by Dunnett's multiple-comparison *post hoc* test as well as calculations of the half-maximal effective concentrations (EC_{50}) and the half maximal inhibitory concentrations (IC_{50}) values were carried out in the GraphPad Prism software (GraphPad Software Inc., San Diego, CA).

5 RESULTS

5.1 Effects of statin diastereomers on regulatory pathways of human cytochrome P450s

5.1.1 Cytotoxicity of statin diastereomers in human cancer cell lines

The cytotoxicity of optical isomers of statins was examined in the human cancer cell lines AZ-AHR, AZ-GR, and LS180. For this purpose, the cells were incubated for 24 h with individual diastereomers (3R5R-, 3R5S-, 3S5R-, or 3S5S-) of three selected statin drugs—AT, FLU, or ROS—at concentrations ranging from 100 pM to 100 μM. The vehicle was DMSO (0.1%, v/v). After the treatments, a conventional MTT assay was performed, and the IC_{50} values were calculated. Based on the results from cytotoxicity testing (Figures 10 and 11), gene reporter assays were conducted at the concentrations of the tested compounds up to 100 μM, with the exception of AT, where the maximal concentration of 10 μM was used for incubation with AZ-AHR and AZ-GR cells.

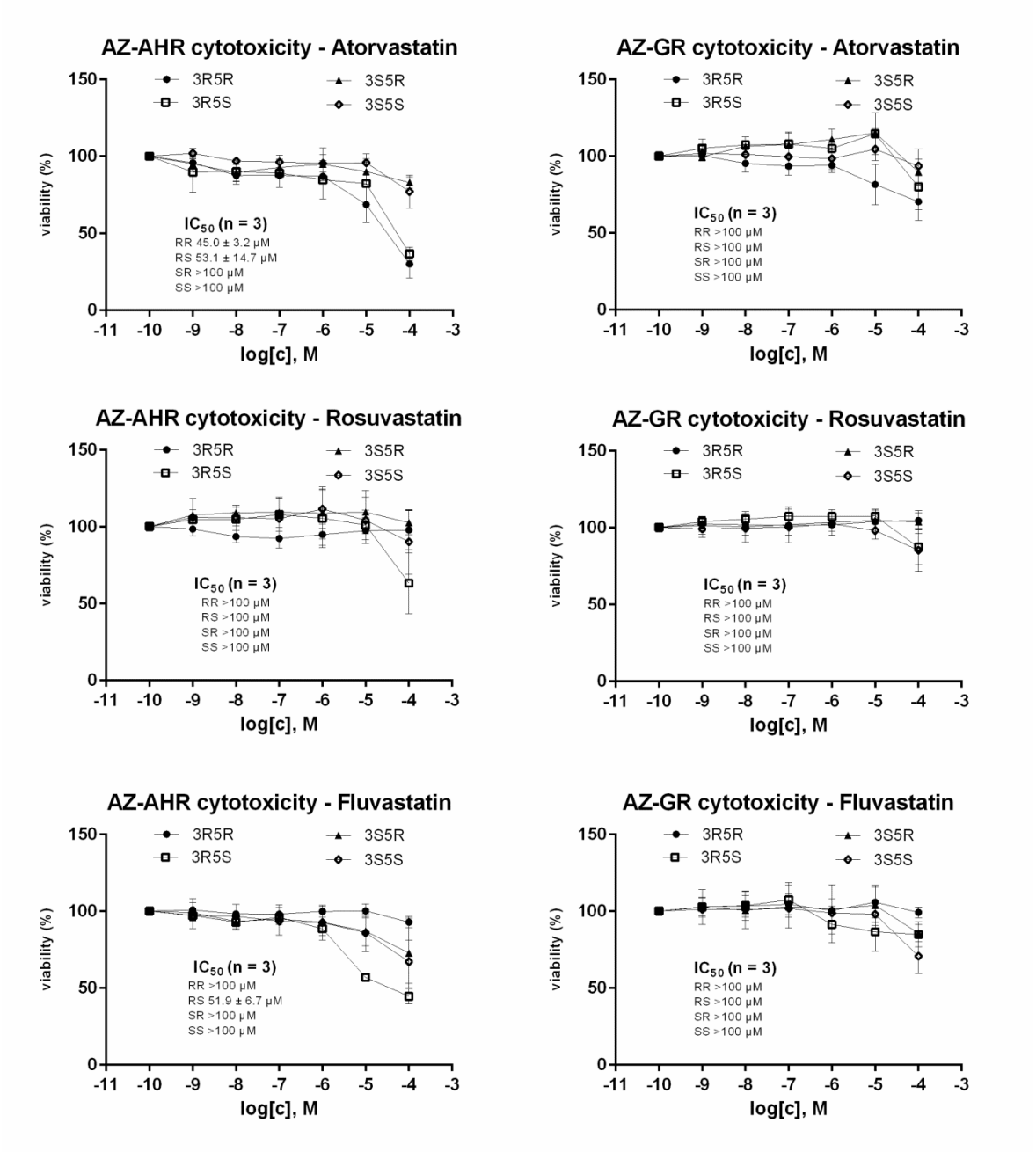

Figure 10: Cytotoxicity of statin diastereomers towards human cancer cell lines AZ-AHR and AZ-GR. The cells were seeded in 96-well plates, stabilised for 16 h, and then incubated for 24 h with single-isomer forms of atorvastatin, rosuvastatin, or fluvastatin at concentrations ranging from 10^{-10} to 10^{-4} M. The vehicle was DMSO (0.1%, v/v). After the treatment, a conventional MTT assay was performed, and absorbance was measured at 540 nm. Treatments were performed in triplicate. The data are shown as mean ± SD from experiments performed on three consecutive passages of cells and are expressed as a percentage of viability of control cells. The values of IC_{50} were calculated where appropriate, and they are indicated in the plots.

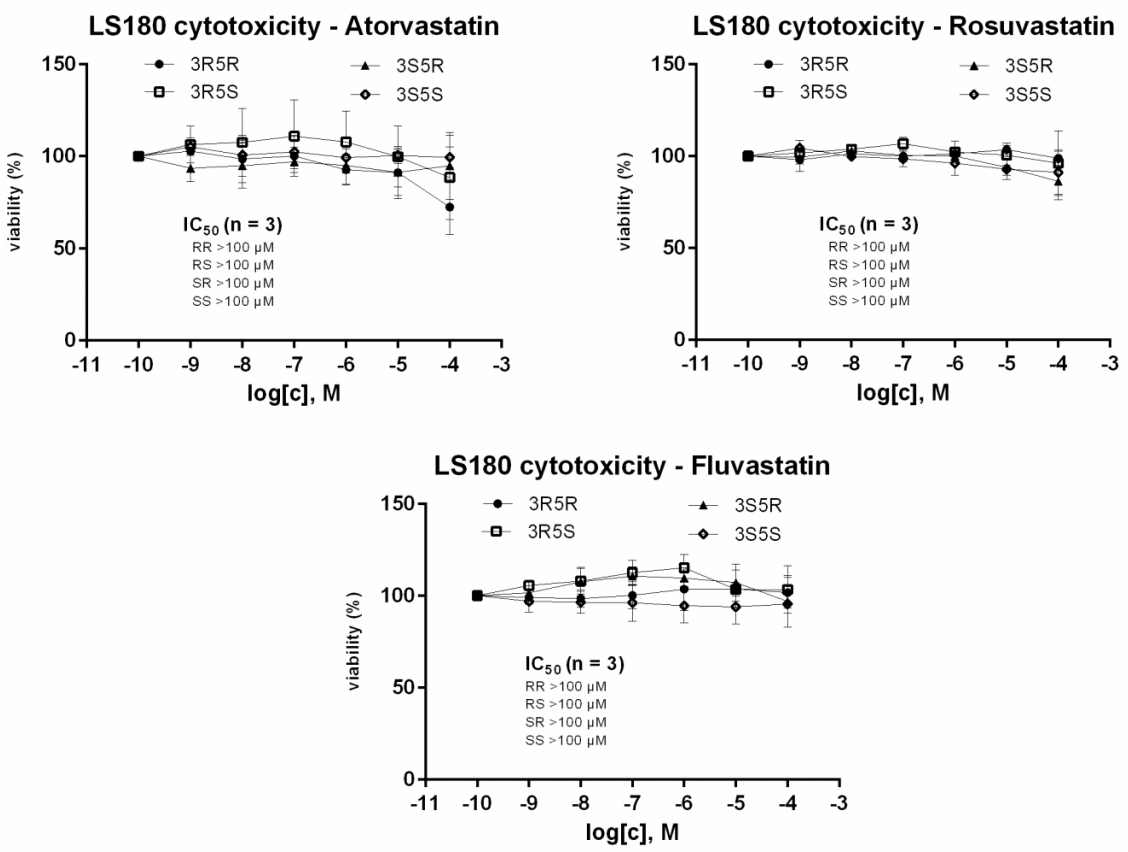

Figure 11: Cytotoxicity of statin diastereomers towards human cancer cell line LS180. The cells were seeded in 96-well plates, stabilised for 16 h, and then incubated for 24 h with single-isomer forms of atorvastatin, rosuvastatin, or fluvastatin at concentrations ranging from 10^{-10} to 10^{-4} M. The vehicle was DMSO (0.1%, v/v). After the treatment, a conventional MTT assay was conducted, and absorbance was measured at 540 nm. Treatments were performed in triplicate. The data are presented as mean ± SD from experiments performed on three consecutive passages of cells and are expressed as a percentage of viability of control cells. The values of IC_{50} were calculated where appropriate and are indicated in the plots.

5.1.2 Effects of statin diastereomers on transcriptional activity of AhR

The effects of individual diastereomers of statins on transcriptional activity of AhR were examined in the AZ-AHR stable reporter cell line derived from HepG2 cells, carrying several dioxin-response elements upstream of a luciferase gene (Novotna *et al.*, 2011). For this purpose, AZ-AHR cells were incubated for 24 h with single-isomer forms of AT, ROS, or FLU in the absence (agonist mode) or presence (antagonist mode) of TCDD (5 nM). The vehicle was DMSO (0.1%, v/v). After that, the cells were lysed, and luciferase activity was measured. Treatment of AZ-AHR cells with TCDD for 24 h resulted in average induction of 1107-fold, as compared with that in DMSO-treated cells. No significant induction of luciferase activity was observed for any AT diastereomer. 3S5S-ROS, but not other optical isomers, dose-dependently increased luciferase activity with average EC_{50} of 17.5 ± 0.4 µM (Figure 12). 3R5R-, 3S5R-, and 3S5S-FLU slightly increased luciferase activity and showed EC_{50} values of 22.0 ± 13.4, 14.4 ± 4.2,

and 14.7 ± 0.9 µM, respectively. Nevertheless, the efficacy of optical isomers of FLU and ROS was below 0.1% of the induction caused by TCDD (Figure 12). TCDD-inducible transcriptional activity of AhR was dose-dependently inhibited by 3R5S-ROS and 3R5S-FLU (Figure 13). The decrease in TCDD-inducible luciferase activity correlated with a decrease in AZ-AHR cell viability (Figure 10). For this reason, the observed effect was likely due to the cytotoxicity of the compounds than to antagonism of AhR. All other forms of ROS, FLU, and all AT diastereomers did not antagonise AhR. Thus, the gene reporter assays in AZ-AHR cells pointed to zero clinical or toxicological potential of statin diastereomers in terms of AhR activation.

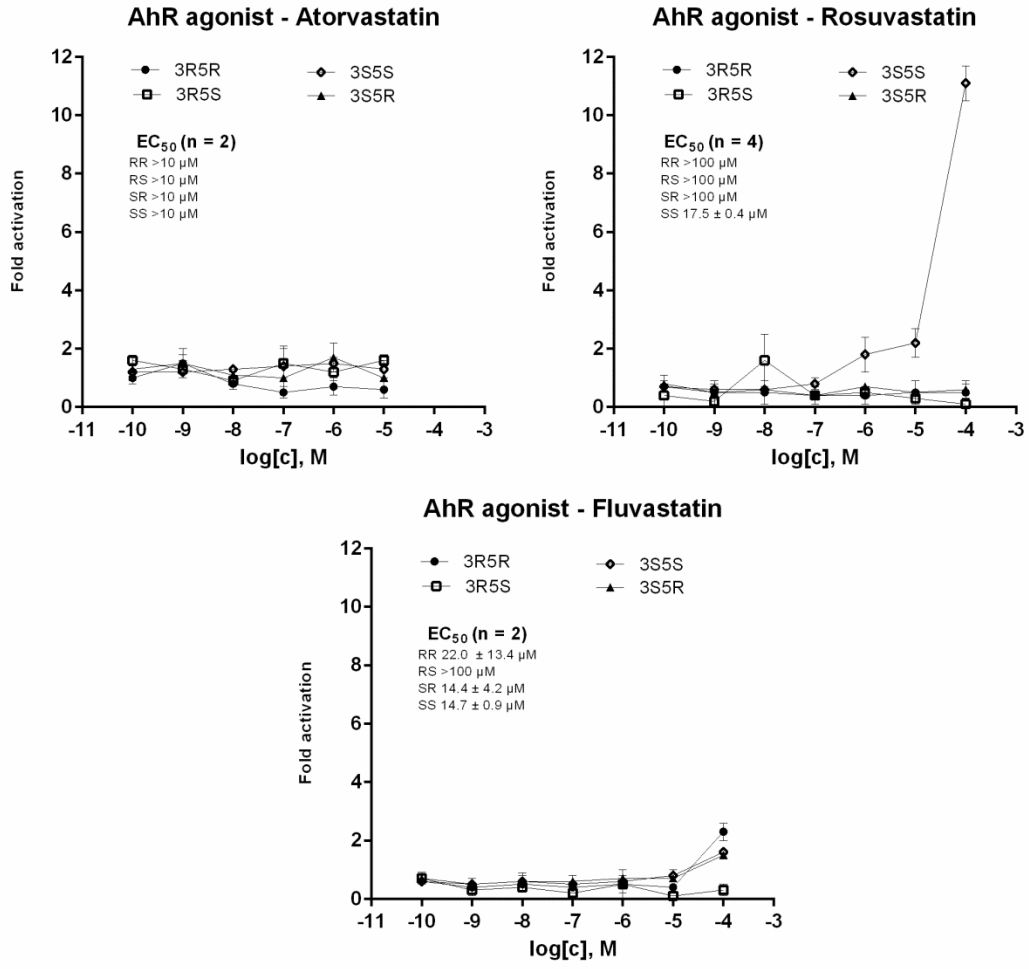

Figure 12: Effects of statin diastereomers on transcriptional activity of human AhR (agonist mode). AZ-AHR cells were seeded in 96-well plates, stabilised for 16 h, and then incubated for 24 h with single-isomer forms of atorvastatin (AT), rosuvastatin (ROS), or fluvastatin (FLU) at concentrations ranging from 10^{-10} to 10^{-5} M (AT) or 10^{-4} M (ROS, FLU). The vehicle was DMSO (0.1%, v/v). After the treatments, the cells were lysed, and luciferase activity was measured. Treatments were carried out in triplicate. Data are expressed as a fold induction of luciferase activity relative to control cells. The values of EC_{50} from *n* independent cell passages were calculated where appropriate, and the average values are indicated in the plots. Representative gene reporter assays are shown.

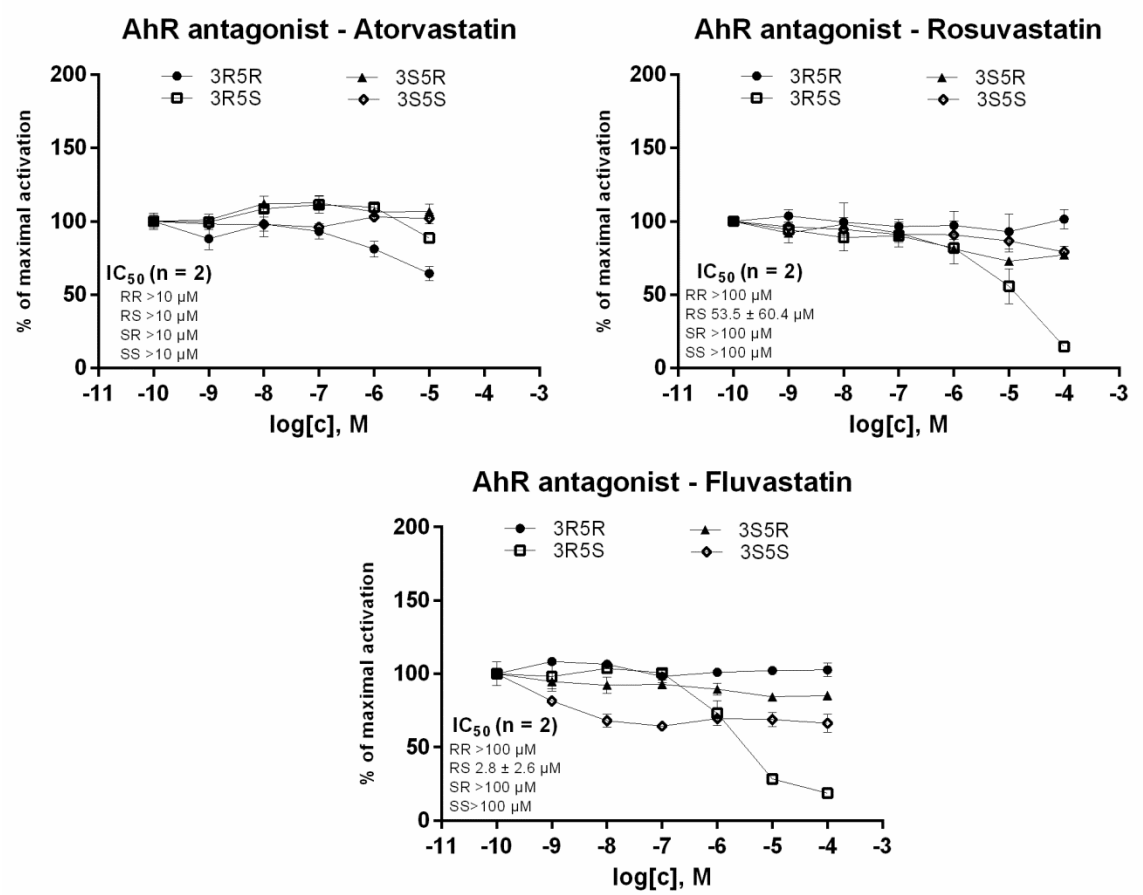

Figure 13: Effects of statin diastereomers on transcriptional activity of human AhR (antagonist mode). AZ-AHR cells were seeded in 96-well plates, stabilised for 16 h and, then incubated for 24 h with single-isomer forms of atorvastatin (AT), rosuvastatin (ROS), or fluvastatin (FLU) at concentrations ranging from 10^{-10} to 10^{-5} M (AT)/10^{-4} M (ROS, FLU) in the presence of dioxin (TCDD; 5 nM). The vehicle was DMSO (0.1%, v/v). After the treatments, the cells were lysed, and luciferase activity was measured. The treatments were performed in triplicate. Data are expressed as a percentage of maximal activation caused by TCDD. The values of IC_{50} from *n* independent cell passages were calculated where appropriate, and the average values are indicated in the plots. Representative gene reporter assays are shown.

5.1.3 Effects of statin diastereomers on transcriptional activity of GR

We studied the effects of statin diastereomers on transcriptional activity of GR in the AZ-GR stable reporter cell line derived from HeLa cells, carrying several glucocorticoid-response elements upstream of a luciferase gene (Novotna *et al.*, 2012). AZ-GR cells were incubated for 24 h with the tested compounds in the absence (agonist mode) or presence (antagonist mode) of DEX (100 nM). The vehicle was DMSO (0.1%, v/v). After the treatments, the cells were lysed, and luciferase activity was measured. The average induction of GR-dependent luciferase activity by model agonist DEX was 32-fold, as compared with that in DMSO-treated cells. None of the tested statins induced GR-dependent luciferase activity (Figure 14). DEX-inducible transcriptional activity of GR was

decreased by 3R5R-AT (10 µM) and 3R5S-ROS (100 µM; Figure 15), but the decrease was not dose-dependent and probably happened due to cytotoxicity of the tested compounds (Figure 10). However, FLU diastereomers, except for 3S5R-FLU, dose-dependently antagonised GR, and significant differences ($p < 0.05$) between IC_{50} values were observed for 3R5R-FLU versus 3R5S-FLU and 3R5S-FLU versus 3S5S-FLU (Figure 15).

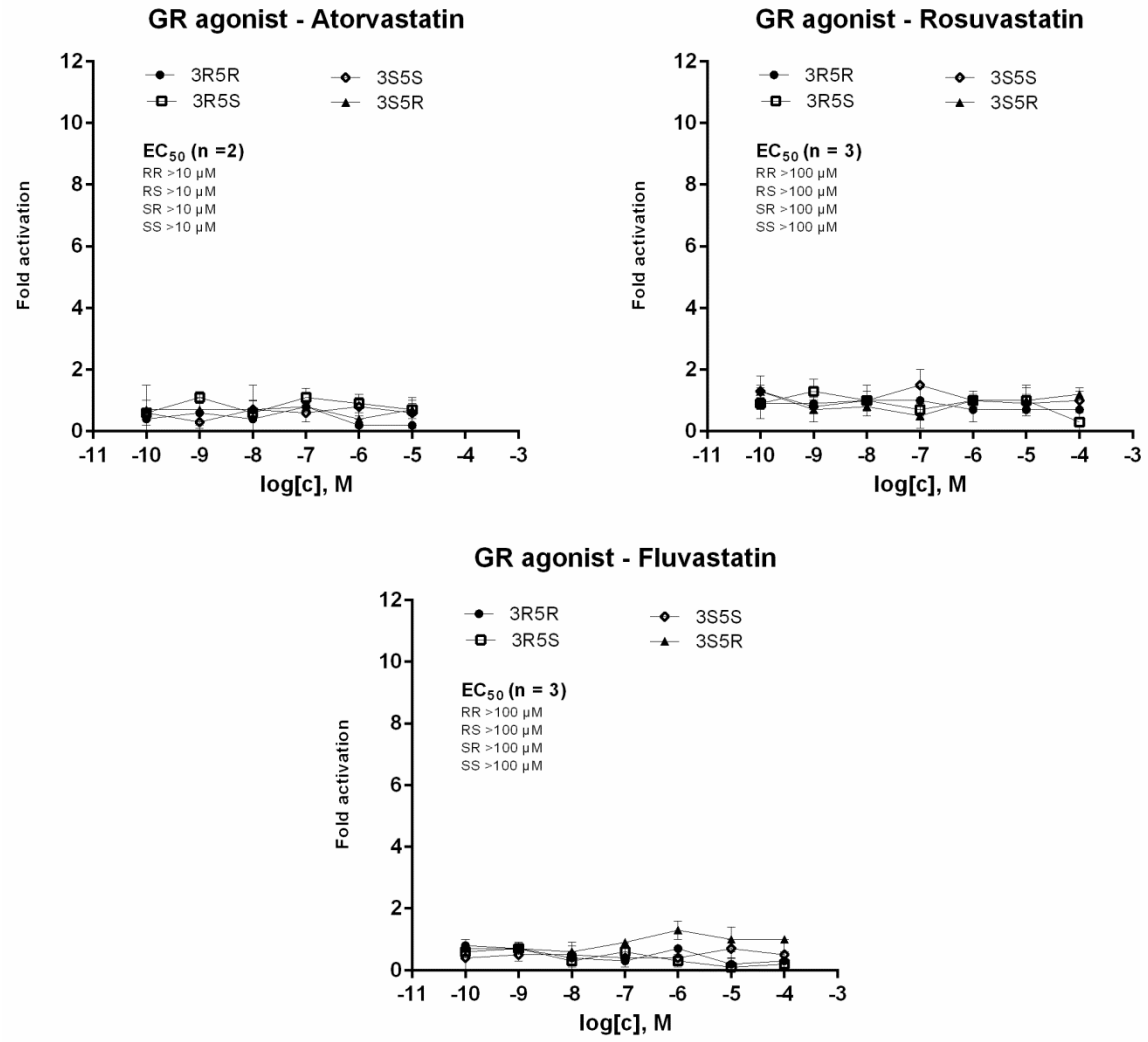

Figure 14: Effects of statin diastereomers on transcriptional activity of human GR (agonist mode). AZ-GR cells were seeded in 96-well plates, stabilised for 16 h, and then incubated for 24 h with single-isomer forms of atorvastatin (AT), rosuvastatin (ROS), or fluvastatin (FLU) at concentrations ranging from 10^{-10} to 10^{-5} M (AT)/10^{-4} M (ROS, FLU). The vehicle was DMSO (0.1%, v/v). After the treatments, the cells were lysed, and luciferase activity was measured. Treatments were performed in triplicate. Data are expressed as a fold induction of luciferase activity over control cells. The values of EC_{50} from *n* independent cell passages were calculated where appropriate, and the average values are indicated in the plots. Representative gene reporter assays are shown.

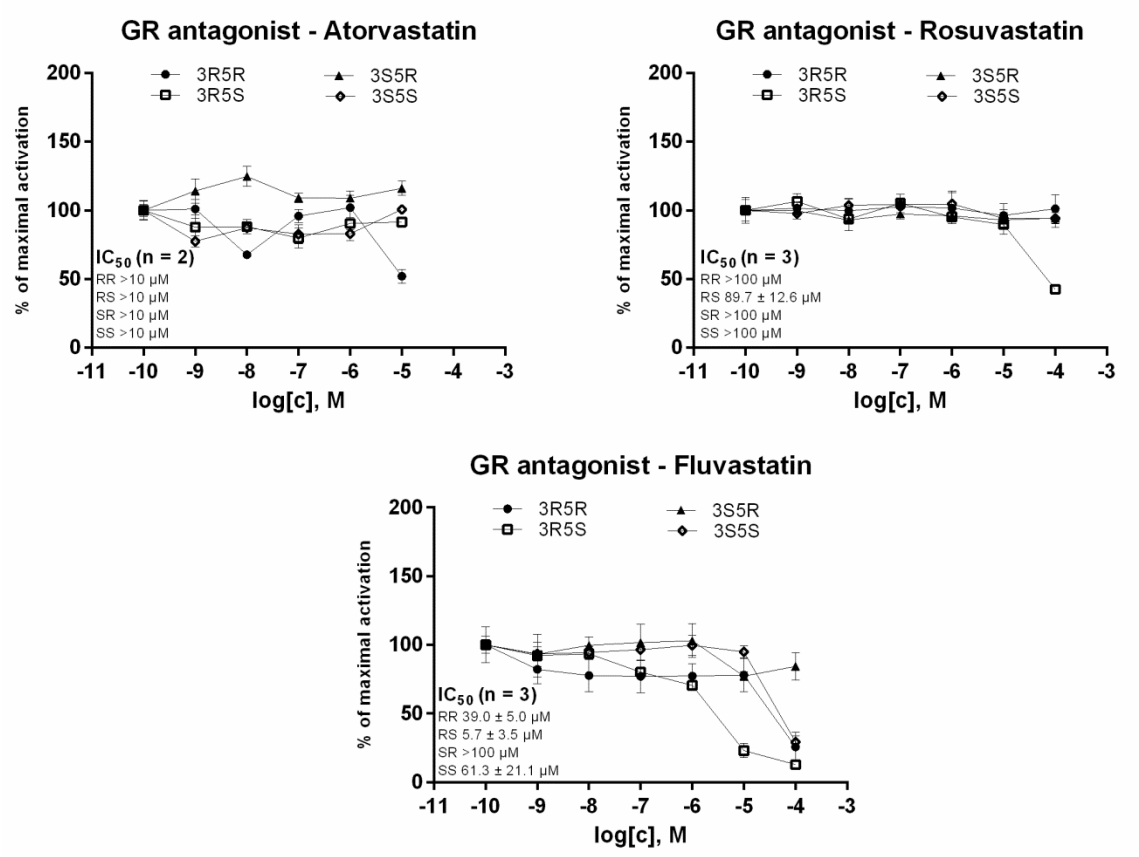

Figure 15: Effects of statin diastereomers on transcriptional activity of human GR (antagonist mode). AZ-GR cells were seeded in 96-well plates, stabilised for 16 h, and then incubated for 24 h with single-isomer forms of atorvastatin (AT), rosuvastatin (ROS), and fluvastatin (FLU) at concentrations ranging from 10^{-10} to 10^{-5} M (AT)/10^{-4} M (ROS, FLU) in the presence of dexamethasone (DEX; 100 nM). The vehicle was DMSO (0.1%, v/v). After the treatments, the cells were lysed, and luciferase activity was measured. Treatments were conducted in triplicate. Data are expressed as a percentage of maximal activation caused by DEX. The values of IC_{50} from *n* independent cell passages were calculated where appropriate, and the average values are indicated in the plots. Representative gene reporter assays are shown.

5.1.4 Effects of statin diastereomers on transcriptional activity of PXR

The effects were studied in human colon adenocarcinoma cells LS180 transiently transfected with the p3A4-luc reporter construct. Next, the cells were incubated for 24 h with the tested compounds in the absence (agonist mode) or presence (antagonist mode) of RIF (10 µM). The vehicle was DMSO (0.1%, v/v). After the treatments, the cells were lysed, and luciferase activity was measured. The average induction of PXR-dependent luciferase activity by model agonist RIF was 10-fold, as compared with that in DMSO-treated cells. The transcriptional activity of PXR was dose-dependently induced by all the tested statins, and the potency and efficacy among individual optical isomers varied significantly (Figure 16). The efficacy values of 3R5R-AT, 3R5S-AT, and 3S5R-AT at the highest concentrations slightly varied, with average induction of luciferase

activity approx. 5-fold. In contrast, the efficacy of 3S5S-AT (100 µM) was much higher, reaching 11-fold induction. The EC_{50} for 3S5S-AT, 3R5S-AT, and 3S5R-AT ranged from 11.6 to 15.0 µM. Of note, the potency of clinically used 3R5R-AT was significantly different ($p < 0.05$) from that of 3R5S-AT. The efficacy values of ROS diastereomers at the highest concentrations slightly varied, with average induction of luciferase activity approx. 3-fold. However, significant differences ($p < 0.05$) between potency indicators (EC_{50}s) of ROS diastereomers were observed against PXR for 3R5R-ROS versus 3S5R-ROS, 3R5S-ROS versus 3S5S-ROS, and 3S5R-ROS versus 3S5S-ROS (Figure 16). The efficacy values of FLU diastereomers at the highest concentrations slightly varied, with average induction of luciferase activity approx. 5-fold (for 3R5R-FLU and 3S5R-FLU) and 3-fold (for 3R5S-FLU and 3S5S-FLU). The potency of optical isomers of FLU was comparable and half-maximal effective concentrations (EC_{50}) ranged from 8.7 to 15.4 µM (Figure 16). Ligand-inducible transcriptional activity of PXR was not affected by any AT diastereomer (Figure 17). RIF-inducible PXR transcriptional activity was not influenced by 3S5R-ROS, whereas 3R5S-ROS yielded an inverse U-shaped curve, just as FLU diastereomers did. In contrast, 3R5R-ROS and 3S5S-ROS yielded a U-shaped curve with a minimum in the concentration range 10^{-8} to 10^{-6} M (60% of RIF value). Combined treatments with RIF and FLU diastereomers revealed inverted U-shaped curves. Dose-dependent augmentation of RIF-inducible luciferase activity (150% of rifampicin value) was observed for all three statins, regardless of optical configuration, at concentrations up to 10^{-6} M, followed by a drop in luciferase activity (85% of RIF value) at statin concentrations of 10^{-4} M (Figure 17).

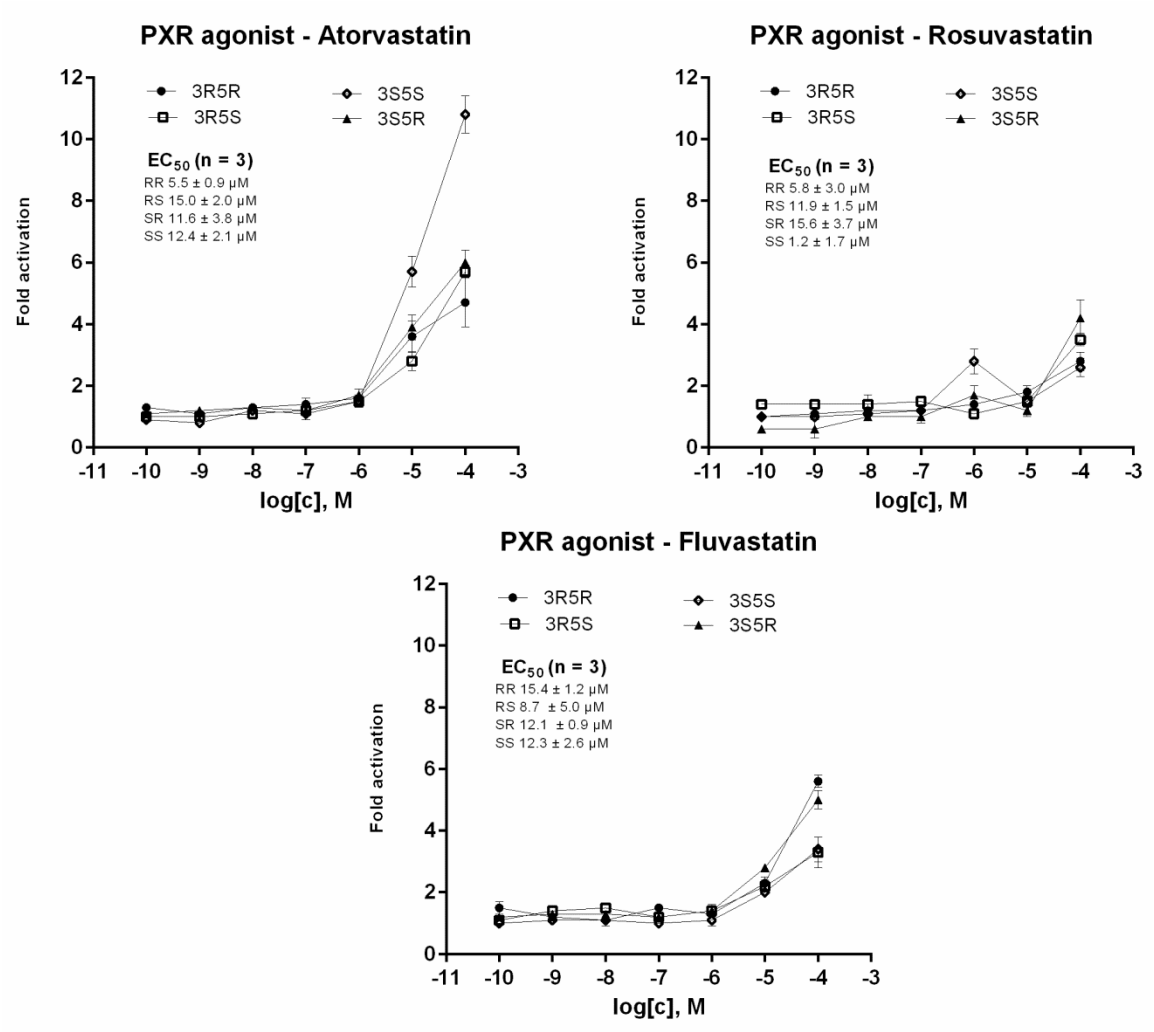

Figure 16: Effects of statin diastereomers on transcriptional activity of human PXR (agonist mode). LS180 cells, transiently transfected with the p3A4-luc reporter, were seeded in 96-well plates, stabilised for 16 h, and then incubated for 24 h with single-isomer forms of atorvastatin, rosuvastatin, and fluvastatin at concentrations ranging from 10^{-10} to 10^{-4} M. The vehicle was DMSO (0.1%, v/v). After the treatments, the cells were lysed, and luciferase activity was measured. Treatments were carried out in triplicate. Data are expressed as a fold induction of luciferase activity over control cells. The values of EC_{50} from *n* independent cell passages were calculated where appropriate, and the average values are indicated in the plots. Representative gene reporter assays are shown.

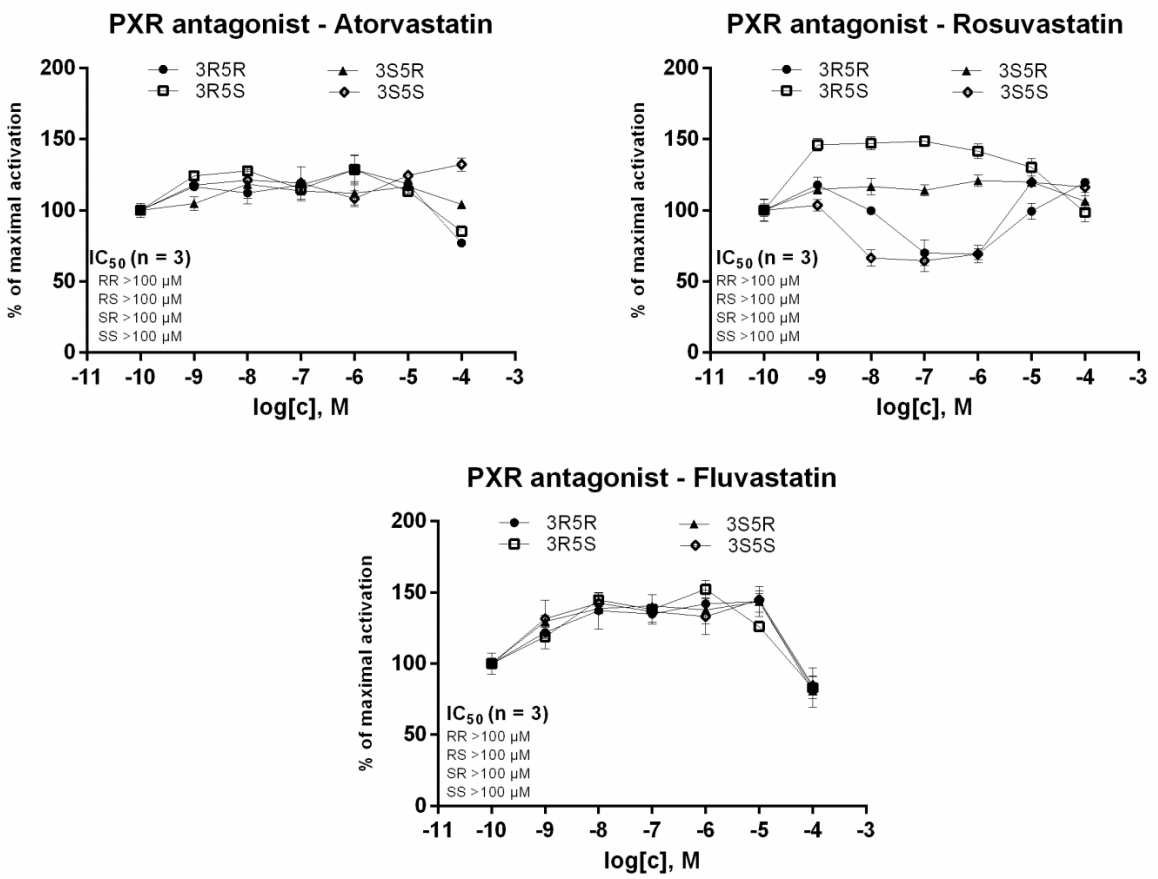

Figure 17: Effects of statin diastereomers on transcriptional activity of human PXR (antagonist mode). LS180 cells, transiently transfected with the p3A4-luc reporter, were seeded in 96-well plates, stabilised for 16 h, and then incubated for 24 h with single-isomer forms of atorvastatin, rosuvastatin, or fluvastatin at concentrations ranging from 10^{-10} to 10^{-4} M in the presence of rifampicin (RIF; 10 µM). The vehicle was DMSO (0.1%, v/v). After that, the cells were lysed, and luciferase activity was measured. Treatments were performed in triplicate. Data are expressed as a percentage of the maximal activation caused by RIF. The values of IC_{50} from *n* independent cell passages were calculated where appropriate, and the average values are indicated in the plots. Representative gene reporter assays are shown.

5.1.5 Effects of statin diastereomers on the expression of drug-metabolizing cytochrome P450s in primary human hepatocytes

The ability of statin diastereomers to induce transcriptionally regulated drug-metabolizing cytochrome P450s was tested in three human hepatocyte cultures (HH59, HH61, and HH63). Hepatocytes were treated for 24 h (for quantification of mRNA) or 48 h (for quantification of proteins) with optical isomers of tested statins, TCDD (5 nM), RIF (10 µM), and vehicle DMSO (0.1%, v/v). Then, mRNA was isolated, and cDNA was synthesised by reverse transcription. The amounts of mRNAs were measured by qPCR and normalised to *GAPDH* as a housekeeping gene. The levels of CYP proteins were determined by Simple Western blotting. Target proteins were identified using primary antibodies, a horseradish peroxidase-conjugated secondary antibody, and a chemiluminescent substrate.

The resulting chemiluminescent signal was quantified, and the CYP signals were normalised to that of β-actin as a loading control.

5.1.5.1 Effects of statin diastereomers on the expression of CYP1A1 and CYP1A2

TCDD strongly induced *CYP1A1/CYP1A2* mRNAs in all the human hepatocyte cultures after 24 h of incubation, and the magnitudes of induction in cultures of HH59, HH61, and HH63 were 98-/110-fold, 339-/143-fold, and 278-/45-fold, respectively. None of the tested statins significantly induced *CYP1A1* or *CYP1A2* mRNA in any human hepatocyte culture (Figure 18A). TCDD strongly induced CYP1A1/CYP1A2 proteins in all human hepatocyte cultures after 48 h of incubation, and the magnitudes of induction in cultures of HH59, HH61, and HH63 were 35-/19-fold, 220-/83-fold, and 35-/50-fold, respectively. In agreement with the mRNAs levels, we did not observe a significant induction of CYP1A1 or CYP1A2 protein by any tested statin diastereomer (Figure 18B). Given the dominant regulation of CYP1A1 and CYP1A2 by AhR, the effects of statins on CYP1A1 and CYP1A2 expression are consistent with their effects on AhR in gene reporter assays (Figure 12).

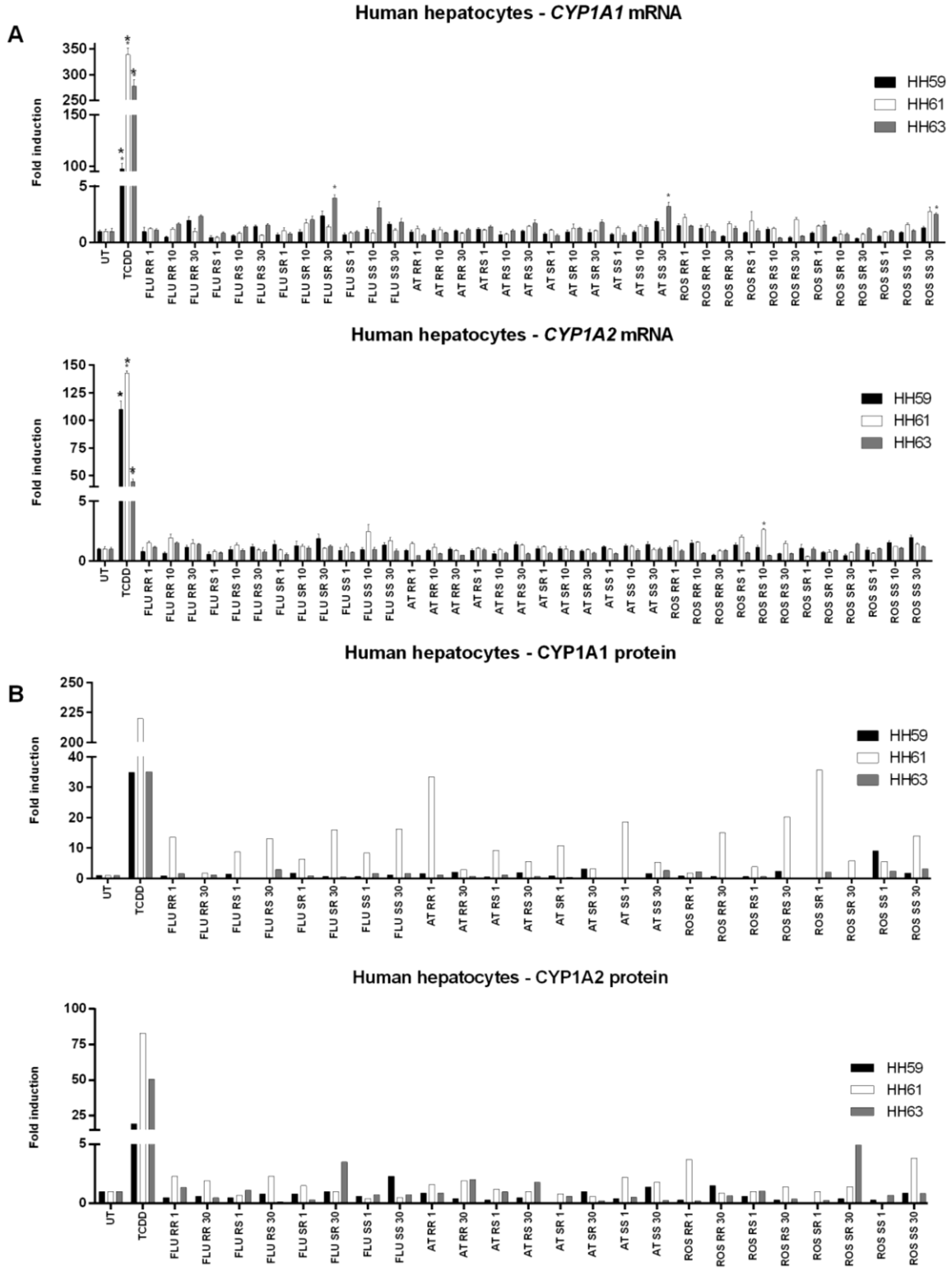

Figure 18: Effects of statin diastereomers on the expression of CYP1A1 and CYP1A2 in primary human hepatocytes. Primary human hepatocytes from three donors (HH59, HH61, and HH63) were used. Panel A: The cells were incubated for 24 h with DMSO (0.1%, v/v), TCDD (5 nM), and individual diastereomers of statins (1, 10, or 30 µM). Bar graphs of qPCR analyses of *CYP1A1* and *CYP1A2* mRNAs are shown. The data are shown as mean ± SD from triplicate measurements and are expressed as a fold induction over vehicle-treated cells. The data were normalised to *GAPDH* mRNA levels; Panel B: The cells were incubated for 48 h with DMSO (0.1%, v/v), TCDD (5 nM), and individual diastereomers of statins (1 or 30 µM). Bar graphs of Simple Western blotting analyses of CYP1A1 and CYP1A2 proteins are shown. The data are expressed as a fold induction over vehicle-treated cells and were normalised to β-actin levels.

5.1.5.2 Effects of statin diastereomers on the expression of CYP2A6

Induction of *CYP2A6* mRNA by RIF after 24 h of incubation was 2.4-fold, 7.4-fold, and 5.7-fold in cultures of HH59, HH61, and HH63, respectively (Figure 19A). Induction of CYP2A6 protein by RIF after 48 h of incubation was 4.8-fold, 7.3-fold, and 2.3-fold in cultures of HH59, HH61, and HH63, respectively (Figure 19B). Induction profiles of CYP2A6 under the influence of FLU diastereomers differed among individual cultures; this phenomenon could be caused by individual differences among the donors. Generally, 3S5S-FLU, 3R5S-FLU, 3S5R-FLU, but not 3R5R-FLU, weakly induced *CYP2A6* mRNA and protein. In comparison with that by ROS and FLU, induction of *CYP2A6* mRNA by AT diastereomers was the highest among all the human hepatocyte cultures, and in some cases, even higher than that caused by RIF, pointing again to inter-individual variability among human hepatocyte donors. The magnitude of CYP2A6 induction by AT diastereomers was as follows (descending order): 3R5R-AT > 3R5S-AT = 3S5R-AT > 3S5S-AT. On the contrary, ROS diastereomers did not significantly induce the expression of *CYP2A6* mRNA and protein, with the exception of a weak increase in *CYP2A6* mRNA in culture of HH61 by 3R5S-ROS (10 and 30 μM).

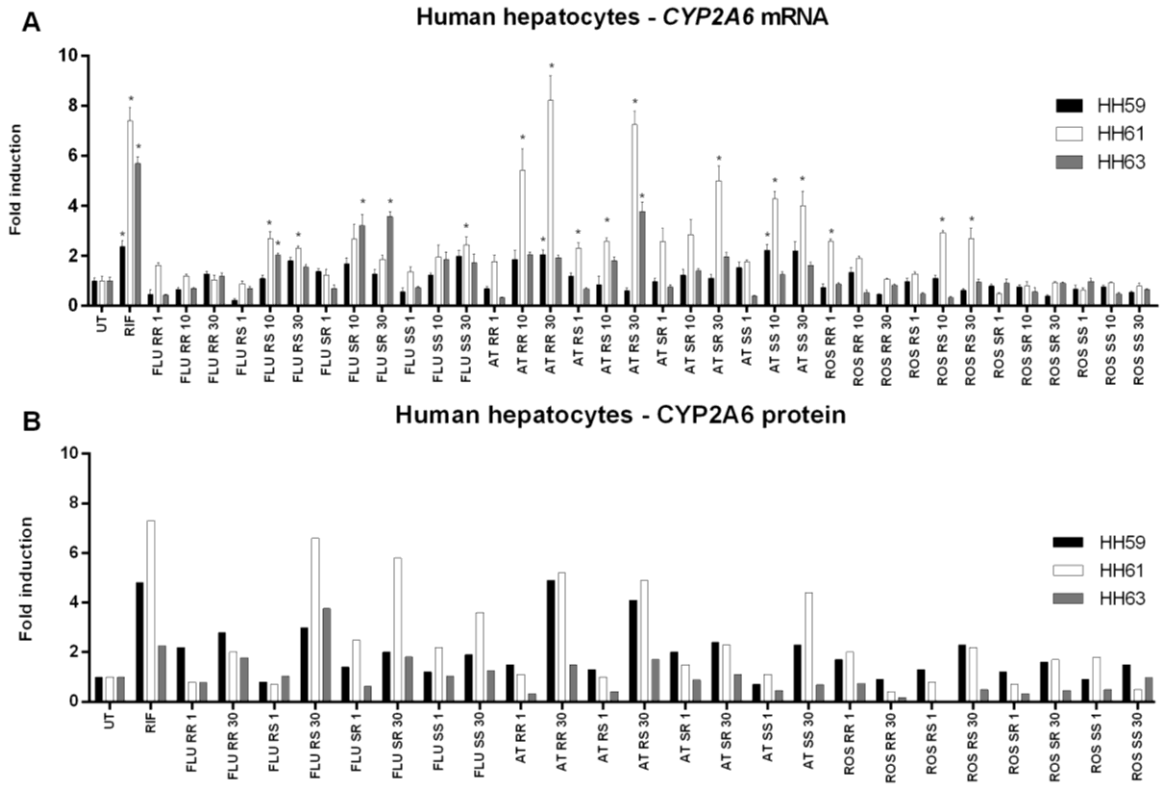

Figure 19: Effects of statin diastereomers on the expression of CYP2A6 in primary human hepatocytes. Primary human hepatocytes from three donors (HH59, HH61, and HH63) were used. Panel A: The cells were incubated for 24 h with DMSO (0.1%, v/v), RIF (10 µM), and individual diastereomers of statins (1, 10, or 30 µM). A bar graph of qPCR analyses of *CYP2A6* mRNA is shown. The data are presented as mean ± SD from triplicate measurements and are expressed as a fold induction over vehicle-treated cells. The data were normalised to *GAPDH* mRNA levels. Panel B: The cells were incubated for 48 h with DMSO (0.1%, v/v), RIF (10 µM), and individual diastereomers of statins (1 or 30 µM). A bar graph of Simple Western blotting analyses of CYP2A6 protein is shown. The data are expressed as a fold induction over vehicle-treated cells and were normalised to β-actin levels. *Significantly different from DMSO-treated cells ($p < 0.05$).

5.1.5.3 Effects of statin diastereomers on the expression of CYP2B6

Induction levels of CYP2B6 mRNA/protein by RIF after 24/48 h of incubation were 5.8-/8.4-fold, 16-/5.9-fold, and 6.9-/7.7-fold in cultures of HH59, HH61, and HH63, respectively (Figure 20A, 20B). The induction profiles of CYP2B6 for the tested statins showed a pattern analogous to that for CYP2A6. CYP2B6 was moderately and dose-dependently induced by 3S5S-FLU, 3R5S-FLU, and 3S5R-FLU, but not 3R5R-FLU, in all the hepatocyte cultures. AT was the strongest inducer of CYP2B6, as compared to ROS and FLU. Dose-dependent induction of *CYP2B6* mRNA and protein by AT optical isomers was as follows (descending order): 3R5R-AT > 3R5S-AT = 3S5S-AT > 3S5R-AT. ROS induced neither *CYP2B6* mRNA nor CYP2B6 protein in any human hepatocyte culture.

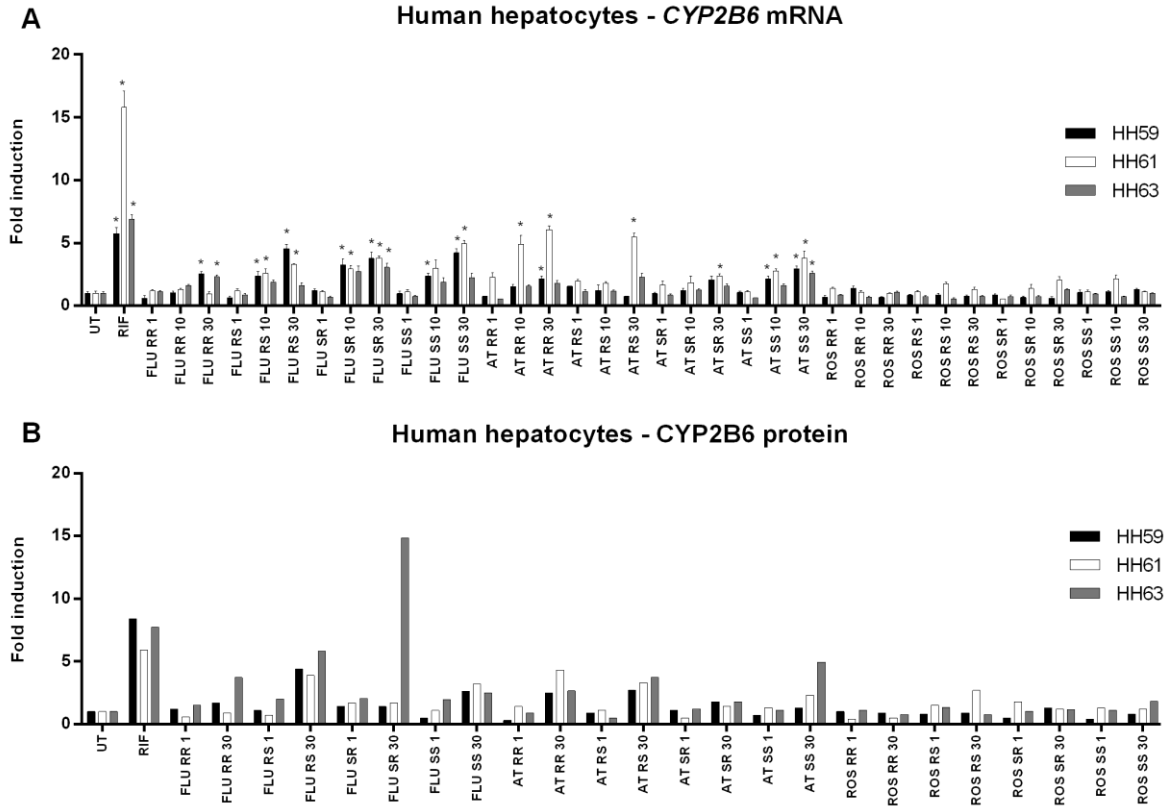

Figure 20: Effects of statin diastereomers on the expression of CYP2B6 in primary human hepatocytes. Primary human hepatocytes from three donors (HH59, HH61, and HH63) were used. Panel A: The cells were incubated for 24 h with DMSO (0.1%, v/v), RIF (10 µM), and individual diastereomers of statins (1, 10, or 30 µM). A bar graph of qPCR analyses of *CYP2B6* mRNA is shown. The data are shown as mean ± SD from triplicate measurements and are expressed as a fold induction over vehicle-treated cells. The data were normalised to *GAPDH* mRNA levels. Panel B: The cells were incubated for 48 h with DMSO (0.1%, v/v), RIF (10 µM), and individual diastereomers of statins (1 or 30 µM). A bar graph of Simple Western blotting analyses of CYP2B6 protein is shown. The data are expressed as a fold induction over vehicle-treated cells and normalised to β-actin levels. *Significantly different from DMSO-treated cells ($p < 0.05$).

5.1.5.4 Effects of statin diastereomers on the expression of CYP2C9

Induction of *CYP2C9* mRNA by RIF after 24 h of incubation in the three human hepatocyte cultures varied from 1.5- to 3-fold (Figure 21). The induction of CYP2C9 protein was not measured because commercial CYP2C9 antibodies were not compatible with the Sally Sue Simple Western System used for the analyses. The effects of optical isomers of the statins under study on *CYP2C9* mRNA expression were rather stimulatory, causing weak induction, with patterns similar to those for *CYP2A6* and *CYP2B6* mRNAs.

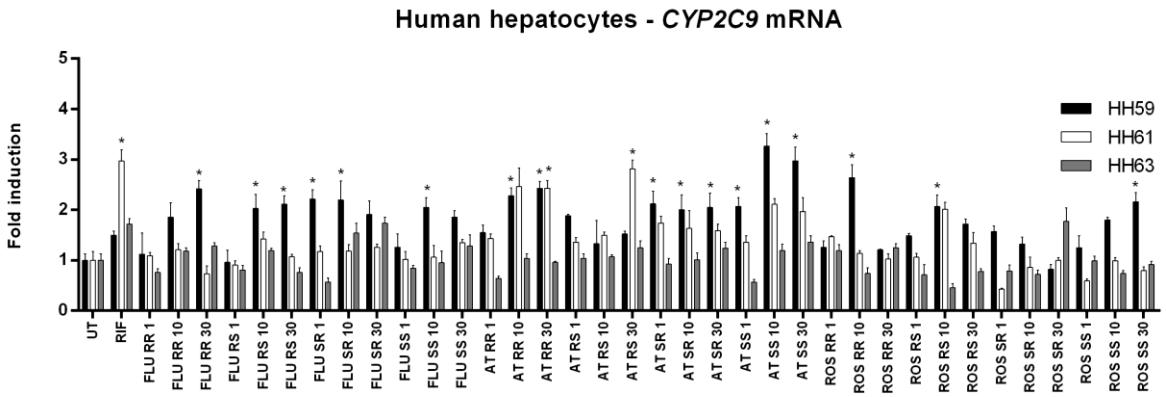

Figure 21: Effects of statin diastereomers on the expression of CYP2C9 in primary human hepatocytes. Primary human hepatocytes from three donors (HH59, HH61, and HH63) were used. The cells were incubated for 24 h with DMSO (0.1%, v/v), RIF (10 µM), and individual diastereomers of statins (1, 10, or 30 µM). A bar graph of qPCR analyses of *CYP2C9* mRNA is shown. The data are presented as mean ± SD from triplicate measurements and are expressed as a fold induction over vehicle-treated cells. The data were normalised to *GAPDH* mRNA levels. *Significantly different from DMSO-treated cells ($p < 0.05$).

5.1.5.5 Effects of statin diastereomers on the expression of CYP3A4

Induction levels of CYP3A4 mRNA/protein by RIF after 24/48 h of incubation were 73-/63-fold, 73-/9-fold, and 11-/18-fold in cultures of HH59, HH61, and HH63, respectively (Figure 22A, 22B). FLU dose-dependently induced *CYP3A4* mRNA and protein in all the human hepatocyte cultures. The effects of 3S5S-FLU, 3R5S-FLU, and 3S5R-FLU were nearly equipotent, while 3R5R-FLU was a much weaker inducer of CYP3A4 than the other diastereomers. All AT optical isomers strongly and dose-dependently induced CYP3A4 mRNA and protein in all the human hepatocyte cultures. The magnitude of CYP3A4 induction differed for individual diastereomers as follows: 3R5R-AT > 3R5S-AT = 3S5S-AT > 3S5R-AT. On the contrary, ROS did not induce *CYP3A4* mRNA, but 3S5R-ROS and 3R5S-ROS up-regulated CYP3A4 protein in two human hepatocyte cultures.

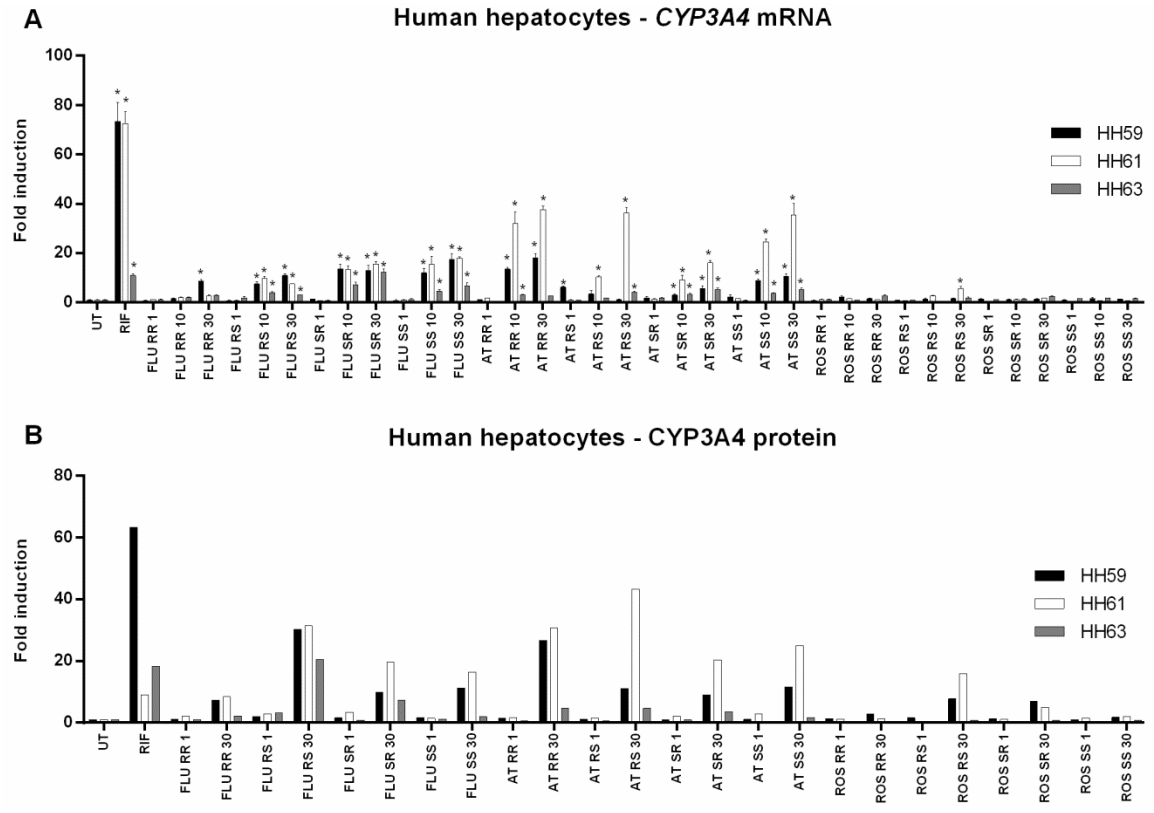

Figure 22: Effects of statin diastereomers on the expression of CYP3A4 in primary human hepatocytes. Primary human hepatocytes from three donors (HH59, HH61, and HH63) were used. Panel A: The cells were incubated for 24 h with DMSO (0.1%, v/v), RIF (10 µM), and individual diastereomers of statins (1, 10, or 30 µM). A bar graph of qPCR analyses of *CYP3A4* mRNA is shown. The data are presented as mean ± SD from triplicate measurements and are expressed as a fold induction over vehicle-treated cells. The data were normalised to *GAPDH* mRNA levels. Panel B: The cells were incubated for 48 h with DMSO (0.1%, v/v), RIF (10 µM), and individual diastereomers of statins (1 or 30 µM). A bar graph of Simple Western analyses of CYP3A4 protein is shown. The data are expressed as a fold induction over vehicle-treated cells and were normalised to β-actin levels. *Significantly different from DMSO-treated cells ($p < 0.05$).

5.1.5.6 Effects of statin diastereomers on the expression of PXR and TAT

GR plays a key role in transcriptional regulation of drug-metabolizing enzymes via multiple mechanisms. For this reason, the expression of tyrosine aminotransferase (*TAT*, an exclusive GR's target gene) and *PXR* (non-exclusive GR's target gene) was also analysed in primary human hepatocytes. These cells are normally cultured in the presence of DEX at a concentration that fully activates GR. As a consequence, the *TAT* and *PXR* genes are induced under these conditions. We tested whether the effects of statin diastereomers on *TAT* expression are inhibitory or synergistic. *TAT* was down-regulated by some of the compounds tested; however, the effects lacked a dose-response pattern and were not consistent among human hepatocyte cultures. The most frequent and pronounced were the effects of FLU diastereomers (Figure 23). This result

is consistent with antagonism of GR by these compounds in the gene reporter assay (Figure 15). Likewise, the tested statins had weak regulatory effects on the expression of *PXR* mRNA; however, the effects varied among hepatocyte cultures and were not dose dependent (Figure 23).

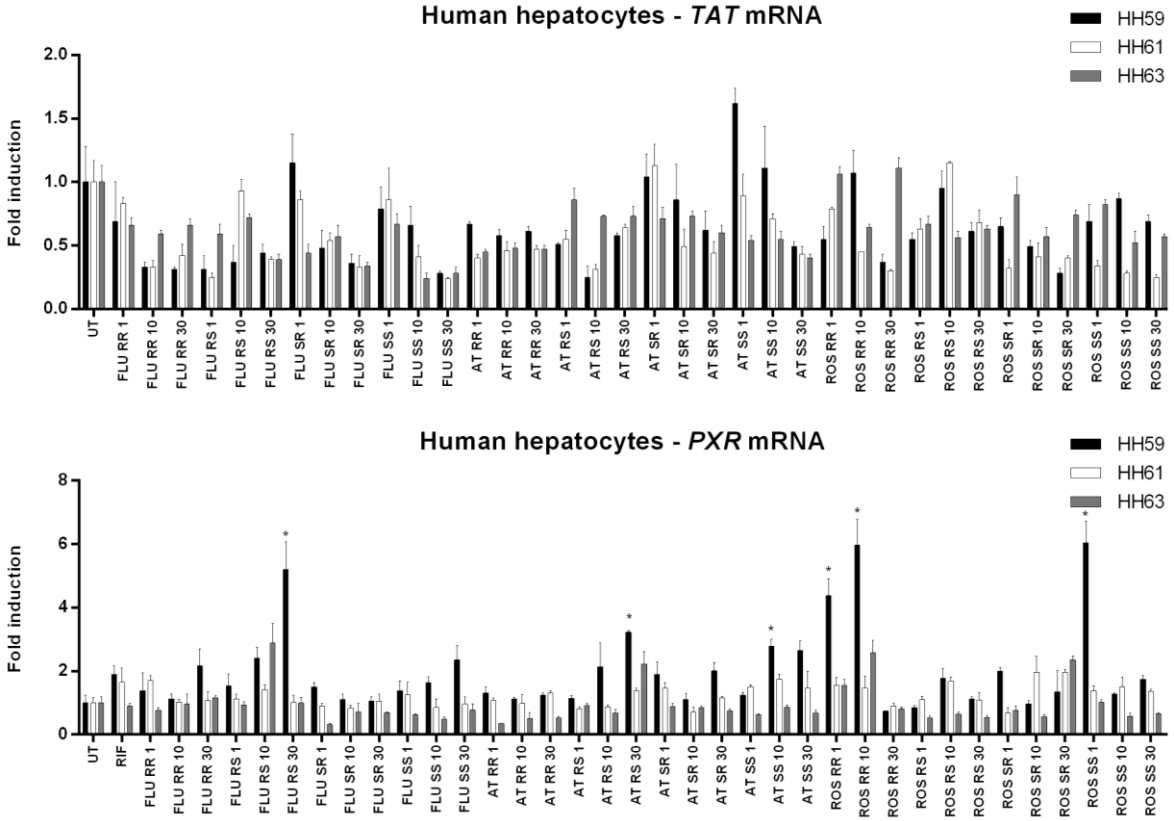

Figure 23: Effects of statin diastereomers on the expression of TAT and PXR in primary human hepatocytes. Primary human hepatocytes from three donors (HH59, HH61, and HH63) were used. The cells were incubated for 24 h with DMSO (0.1%, v/v), RIF (10 µM), and individual diastereomers of statins (1, 10, or 30 µM). A bar graph of qPCR analyses of *TAT* and *PXR* mRNAs is shown. The data are mean ± SD from triplicate measurements and are expressed as a fold induction over vehicle-treated cells. The data were normalised to *GAPDH* mRNA levels. *Significantly different from DMSO-treated cells ($p < 0.05$).

5.1.6 Effects of statin diastereomers on the binding of PXR to DNA

The effects of statins on the formation of the PXR–RXRα–DNA complex were studied by an EMSA. Direct binding of the PXR–RXRα heterodimeric complex to the response elements in the gene promoter is needed for PXR-mediated gene activation. Maximal induction of the *CYP3A4* gene expression apparently requires an additional DR3 nuclear receptor-binding element 1(dNR1; -7733/-7719) in a distal xenobiotic-responsive enhancer module (Goodwin *et al.*, 1999; Lehmann *et al.*, 1998). Nuclear extracts from LS174T cells treated with DMSO (0.1%, v/v), RIF (10 µM), and individual diastereomers of statins at concentration

10 μM were incubated with a biotin-labelled double-stranded oligonucleotide corresponding to the DR3 PXR response element in the *CYP3A4* promoter, and the products were resolved on a non-denaturing gel. The specificity of PXR–RXRα binding was confirmed by competition with an unlabelled double-stranded DR3 oligonucleotide. Positive control RIF strongly stimulated formation of the PXR–RXRα DNA-binding complex (Figure 24A). All the tested compounds increased binding of PXR–RXRα to the DR3 module as compared to vehicle. In many samples, the intensity of bands was similar to that for RIF. Because this method is semi-quantitative, the intensity of individual bands between diastereomers was not quantified. Immunoblot analysis confirmed that equal amounts of the PXR protein were used in the EMSA (Figure 24B).

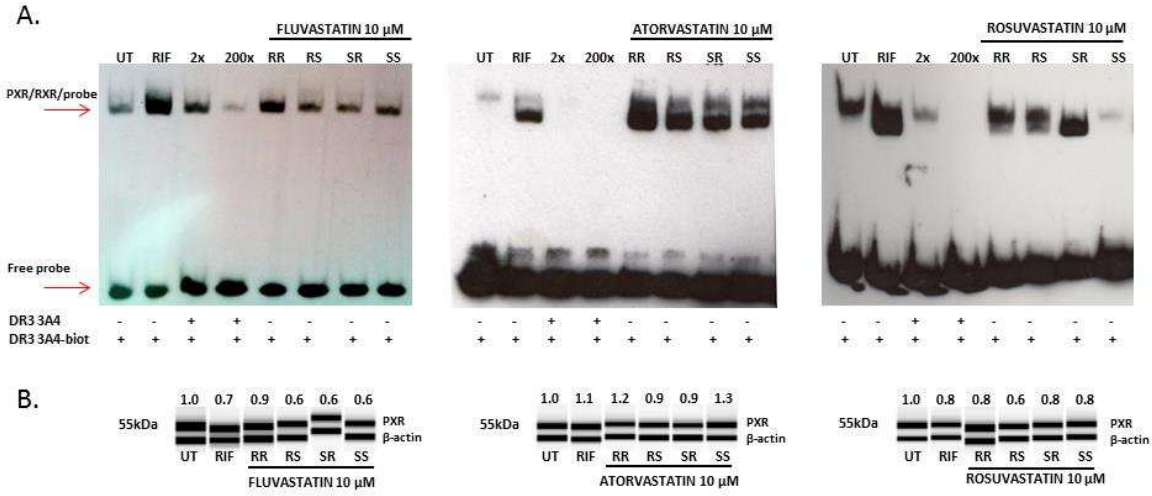

Figure 24: Effects of statin diastereomers on the binding of the PXR–RXR complex to the DR3 motif of the human CYP3A4 gene promoter. Nuclear fractions of LS174T cells from three independent cell passages were incubated for 2 h at 30 °C with DMSO (0.1%, v/v), RIF (10 μM), and individual statin diastereomers at concentration 10 μM. The treated nuclear extracts were incubated with a biotin-labelled CYP3A4-DR3 probe and subjected to electrophoresis in a 5% polyacrylamide gel. Panel A: Formation of the complex of the *CYP3A4* DR3 response element with the PXR–RXRα heterodimer. Panel B: A Simple Western blot showing equal expression levels of PXR in nuclear extracts used for the EMSA (normalised to β-actin levels).

5.2. Effects of dihydropyridine enantiomers on regulatory pathways of human cytochrome P450s

5.2.1 Cytotoxicity of dihydropyridine enantiomers in human cancer cell lines

Prior to the gene reporter assays, cytotoxicity of optical isomers of dihydropyridines was assessed in AZ-AHR, AZ-GR, and LS180 cell lines. The cells were incubated for 24 h with individual (+) and (-) enantiomers of BENI, FELO, and ISRA at concentrations ranging from 100 pM to 50 μM. The vehicle was DMSO (0.1%, v/v). After the treatments, a conventional MTT assay was performed, and the values of IC_{50} were calculated, where appropriate. According to the results of cytotoxicity testing (Figures 25 and 26), the gene reporter assays were conducted at concentrations of the tested compounds up to 50 μM.

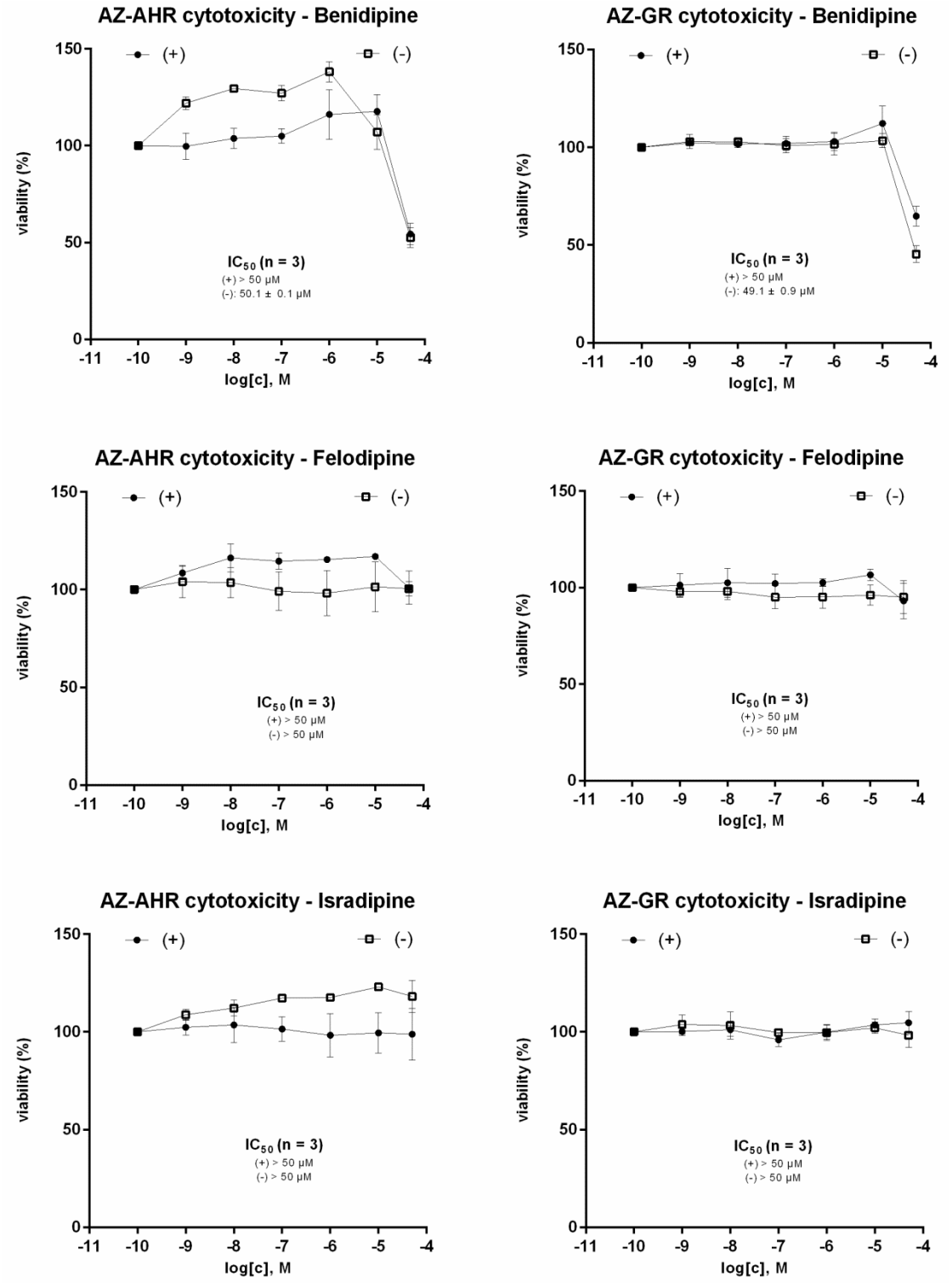

Figure 25: Cytotoxicity of dihydropyridine enantiomers towards human cancer cell lines AZ-AHR and AZ-GR. The cells were seeded in 96-well plates, stabilised for 16 h, and then incubated for 24 h with enantiopure forms of benidipine, felodipine, or isradipine at concentrations ranging from 100 pM to 50 µM. The vehicle was DMSO (0.1%, v/v). After the treatments, a conventional MTT assay was performed, and absorbance was measured at 540 nm. Treatments were performed in triplicate. The data are shown as mean ± SD from experiments performed on three consecutive passages of cells and are expressed as a percentage of viability of control cells. The IC_{50} values were calculated where appropriate and are indicated in the plots.

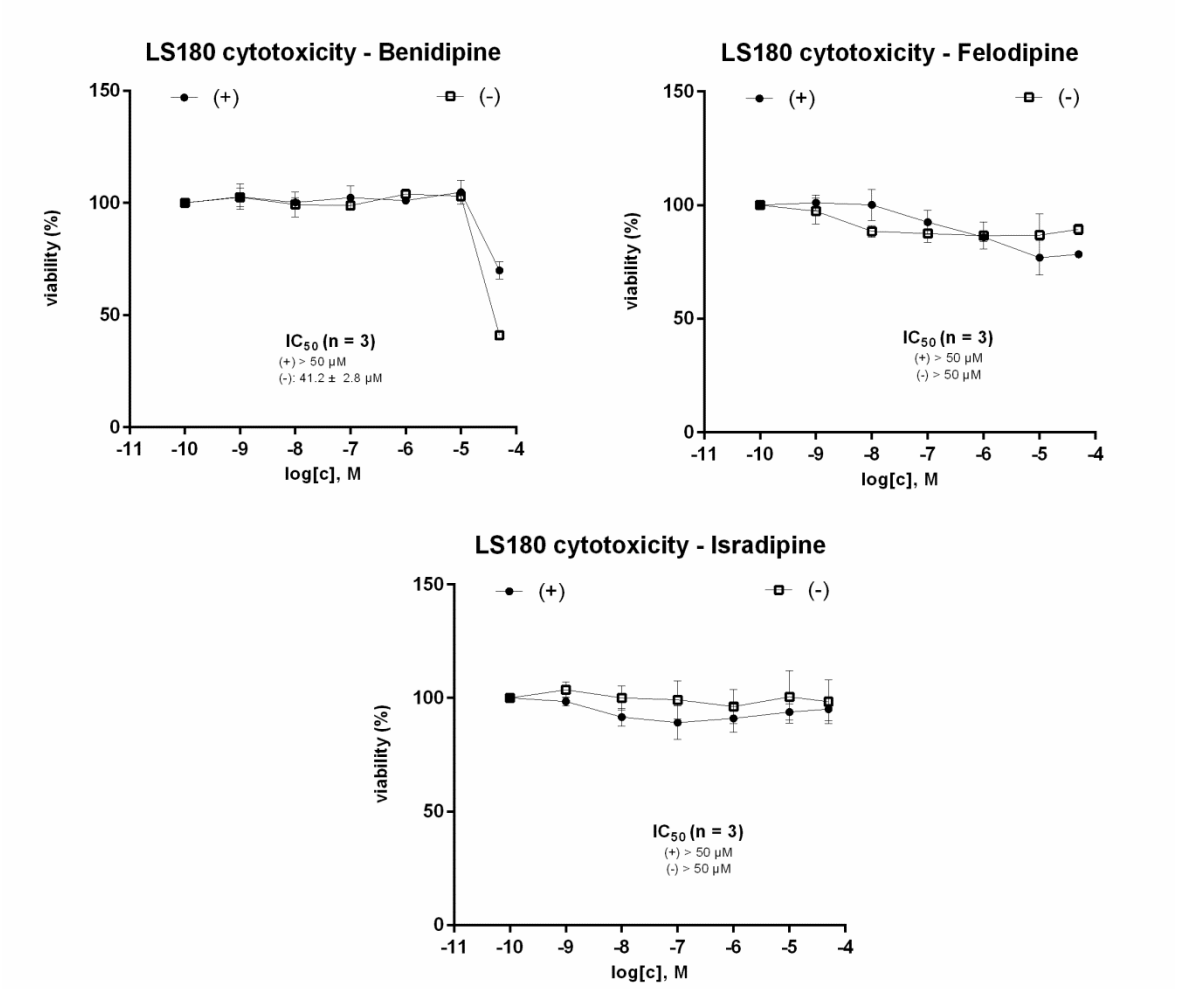

Figure 26: Cytotoxicity of dihydropyridine enantiomers towards human cancer cell line LS180. The cells were seeded in 96-well plates, stabilised for 16 h, and then incubated for 24 h with enantiopure forms of benidipine, felodipine, or isradipine at concentrations ranging from 100 pM to 50 µM. The vehicle was DMSO (0.1%, v/v). After the treatments, a conventional MTT assay was performed, and absorbance was measured at 540 nm. Treatments were administered in triplicate. The data are shown as mean ± SD from experiments performed on three consecutive passages of cells and are expressed as a percentage of viability of control cells. The values of IC_{50} were calculated where appropriate and are indicated in the plots.

5.2.2 Effects of dihydropyridine enantiomers on transcriptional activity of AhR

Transcriptional activity of AhR was assessed in the human gene reporter cell line AZ-AHR incubated for 24 h with individual (+) or (-) enantiomers of BENI, FELO, or ISRA at concentrations ranging from 100 pM to 50 µM in the absence (agonist mode) or presence (antagonist mode) of TCDD (5 nM). The vehicle was DMSO (0.1%, v/v). The average induction of AhR-dependent luciferase activity by TCDD was 685-fold, as compared with that in DMSO-treated cells. (-)-BENI dose-dependently increased luciferase activity, with EC_{50} 11.7 µM, whereas no significant induction of luciferase activity was observed for (+)-BENI. Dose-

dependent activation of AhR was caused by (+)-FELO, (-)-FELO, (+)-ISRA, and (-)-ISRA, with average EC_{50} values 6.5, 12.1, 11.3, and 11.4 μM, respectively. Although the potency levels of (+)-FELO and (-)-FELO were significantly different ($p < 0.05$), the potency levels of (+)-ISRA and (-)-ISRA were similar. Moreover, the efficacy values of (+)-FELO and (+)-ISRA were much higher as compared to their (-) enantiomers (Figure 27). Combined treatments of AZ-AHR cells with the AhR agonist TCDD (5 nM) and enantiomers of FELO and ISRA yielded inversed U-shaped curves. Dose-dependent augmentation of TCDD-inducible luciferase activity was observed for each optical isomer, at concentrations up to 1 μM, followed by a decline of luciferase activity with rising concentrations of dihydropyridines up to 50 μM. Unlike (+)-BENI, (-)-BENI did not augment TCDD-induced luciferase activity. The decrease in luciferase activity at higher concentrations of (+)-BENI and (-)-BENI was caused partly by their cytotoxicity (Figure 28). Therefore, the tested dihydropyridines dose-dependently and enantio-specifically activated AhR. Both the potency and efficacy of the compounds were much lower as compared to TCDD.

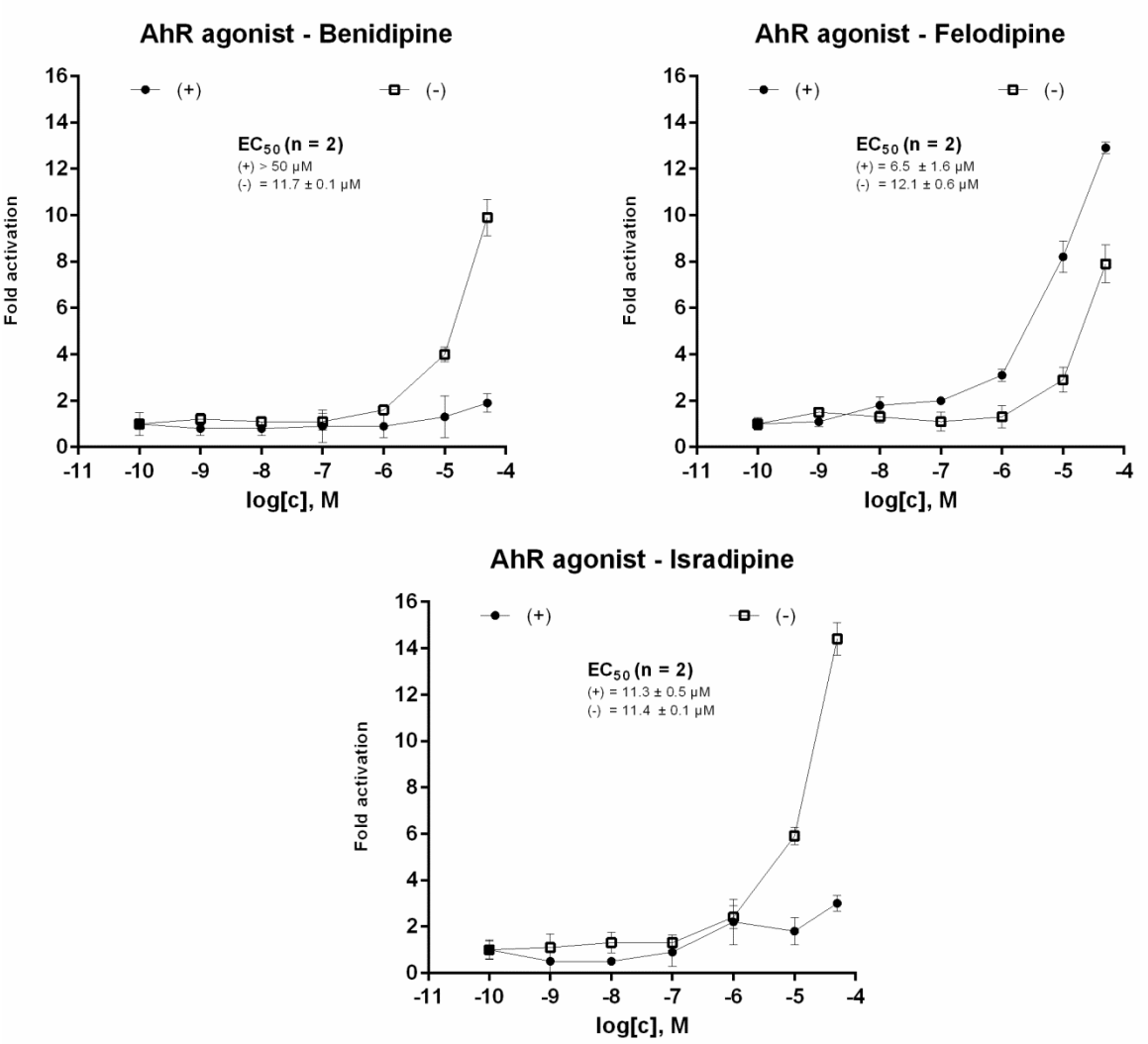

Figure 27: Effects of dihydropyridine enantiomers on transcriptional activity of human AhR (agonist mode). AZ-AHR cells were seeded in 96-well plates and stabilised for 16 h, and then incubated for 24 h with enantiopure forms of benidipine, felodipine, or isradipine at concentrations ranging from 100 pM to 50 µM. The vehicle was DMSO (0.1%, v/v). After the treatments, the cells were lysed, and luciferase activity was measured. Treatments were conducted in triplicate. Data are expressed as a fold induction of luciferase activity over control cells. The EC_{50} values from *n* independent cell passages were calculated where appropriate, and the average values are indicated in the plots. Representative gene reporter assays are shown.

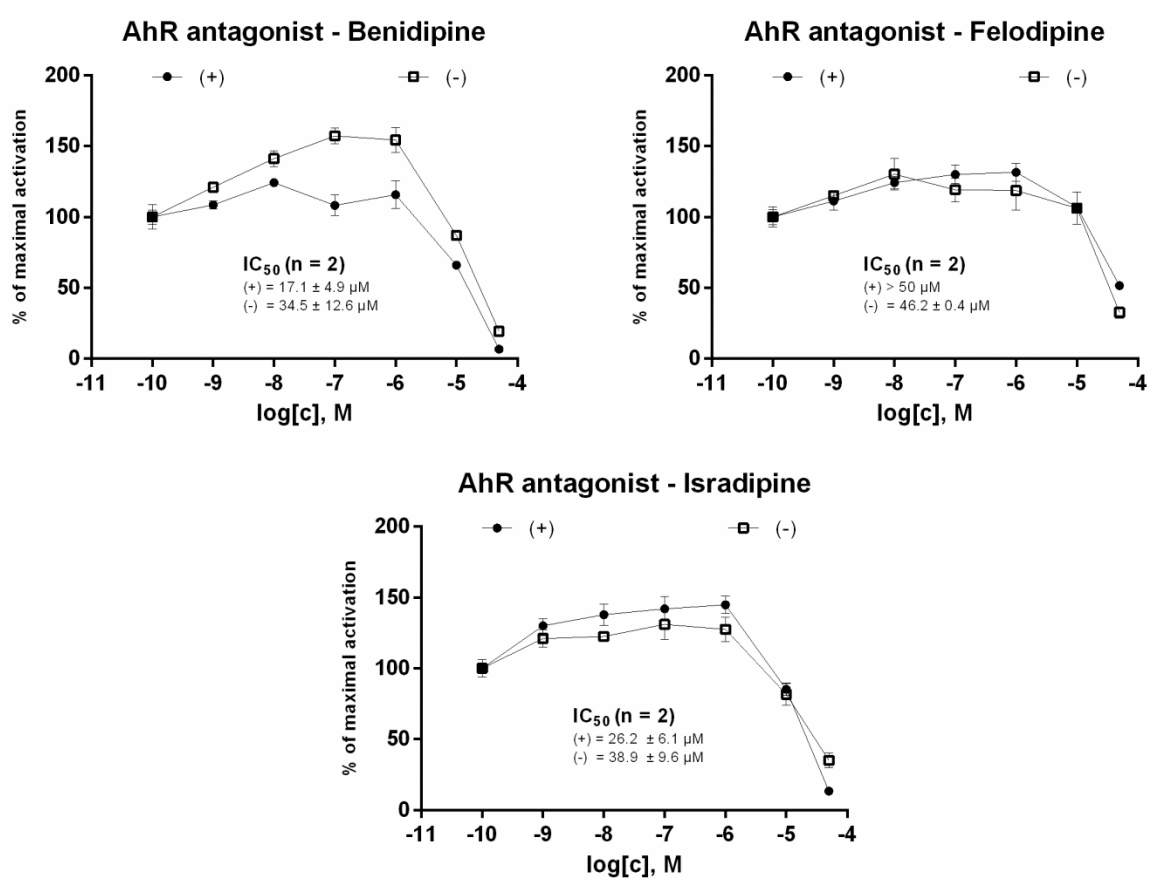

Figure 28: Effects of dihydropyridine enantiomers on transcriptional activity of human AhR (antagonist mode). AZ-AHR cells were seeded in 96-well plates, stabilised for 16 h, and then incubated for 24 h with enantiopure forms of benidipine, felodipine, or isradipine at concentrations ranging from 100 pM to 50 µM in the presence of dioxin (TCDD; 5 nM). The vehicle was DMSO (0.1%, v/v). After the treatments, the cells were lysed, and luciferase activity was measured. Treatments were performed in triplicate. Data are expressed as a percentage of the maximal activation caused by TCDD. The values of IC_{50} from *n* independent cell passages were calculated where appropriate, and the average values are indicated in the plots. Representative gene reporter assays are shown.

5.2.3 Effects of dihydropyridine enantiomers on transcriptional activity of GR

Transcriptional activity of GR was tested in the human gene reporter cell line AZ-GR incubated for 24 h with the tested compounds in the absence (agonist mode) or presence (antagonist mode) of DEX (100 nM). The vehicle was DMSO (0.1%, v/v). The average induction of GR-dependent luciferase activity by model agonist DEX was 25-fold, as compared with that in DMSO-treated cells. None of the tested dihydropyridines induced GR-dependent luciferase activity (Figure 29). However, they all dose-dependently decreased DEX-inducible transcriptional activity of GR (Figure 30). Nonetheless, there were no significant differences between optical isomers of individual dihydropyridines. Moreover, the cytotoxic effects of both optical isomers of BENI should be considered.

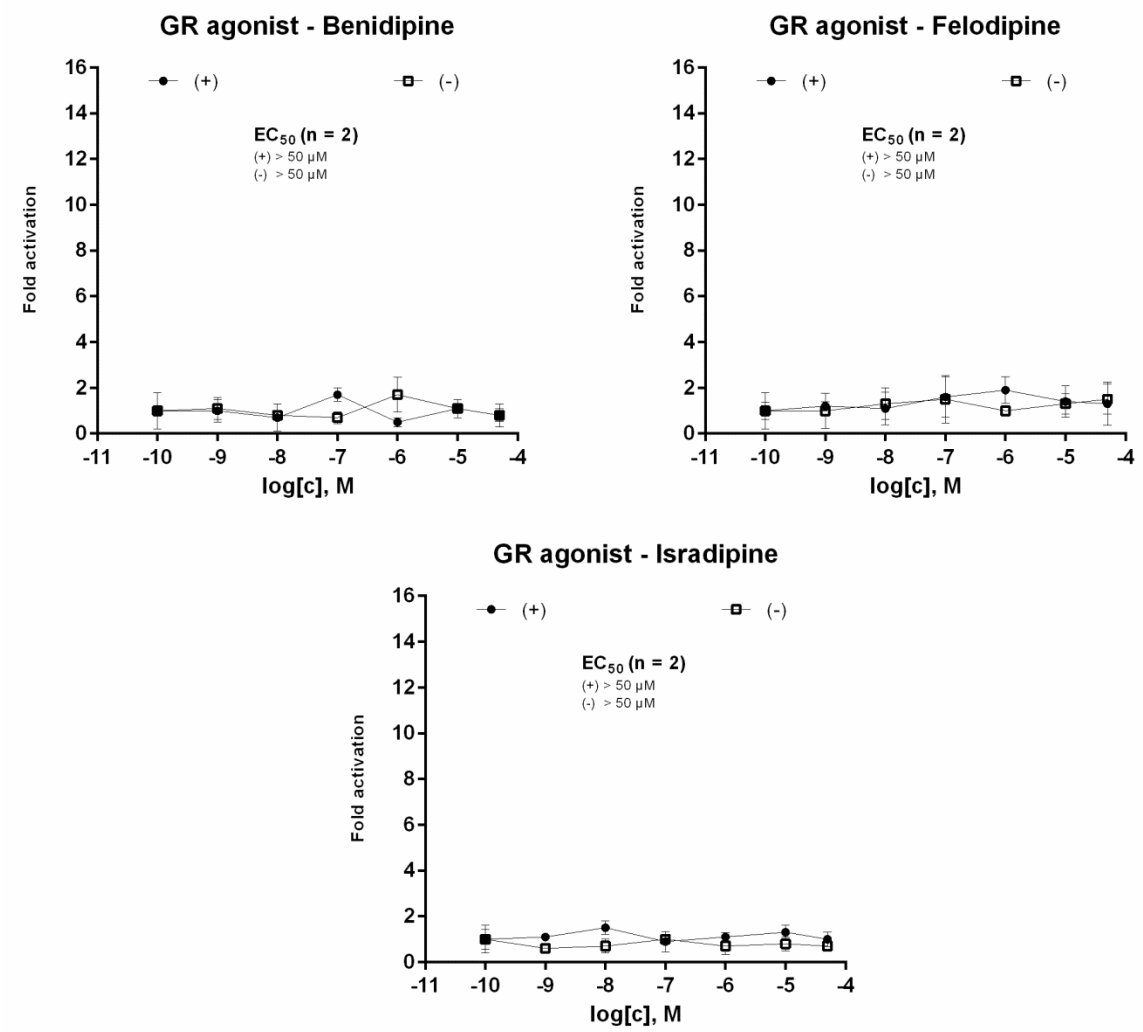

Figure 29: Effects of dihydropyridine enantiomers on transcriptional activity of human GR (agonist mode). AZ-GR cells were seeded in 96-well plates and stabilised for 16 h, and then incubated for 24 h with enantiopure forms of benidipine, felodipine, or isradipine at concentrations ranging from 100 pM to 50 µM. The vehicle was DMSO (0.1%, v/v). After the treatments, the cells were lysed, and luciferase activity was measured. Treatments were carried out in triplicate. Data are expressed as a fold induction of luciferase activity over control cells. The EC_{50} values from *n* independent cell passages were calculated where appropriate, and the average values are indicated in the plots. Representative gene reporter assays are shown.

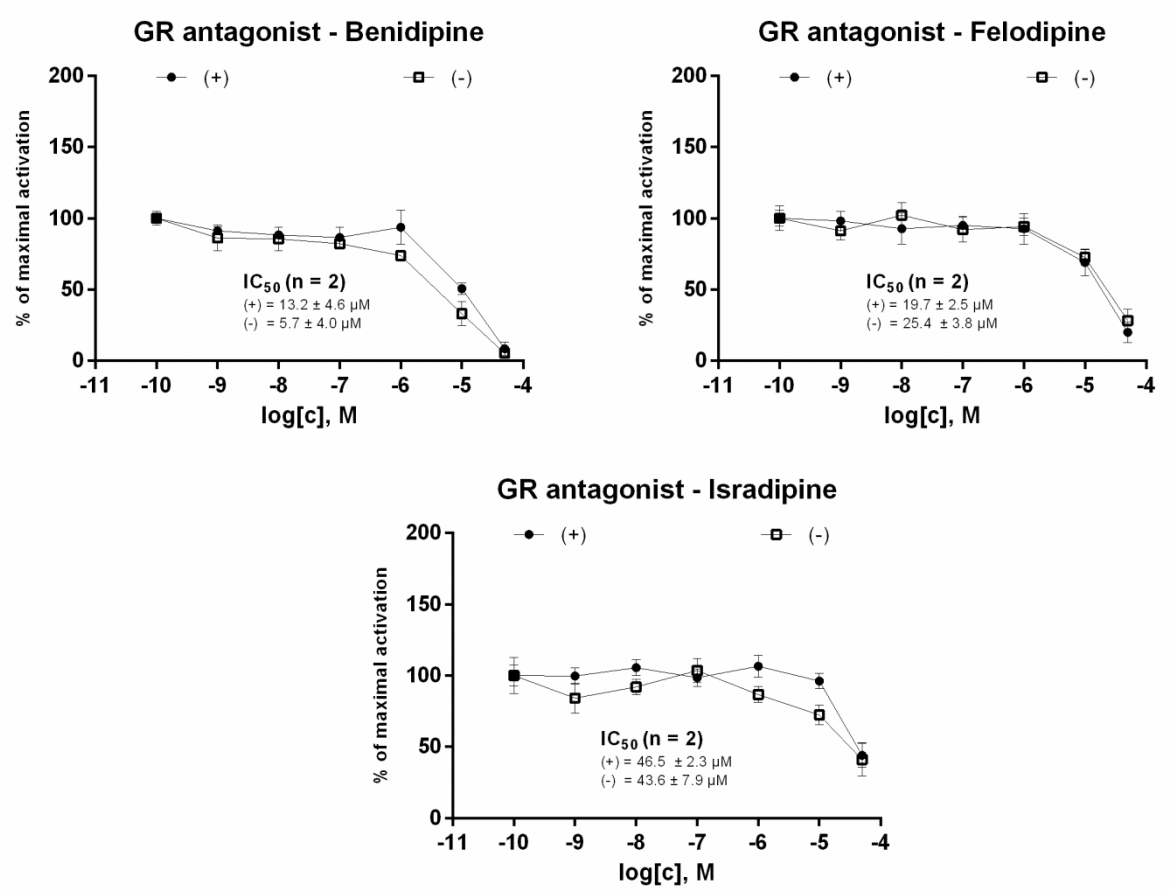

Figure 30: Effects of dihydropyridine enantiomers on transcriptional activity of human GR (antagonist mode). AZ-GR cells were seeded in 96-well plates, stabilised for 16 h, and then incubated for 24 h with enantiopure forms of benidipine, felodipine, or isradipine at concentrations ranging from 100 pM to 50 µM in the presence of DEX (100 nM). The vehicle was DMSO (0.1%, v/v). After the treatments, the cells were lysed and luciferase activity was measured. Treatments were performed in triplicate. Data are expressed as a percentage of the maximal activation caused by TCDD (antagonist mode). The values of IC_{50} from *n* independent cell passages were calculated where appropriate, and the average values are indicated in the plots. Representative gene reporter assays are shown.

5.2.4 Effects of dihydropyridine enantiomers on transcriptional activity of PXR

Transcriptional activity of PXR was tested in human colon adenocarcinoma cells LS180 transiently transfected with the p3A4-luc reporter construct, incubated for 24 h with the tested compounds in the absence (agonist mode) or presence (antagonist mode) of model PXR agonist RIF (10 µM). The vehicle was DMSO (0.1%, v/v). The average induction of luciferase activity by RIF was 9.5-fold, as compared with that in DMSO-treated cells. Transcriptional activity of PXR was dose-dependently induced by all the dihydropyridine enantiomers up to 10 µM concentrations, followed by a plunge of luciferase activity at 50 µM (Figure 31). The efficacy levels of individual compounds were comparable, and magnitudes of the induction ranged from 47% to 80% relative to RIF. The EC_{50} values of ISRA and FELO varied between 0.4 and 1.2 µM. Furthermore, (+)-BENI and

(-)-BENI were ~10-fold more potent, and EC_{50} of (+)-BENI and (-)-BENI was 0.06 and 0.09 µM, respectively. RIF-inducible transcriptional activity of PXR was decreased by all the tested dihydropyridines without significant differences among individual optical isomers (Figure 32). The cytotoxic effects of both optical isomers of BENI should be considered.

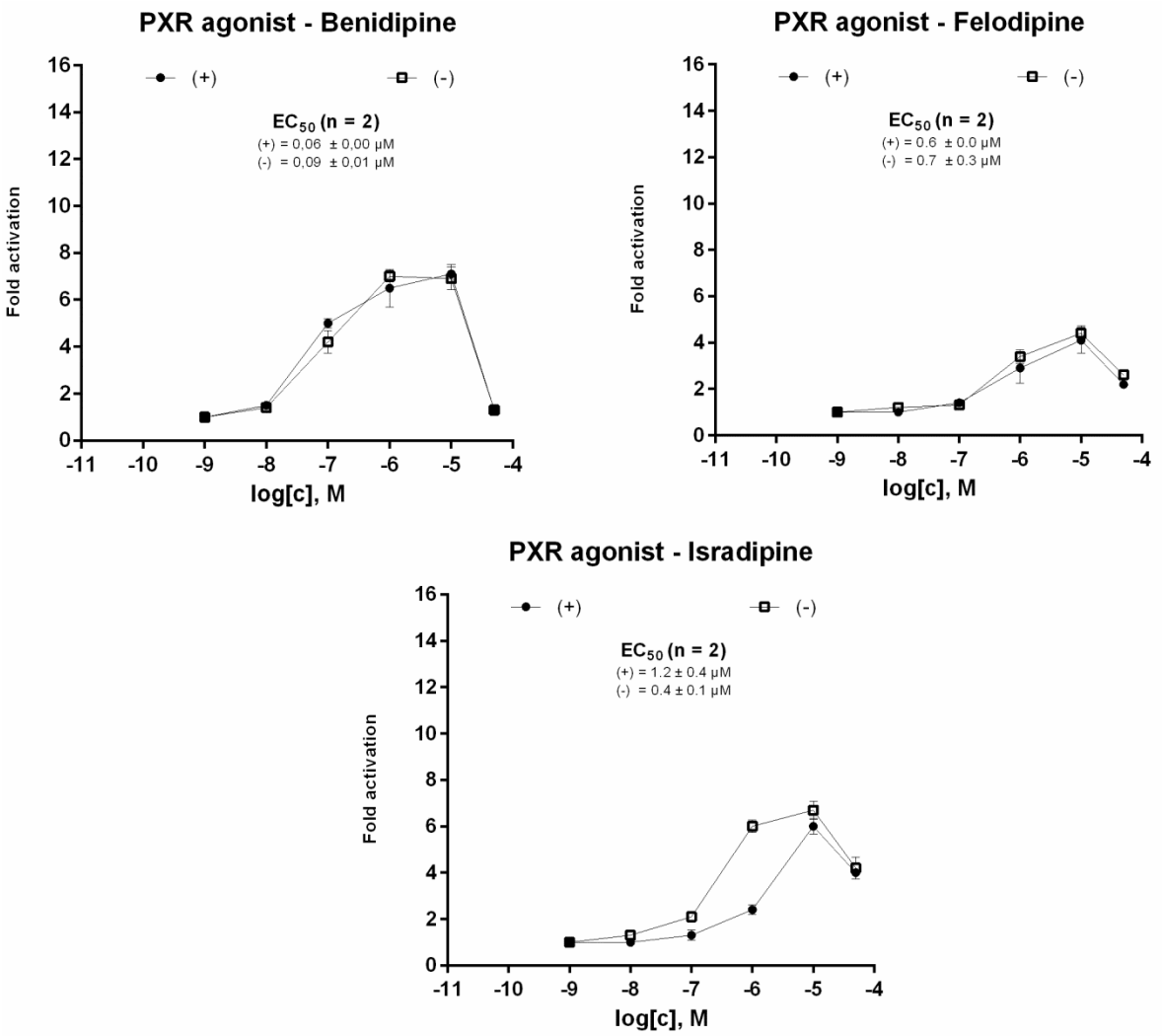

Figure 31: Effects of dihydropyridine enantiomers on transcriptional activity of human PXR (agonist mode). LS180 cells, transiently transfected with the p3A4-luc reporter, were seeded in 96-well plates, stabilised for 16 h, and then incubated for 24 h with enantiopure forms of benidipine, felodipine, or isradipine at concentrations ranging from 100 pM to 50 µM. The vehicle was DMSO (0.1%, v/v). After the treatments, the cells were lysed, and luciferase activity was measured. The treatments were conducted in triplicate. Data are expressed as a fold induction of luciferase activity over control cells. The values of EC_{50} from *n* independent cell passages were calculated where appropriate, and the average values are indicated in the plots. Representative gene reporter assays are shown.

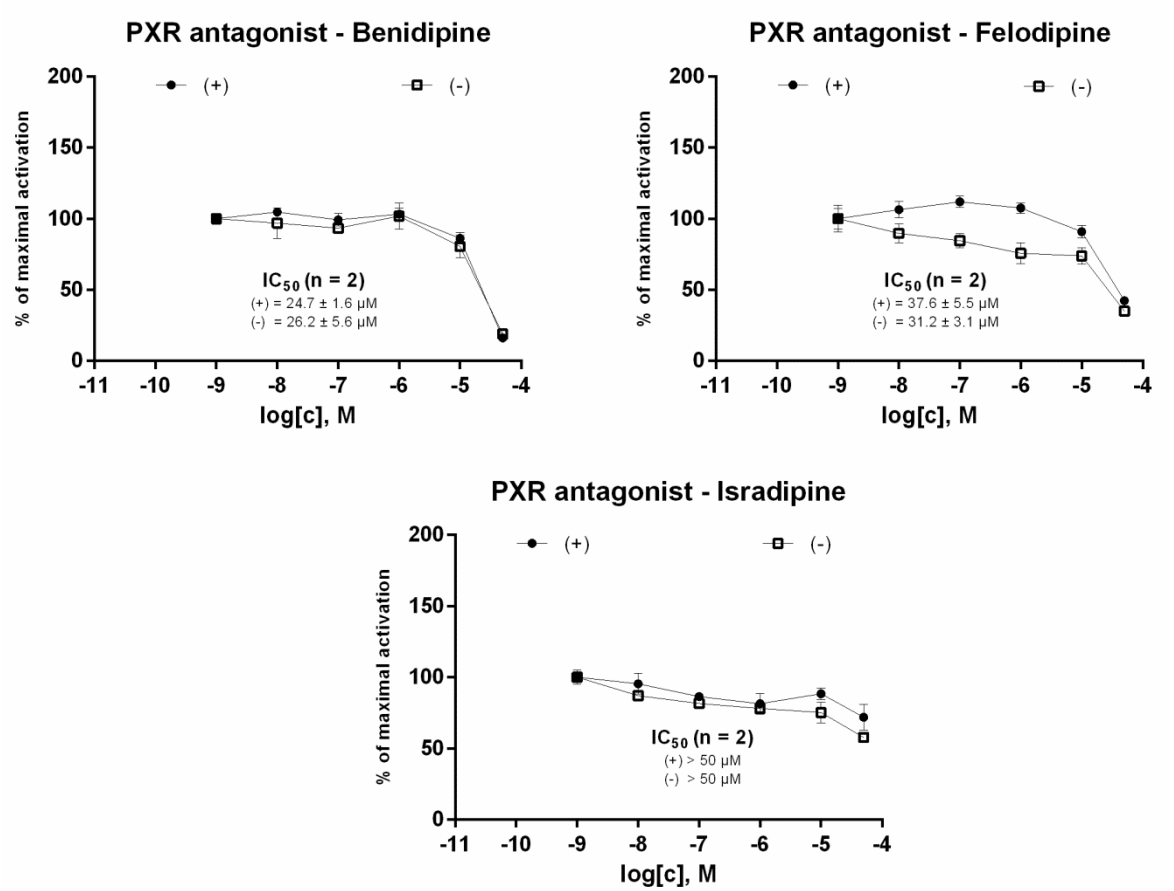

Figure 32: Effects of dihydropyridine enantiomers on transcriptional activity of human PXR (antagonist mode). LS180 cells, transiently transfected with p3A4-luc reporter, were seeded in 96-well plates, stabilised for 16 h, and then incubated for 24 h with enantiopure forms of benidipine, felodipine, or isradipine at concentrations ranging from 100 pM to 50 µM in the presence of RIF (10 µM). The vehicle was DMSO (0.1%, v/v). After the treatments, the cells were lysed, and luciferase activity was measured. Treatments were performed in triplicate. Data are expressed as a percentage of maximal activation caused by TCDD. The values of IC_{50} from *n* independent cell passages were calculated where appropriate, and the average values are indicated in the plots. Representative gene reporter assays are shown.

5.2.5 Effects of dihydropyridine enantiomers on the expression of CYP1A1 and CYP3A4 mRNAs and proteins in human cancer cell line HepG2

Because the tested dihydropyridines activated AhR and PXR in gene reporter assays, we next studied the effects of dihydropyridines on the expression of *CYP1A1* and *CYP3A4*, which are target genes for AhR and PXR, respectively. To this end, HepG2 cells were incubated for 24 h (mRNA analyses) and 48 h (protein analyses) with the tested compounds (1 or 10 µM) and model inducers. The average induction levels of CYP1A1 mRNA and protein by TCDD were 325-fold and 21-fold, respectively. All the tested dihydropyridines dose-dependently induced *CYP1A1* mRNA (Figure 33A). Enantiospecific patterns of induction were consistent with those from gene reporter assays in AZ-AHR cells, i.e. the magnitude of induction was as follows (descending order):

(-)-BENI > (+)-BENI; (-)-ISRA > (+)-ISRA; (+)-FELO > (-)-FELO. Considerable induction of the CYP1A1 protein was achieved only with 10 µM (-)-BENI (Figure 33B). RIF weakly induced (approx. 1.5-fold) *CYP3A4* mRNA (Figure 34A), but not protein (Figure 34B). Among the compounds tested, only (+)-BENI slightly induced *CYP3A4* mRNA, whereas the CYP3A4 protein was not induced by any dihydropyridine. These results are consistent with gene reporter assays, where BENI was identified as the most potent PXR activator (Figure 31).

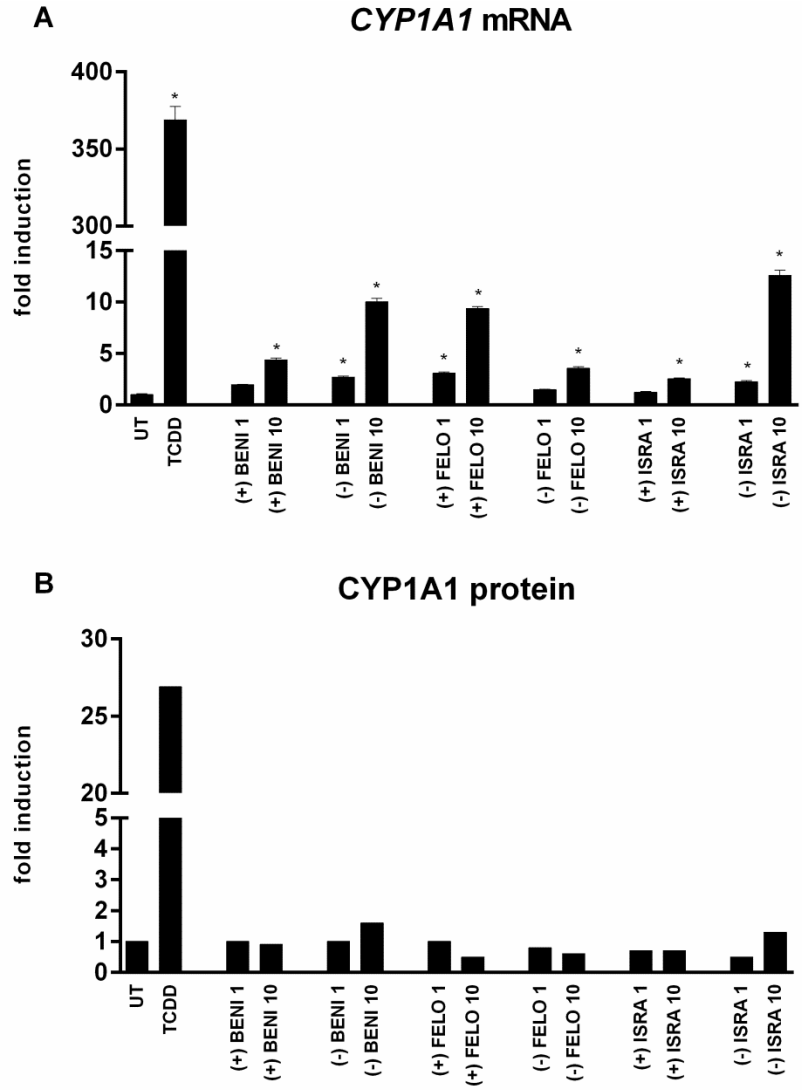

Figure 33: Effects of dihydropyridine enantiomers on CYP1A1 mRNA and protein levels in HepG2 cells. HepG2 cells were seeded in 6-well plates and stabilised for 16 h. All the experiments were performed on three consecutive cell passages. Panel A: The cells were incubated for 24 h with DMSO (0.1%, v/v), TCDD (5 nM), and enantiopure forms of benidipine, felodipine, or isradipine (1 or 10 µM). A representative bar graph of qPCR analyses of *CYP1A1* mRNA is shown. The data are presented as mean ± SD from triplicate measurements and are expressed as a fold induction over vehicle-treated cells. The data were normalised to *GAPDH* mRNA levels. Panel B: The cells were incubated for 48 h with DMSO (0.1%, v/v), TCDD (5 nM), and enantiopure forms of benidipine, felodipine, or isradipine (1 or 10 µM). A representative bar graph of Simple Western analyses of CYP1A1 protein is shown. The data are expressed as a fold induction over vehicle-treated cells and were normalised to β-actin levels. *Significantly different from DMSO-treated cells ($p < 0.05$).

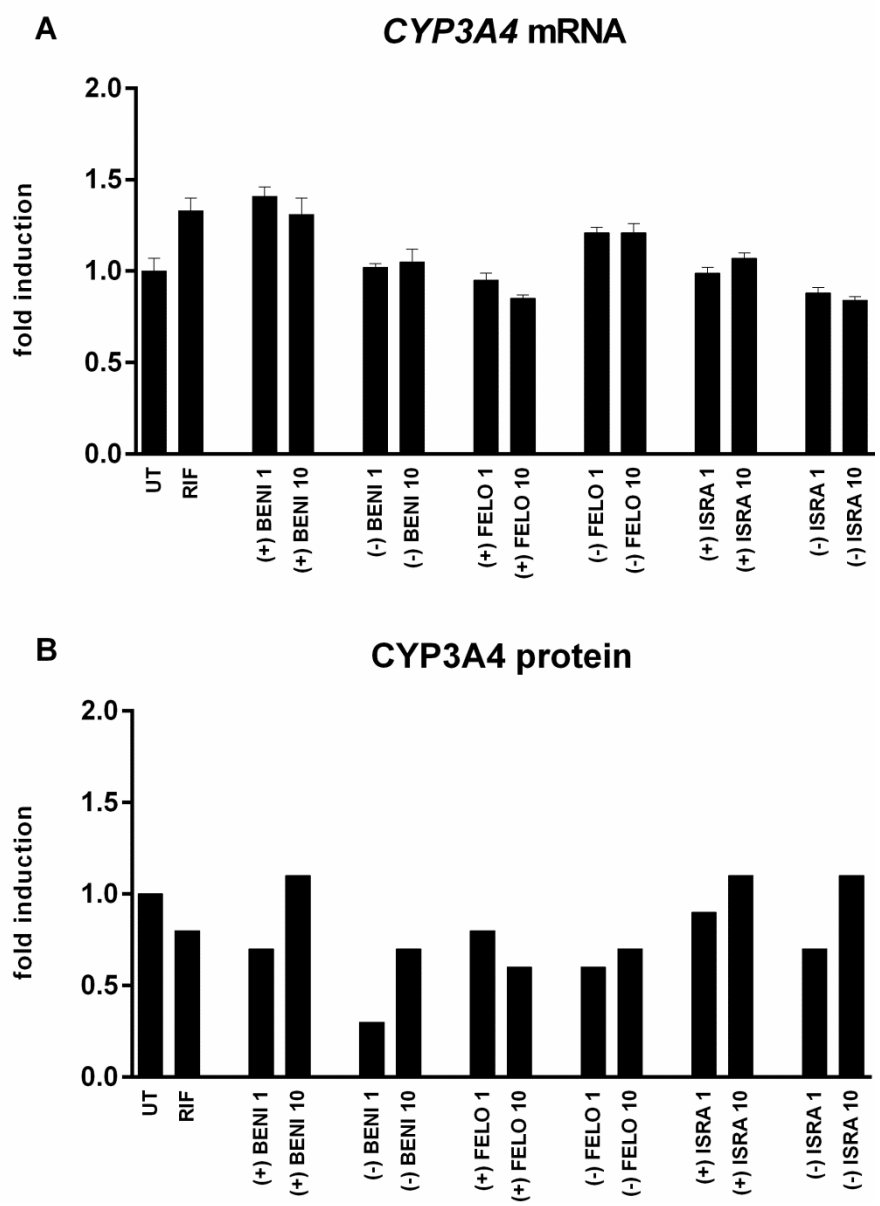

Figure 34: Effects of dihydropyridine enantiomers on CYP3A4 mRNA and protein levels in HepG2 cells. HepG2 cells were seeded in 6-well plates and stabilised for 16 h. All the experiments were performed on three consecutive cell passages. Panel A: The cells were incubated for 24 h with DMSO (0.1%, v/v), RIF (10 µM), and enantiopure forms of benidipine, felodipine, or isradipine (1 or 10 µM). A representative bar graph of qPCR analyses of *CYP3A4* mRNA is shown. The data are presented as mean ± SD from triplicate measurements and are expressed as a fold induction over vehicle-treated cells. The data were normalised to *GAPDH* mRNA levels. Panel B: The cells were incubated for 48 h with DMSO (0.1%, v/v), RIF (10 µM), and enantiopure forms of benidipine, felodipine, or isradipine (1 or 10 µM). A representative bar graph of Simple Western analyses of CYP3A4 protein is shown. The data are expressed as a fold induction over vehicle-treated cells and were normalised to β-actin levels.

5.2.6 Effects of dihydropyridine enantiomers on the expression of drug-metabolizing cytochrome P450s in primary human hepatocytes

The ability of dihydropyridine enantiomers to induce transcriptionally regulated drug-metabolizing CYPs was tested in three human hepatocyte cultures (HH64, HH65, and HH66). Hepatocytes were treated for 24 h (for determination of mRNA) or 48 h (for quantification of proteins) with optical isomers of dihydropyridines (1, 10, or 30 µM), TCDD (5 nM), RIF (10 µM), and vehicle DMSO (0.1%, v/v).

5.2.6.1 Effects of dihydropyridine enantiomers on the expression of CYP1A1 and CYP1A2

TCDD strongly induced CYP1A1/CYP1A2 mRNAs (662-/940-fold, 38-/40-fold, and 35-/45-fold) and proteins (7.8-/8.5-fold, 23-/20-fold, and 19-/13-fold) in cultures of HH64, HH65, and HH66, respectively (Figures 35 and 36). The induction profiles of *CYP1A1* and *CYP1A2* mRNAs under the influence of dihydropyridines yielded similar patterns; with the exception of (+)-FELO, all the tested compounds weakly and dose-dependently induced *CYP1A* genes with the following magnitude (descending order): (-)-ISRA > (-)-BENI > (+)-ISRA ~ (+)-BENI ~ (-)-FELO (Figures 35 and 36, upper panels). The data are mostly consistent with the results on *CYP1A1* mRNA induction in HepG2 cells and AhR in gene reporter assays. Nonetheless, inter-individual variability among hepatocytes from different donors and metabolic competence of human hepatocytes should be considered. Optical isomers of dihydropyridines did not significantly induce the expression of the CYP1A1 or CYP1A2 protein, with the exception of weak up-regulation of CYP1A1 protein in culture HH66 by FELO enantiomers (Figures 35 and 36, lower panels).

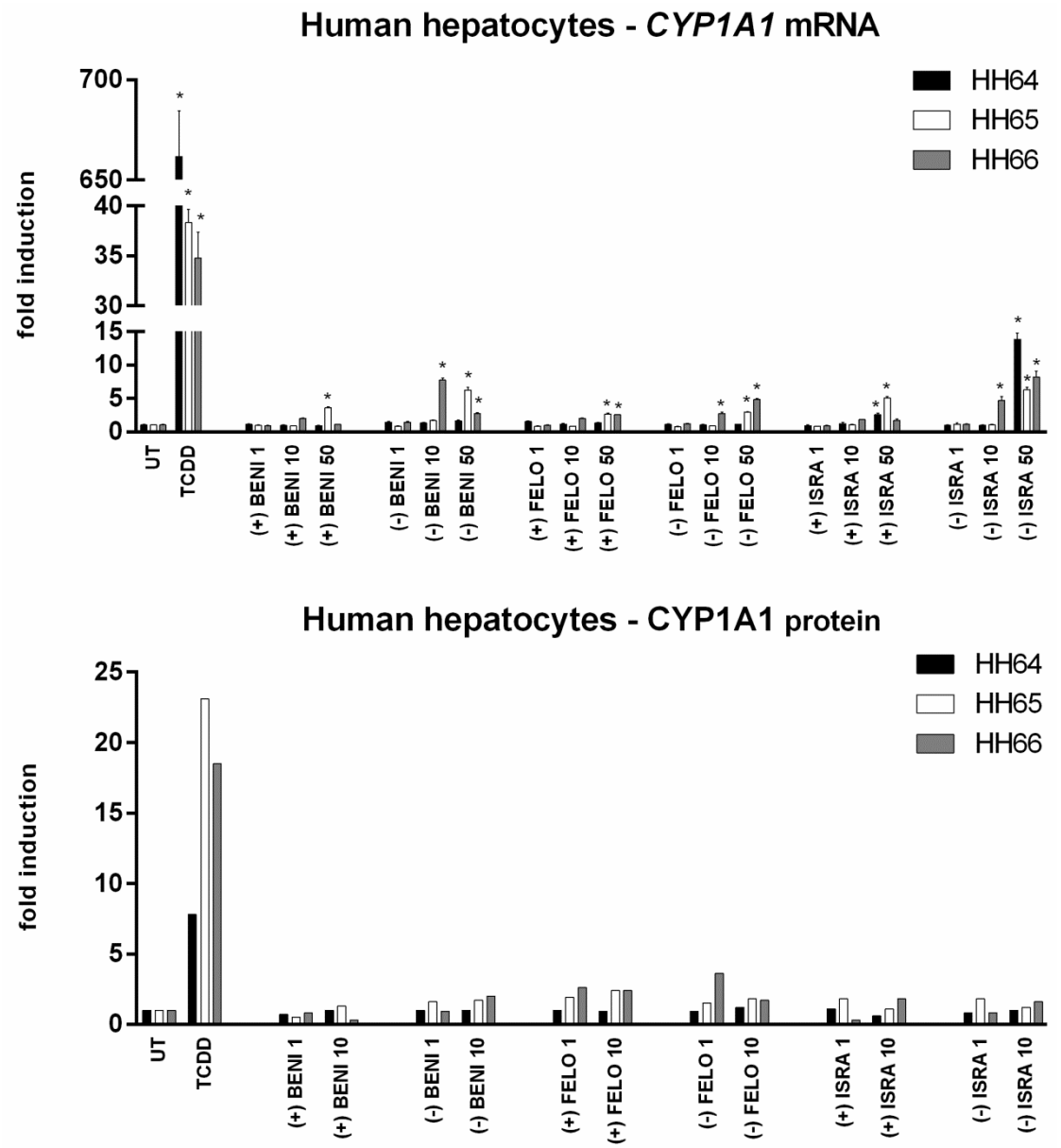

Figure 35: Effects of dihydropyridine enantiomers on the expression of CYP1A1 in primary human hepatocytes. Primary human hepatocytes from three donors (HH64, HH65, and HH66) were used. Upper panel: The cells were incubated for 24 h with DMSO (0.1%, v/v), TCDD (5 nM), and individual enantiomers of dihydropyridines (1, 10, or 30 µM). A bar graph of qPCR analyses of *CYP1A1* mRNAs is shown. The data are presented as mean ± SD from triplicate measurements and are expressed as a fold induction over vehicle-treated cells. The data were normalised to *GAPDH* mRNA levels. Lower panel: The cells were incubated for 48 h with DMSO (0.1%, v/v), TCDD (5 nM), and individual enantiomers of dihydropyridines (1 or 10 µM). A bar graph of Simple Western analyses of CYP1A1 protein is shown. The data are expressed as a fold induction over vehicle-treated cells and were normalised to β-actin levels. *Significantly different from DMSO-treated cells ($p < 0.05$).

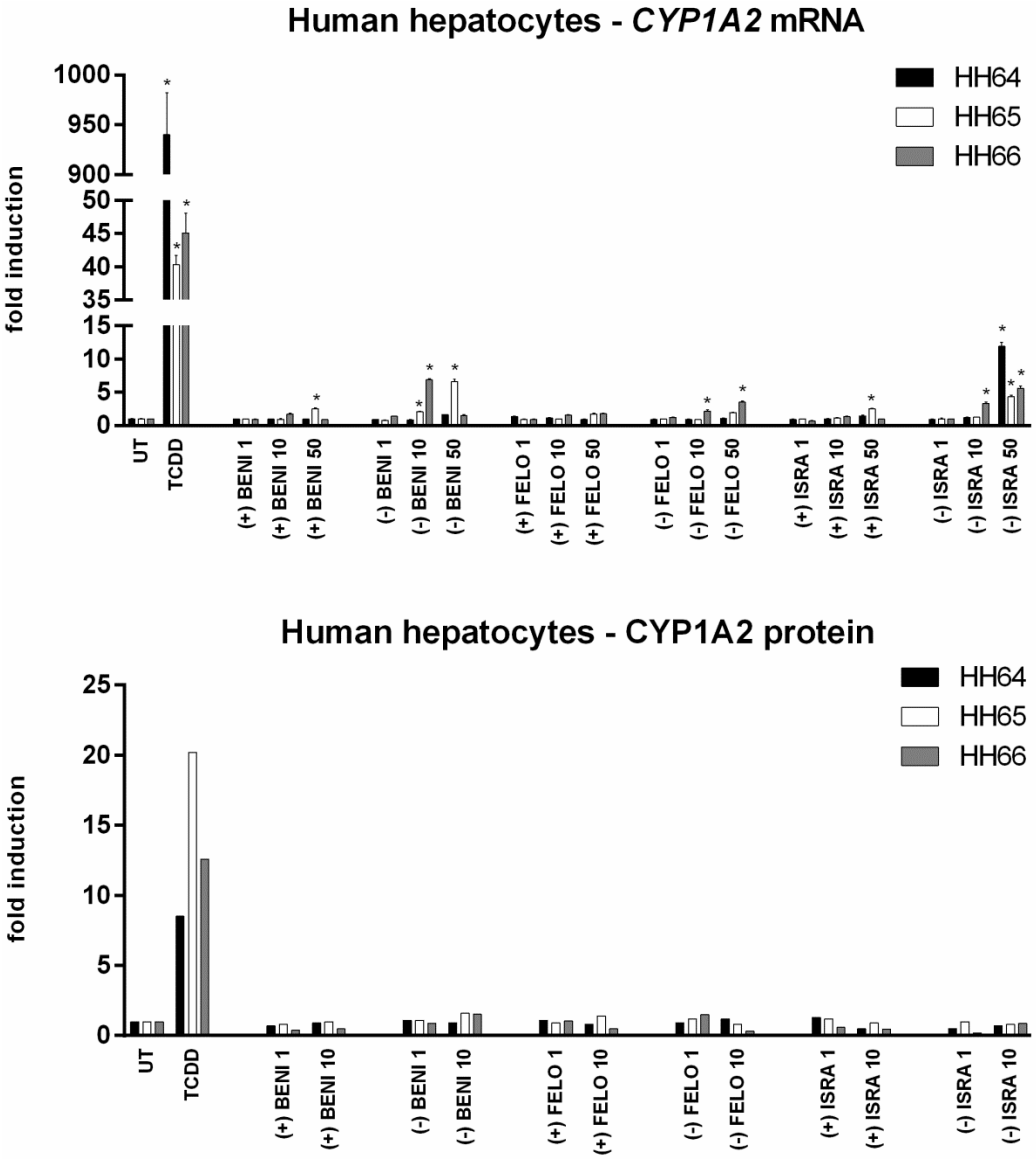

Figure 36: Effects of dihydropyridine enantiomers on the expression of CYP1A2 in primary human hepatocytes. Primary human hepatocytes from three donors (HH64, HH65, and HH66) were used. Upper panel: The cells were incubated for 24 h with DMSO (0.1%, v/v), TCDD (5 nM), and individual enantiomers of dihydropyridines (1, 10, or 30 µM). A bar graph of qPCR analyses of *CYP1A2* mRNAs is shown. The data are presented as mean ± SD from triplicate measurements and are expressed as a fold induction over vehicle-treated cells. The data were normalised to *GAPDH* mRNA levels. Lower panel: The cells were incubated for 48 h with DMSO (0.1%, v/v), TCDD (5 nM), and individual enantiomers of dihydropyridines (1 or 10 µM). A bar graph of Simple Western analyses of CYP1A2 protein is shown. The data are expressed as a fold induction over vehicle-treated cells and were normalised to β-actin levels. *Significantly different from DMSO-treated cells ($p < 0.05$).

5.2.6.2 Effects of dihydropyridine enantiomers on the expression of CYP2A6

Induction levels of CYP2A6 mRNA/protein by RIF after 24/48 h of incubation were 5.2-/1.5-fold and 13-/6.5-fold in cultures of HH64 and HH65, respectively (Figure 37). Given the weak induction of *CYP2A6* mRNA by RIF after 24 h (1.6-fold) in culture HH66, the CYP2A6 protein was not analysed in that culture. Induction profiles of CYP2A6 mRNA and protein by dihydropyridine enantiomers

varied among individual cultures, most likely due to individual differences among the donors. Overall, the effects on the expression of CYP2A6 mRNA and protein by FELO enantiomers were equipotent. In case of ISRA enantiomers, stronger induction of *CYP2A6* mRNA was observed for (+)-ISRA, but the data were not consistent with its effect on the CYP2A6 protein. The induction profiles of CYP2A6 by BENI enantiomers differed among the hepatocyte cultures and were not dose-dependent.

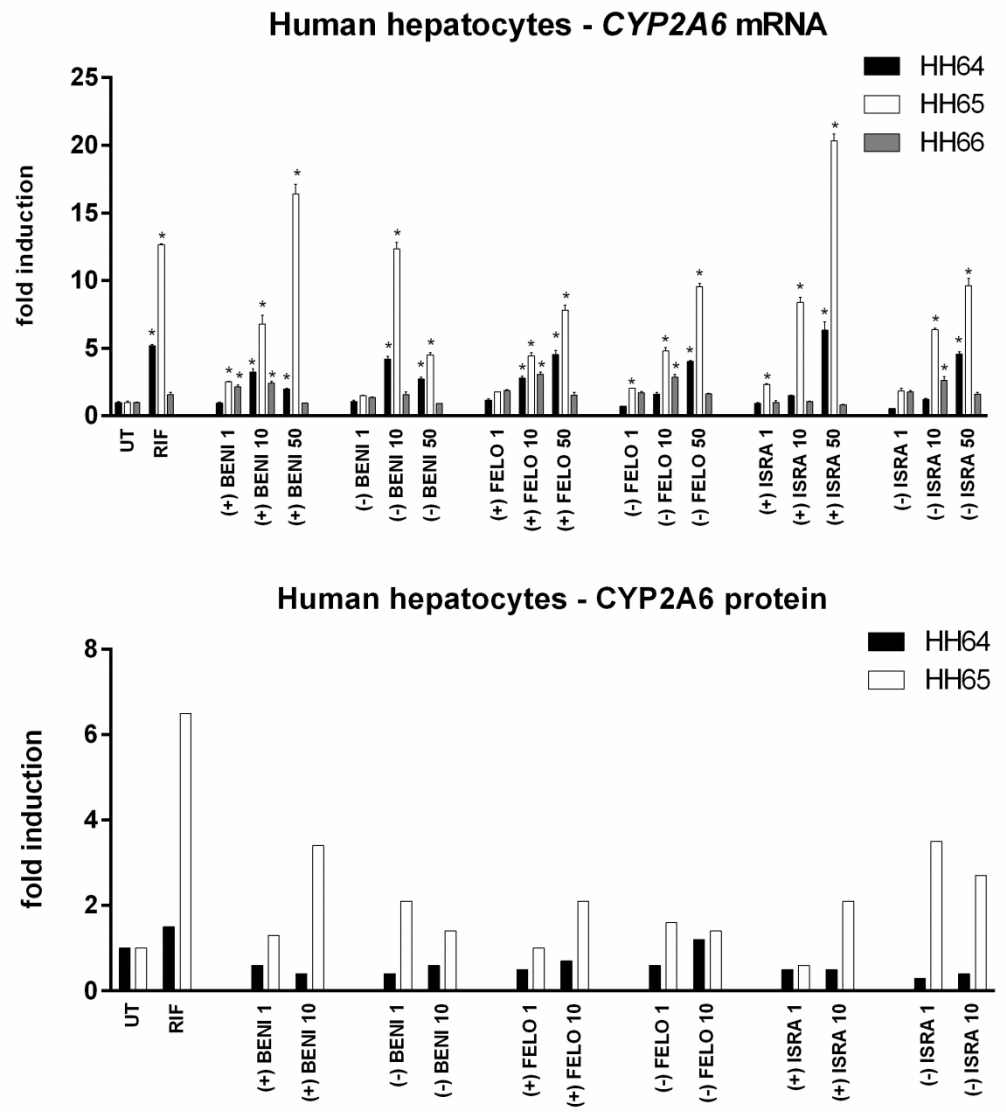

Figure 37: Effects of dihydropyridine enantiomers on the expression of CYP2A6 in primary human hepatocytes. Primary human hepatocytes from two (or three) different donors (HH64 and HH65 [or HH66]) were used for protein (or mRNA) analyses, respectively. Upper panel: The cells were incubated for 24 h with DMSO (0.1%, v/v), RIF (10 µM), and individual enantiomers of dihydropyridines (1, 10, or 30 µM). A bar graph of qPCR analyses of *CYP2A6* mRNAs is shown. The data are presented as mean ± SD from triplicate measurements and are expressed as a fold induction over vehicle-treated cells. The data were normalised to *GAPDH* mRNA levels. Lower panel: The cells were incubated for 48 h with DMSO (0.1%, v/v), RIF (10 µM), and individual enantiomers of dihydropyridines (1 or 10 µM). A bar graph of Simple Western analyses of CYP2A6 protein is shown. The data are expressed as a fold induction over vehicle-treated cells and were normalised to β-actin levels. *Significantly different from DMSO-treated cells ($p < 0.05$).

5.2.6.3 Effects of dihydropyridine enantiomers on the expression of CYP2B6

Induction levels of CYP2B6 mRNA/protein by RIF after 24/48 h of incubation were 20-/5.8-fold, 25-/3.3-fold, and 5.8-/3.3-fold in cultures of HH64, HH65, and HH66, respectively. All dihydropyridines dose-dependently induced *CYP2B6* mRNA in all the hepatocyte cultures (Figure 38, upper panel). Their effects were nearly equipotent, and they were not enantiospecific. Comparable induction profiles were observed for the CYP2B6 protein (Figure 38, lower panel).

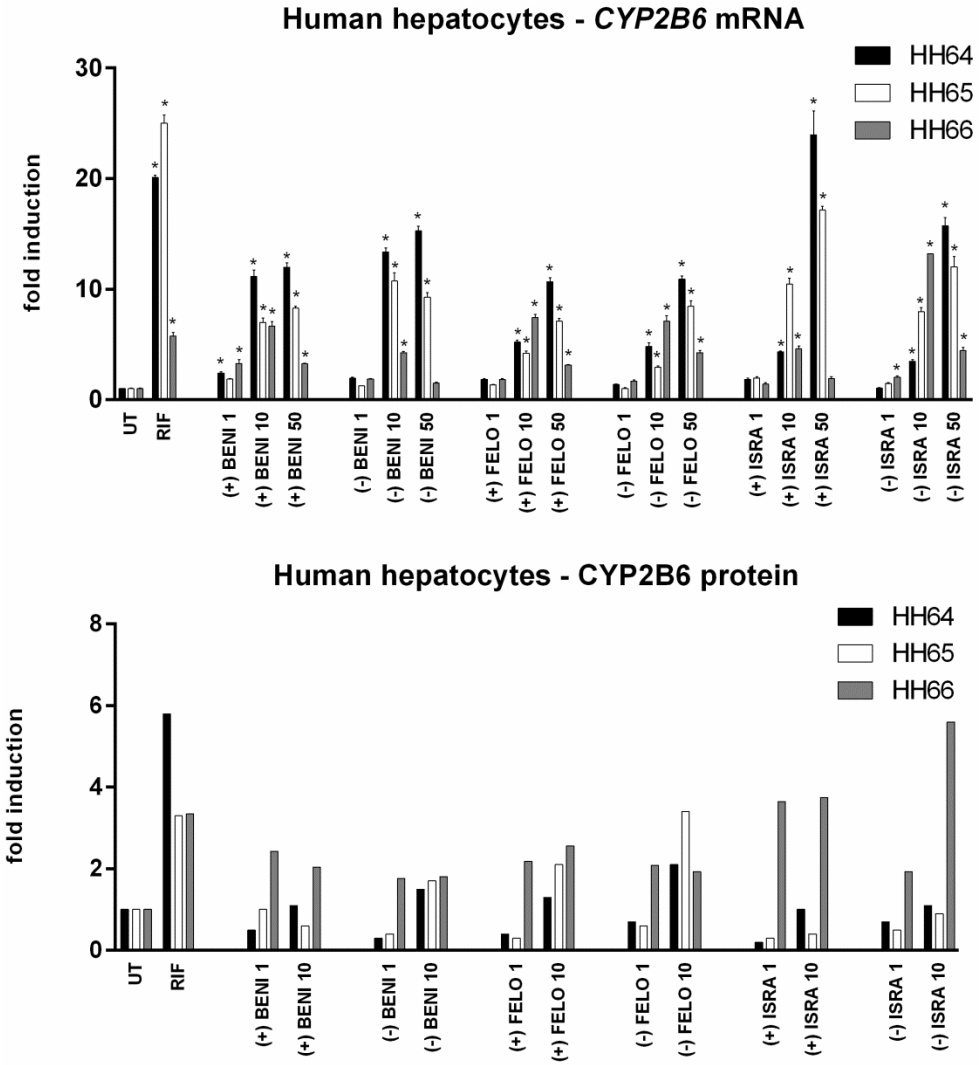

Figure 38: Effects of dihydropyridine enantiomers on the expression of CYP2B6 in primary human hepatocytes. Primary human hepatocytes from three donors (HH64, HH65, and HH66) were used. Upper panel: The cells were incubated for 24 h with DMSO (0.1%, v/v), RIF (10 µM), and individual enantiomers of dihydropyridines (1, 10, or 30 µM). A bar graph of qPCR analyses of *CYP2B6* mRNAs is shown. The data are presented as mean ± SD from triplicate measurements and are expressed as a fold induction over vehicle-treated cells. The data were normalised to *GAPDH* mRNA levels. Lower panel: The cells were incubated for 48 h with DMSO (0.1%, v/v), RIF (10 µM), and individual enantiomers of dihydropyridines (1 or 10 µM). A bar graph of Simple Western analyses of CYP2B6 protein is shown. The data are expressed as a fold induction over vehicle-treated cells and were normalised to β-actin levels. *Significantly different from DMSO-treated cells ($p < 0.05$).

5.2.6.4 Effects of dihydropyridine enantiomers on the expression of CYP2C9

Induction of *CYP2C9* mRNA by RIF after 24 h of incubation in three human hepatocyte cultures varied from 0.9- to 2.3-fold (Figure 39). Because the induction of *CYP2C9* mRNA was weak, we did not analyse induction of the CYP2C9 protein. The effects of optical isomers of the analysed dihydropyridines on *CYP2C9* mRNA expression were rather positive.

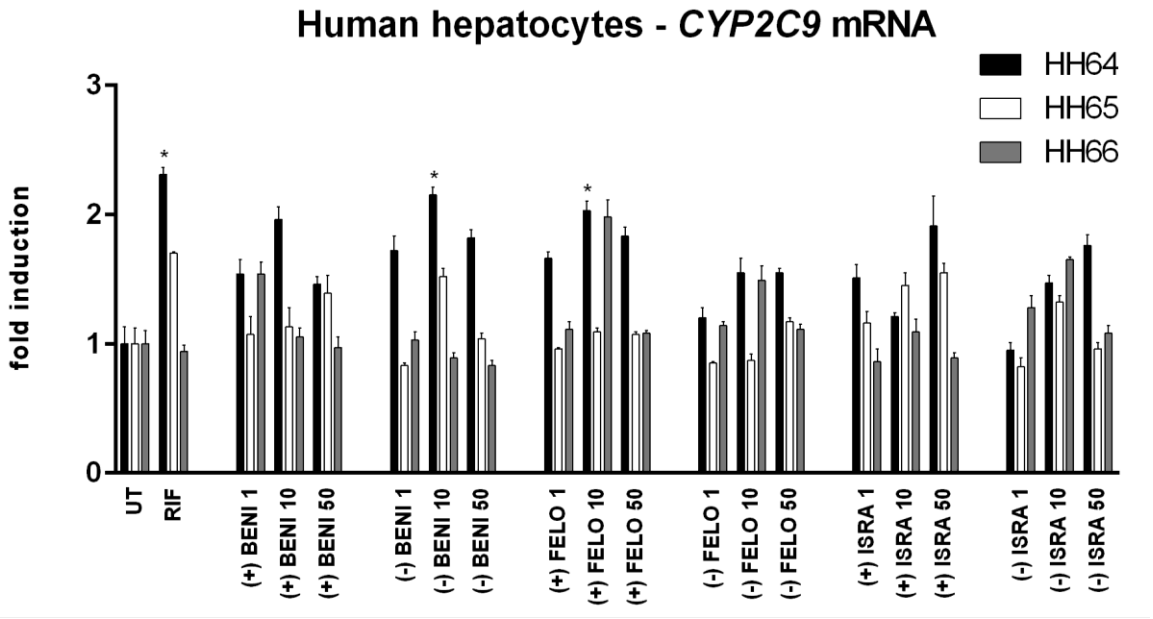

Figure 39: Effects of dihydropyridine enantiomers on the expression of CYP2C9 in primary human hepatocytes. Primary human hepatocytes from three donors (HH64, HH65, and HH66) were used. The cells were incubated for 24 h with DMSO (0.1%, v/v), RIF (10 µM), and individual enantiomers of dihydropyridines (1, 10, or 30 µM). A bar graph of qPCR analyses of *CYP2C9* mRNAs is shown. The data are presented as mean ± SD from triplicate measurements and are expressed as a fold induction over vehicle-treated cells. The data were normalised to *GAPDH* mRNA levels. *Significantly different from DMSO-treated cells ($p < 0.05$).

5.2.6.5 Effects of dihydropyridine enantiomers on the expression of CYP3A4

Induction levels of CYP3A4 mRNA/protein by RIF after 24/48 h of incubation were 59-/4.4-fold, 81-/36-fold, and 6.5-/7.1-fold in cultures of HH64, HH65, and HH66, respectively (Figure 40). All the dihydropyridines dose-dependently induced *CYP3A4* mRNA in all the hepatocyte cultures, and their effects were not enantiospecific (Figure 40, upper panel). The magnitude of *CYP3A4* induction was as follows (descending order): (+/-)-BENI ~ (+)-ISRA > (+/-)-FELO ~ (-)-ISRA. We detected only moderate induction of CYP3A4 at the protein level, except for strong up-regulation of CYP3A4 protein by BENI enantiomers in hepatocyte culture HH65 (Figure 40, lower panel).

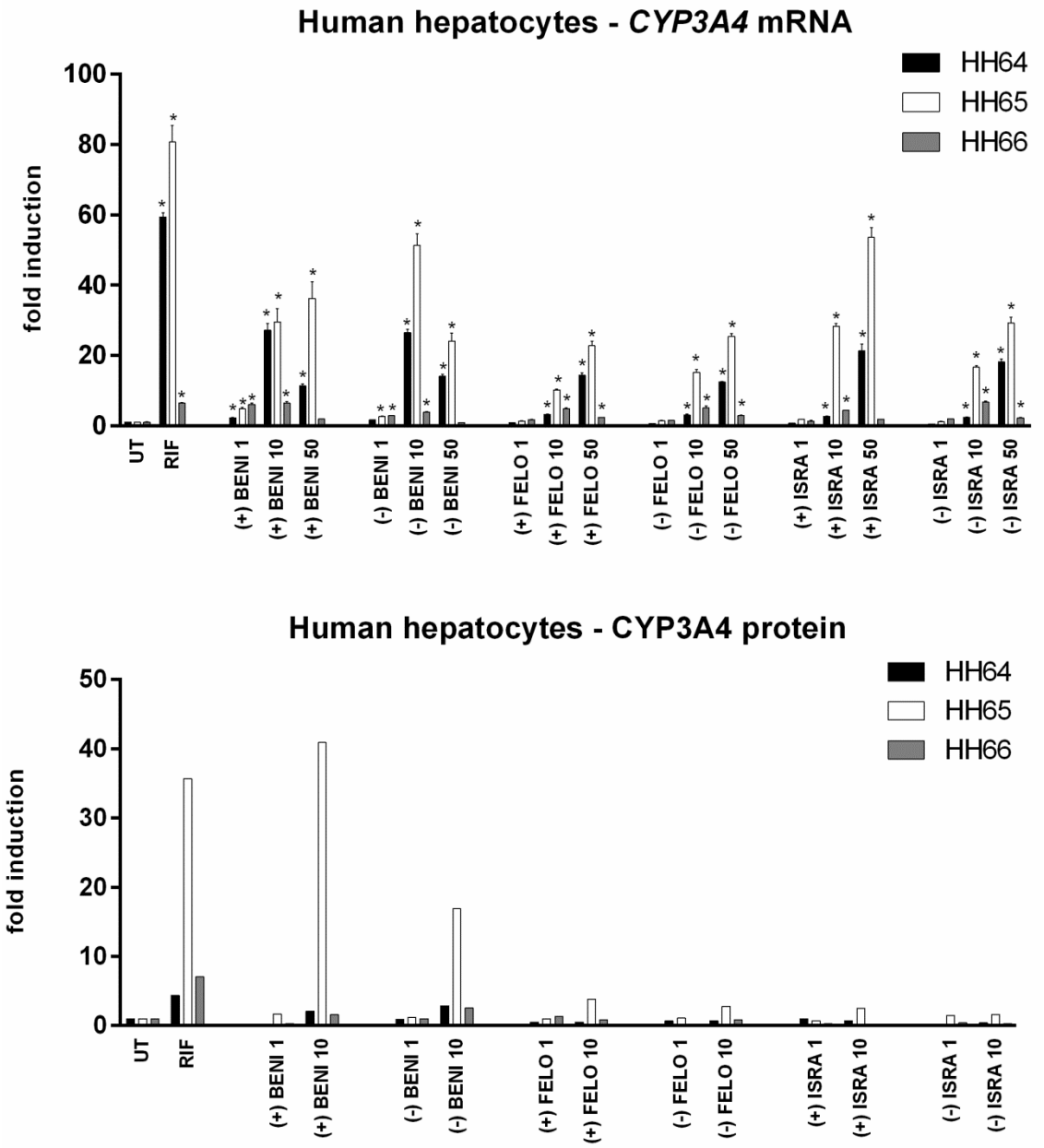

Figure 40: Effects of dihydropyridine enantiomers on the expression of CYP3A4 in primary human hepatocytes. Primary human hepatocytes from three donors (HH64, HH65, and HH66) were used. Upper panel: The cells were incubated for 24 h with DMSO (0.1%, v/v), RIF (10 µM), and individual enantiomers of dihydropyridines (1, 10, or 30 µM). A bar graph of qPCR analyses of *CYP3A4* mRNAs is shown. The data are mean ± SD from triplicate measurements and are expressed as a fold induction over vehicle-treated cells. The data were normalised to *GAPDH* mRNA levels. Lower panel: The cells were incubated for 48 h with DMSO (0.1%, v/v), RIF (10 µM), and individual enantiomers of dihydropyridines (1 or 10 µM). A bar graph of Simple Western analyses of CYP3A4 protein is shown. The data are expressed as a fold induction over vehicle-treated cells and were normalised to β-actin levels. *Significantly different from DMSO-treated cells ($p < 0.05$).

5.2.7 Effects of dihydropyridine enantiomers on binding of AhR to DNA

In a final series of experiments, the ability of dihydropyridine enantiomers to transform human AhR into its DNA-binding conformation was tested by an EMSA. Human breast cancer cells MCF-7 were treated for 2 h with the compounds under study (10 µM), TCDD (5 nM), and DMSO (0.1%, v/v). After that, nuclear extracts were prepared and incubated with a biotin-labelled probe containing an AhR-binding site and were subjected to electrophoresis in a 5% polyacrylamide gel. All the tested compounds induced the binding of AhR to DNA, and the effects were enantiospecific. The strength of binding was as follows (descending order): (+)-BENI > (-)-BENI; (-)-FELO > (+)-FELO; (+)-ISRA > (-)-ISRA (Figure 41). Moreover, semi-quantitative enantiospecific effects of dihydropyridines in the EMSA were opposite to their effects on *CYP1A* mRNA expression and AhR transcriptional activity.

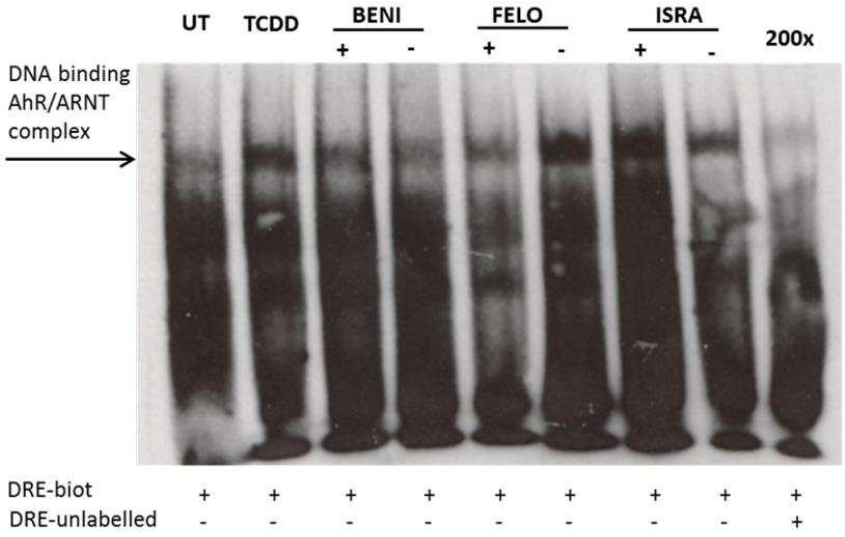

Figure 41: Effects of dihydropyridine enantiomers on binding of AhR to DNA. MCF-7 cells at 100% confluence were incubated for 2 h with vehicle (DMSO, 0.1%, v/v), TCDD (5 nM), and individual enantiomers of dihydropyridines (10 µM). Nuclear extracts were incubated with a biotin-labelled probe containing an AhR-binding site and were analysed by electrophoresis in a 5% polyacrylamide gel. The experiments were performed on three independent cell passages. A representative EMSA analysis is shown.

6 DISCUSSION

In the present thesis, I examined the effects of optical isomers of two groups of popular chiral drugs—statins, as cholesterol-lowering agents, and dihydropyridine CCBs, as antihypertensive drugs—on the human xenoreceptor-P450 signalling pathway. *In vitro* models of primary human hepatocyte cultures and human cancer cell lines were used. Experimental approaches included gene reporter assays, qPCR, western blotting, and EMSAs.

I showed for the first time that AT, FLU, and ROS enantiospecifically induce CYP2A6, CYP2B6, and CYP3A4 in human hepatocytes, and that they enantiospecifically influence transcriptional activities of PXR and GR (Korhoňová *et al*., 2015: Appendix I). The statins investigated in this study are clinically used as pure enantiomers, i.e. 3R5R-AT, 3R5S-ROS, and 3R5S-FLU. These statins were introduced into clinical practice directly as single-isomer drugs, but the data on induction of CYPs by their optical isomers were absent. However, several DDIs, which could be caused by induction of CYPs, were reported in patients using statins simultaneously with other drugs, e.g. anti-hypertensive losartan (Meadowcroft *et al.*, 1999), anti-diabetic repaglinide (Sekhar and Reddy, 2012), and CCBs nifedipine (Lee *et al.*, 2015), and verapamil (Choi *et al.*, 2008). Indeed, there are numerous reports indicating activation of PXR and induction of PXR-regulated CYPs by statins (Hoffart *et al.*, 2012; Howe *et al.*, 2011; Kocarek *et al.*, 2002; Plee-Gautier *et al.*, 2012; Yamasaki *et al.*, 2009). Therefore, it was justified to carry out a comparative study of clinically used *versus* other optical isomers of statins regarding their ability to activate xenoreceptors and to induce drug-metabolizing cytochrome P450s.

I found that the analysed statins do not activate AhR and accordingly do not induce CYP1A1 and CYP1A2 in primary human hepatocytes and cancer cell lines. In contrast, all the optical isomers of all the tested statins induced formation of the PXR–DNA complex and differently induced *CYP2* and *CYP3* genes. Optical isomers of ROS were the least active and only fairly activated PXR in gene reporter assays, without significant differences between diastereomers. ROS did not induce *CYP2* and *CYP3* genes, with the exception of significant induction of the CYP3A4 protein, but not *CYP3A4* mRNA, by 3R5S-ROS and 3S5R-ROS

in human hepatocytes. The optical isomer 3R5S-ROS also induced *CYP2A6* mRNA. Because the clinically approved optical isomer is 3R5S-ROS, the induction of CYP2A6 and CYP3A4 is undesirable. The induction profiles of CYP2A6, CYP2B6, and CYP3A4 by AT optical isomers were similar, and the potency of individual optical isomers was as follows (descending order): 3R5R-AT > 3R5S-AT = 3S5R-AT > 3S5S-AT. EC_{50} in PXR-gene reporter assays was the lowest for 3R5R-AT (~ 50% of other AT diastereomers). This finding is consistent with the fact that families CYP2 and CYP3 are predominantly regulated by PXR. In contrast, the magnitude of luciferase induction was the highest for 3S5S-AT, which was the weakest inducer of *CYP2* and *CYP3* genes. Because the clinically approved form of AT is 3R5R-AT, it is unfortunate that it is the most effective PXR activator and the most potent CYP2 and CYP3 inducer among the four existing AT diastereomers. The optical isomers 3R5R-FLU, 3R5S-FLU, and 3S5S-FLU exerted a dose-dependent antagonistic activity against GR in gene reporter assays. Anti-glucocorticoid activity of 3R5R-FLU and 3S5S-FLU was also confirmed in human hepatocytes, where these compounds down-regulated *TAT* mRNA. The magnitude of induction of CYP2A6, CYP2B6, and CYP3A4 by 3R5R-FLU was much weaker than that of the other three optical isomers. Given the complex and multiple regulatory roles of GR in xenobiotic metabolic pathways, the resulting induction of CYPs by FLU comprises both agonistic effects on PXR and antagonistic effects on GR. Overall, it was demonstrated that the potential of AT, ROS, and FLU to cause CYP induction-based DDIs is higher for clinically used optical isomers, than that of their respective diastereomers.

The second achievement of the present thesis is characterisation of the effects of chiral dihydropyridine-type CCBs, including BENI, FELO, and ISRA on the expression and enzymatic activities of human CYPs. Despite over 40 different existing dihydropyridine CCBs, only four members have been tested so far for interactions with the PXR–CYP3A4 signalling pathway, including nifedipine, nicardipine, ISRA, and clevidipine (Drocourt *et al.*, 2001; Zhang *et al.*, 2006). Presumably, the activation of PXR and induction of CYP3A4 seems to be a general feature applicable to other dihydropyridine CCBs; therefore, an extensive study including all the existing derivatives is not necessary. On the contrary, dihydropyridine CCBs possess at least one chiral centre in their

molecule; consequently, the research into enantiospecific effects of dihydropyridine CCBs on xenoreceptors and cytochrome P450s is worthwhile.

I found that all tested CCBs activate PXR with similar efficacy and the magnitudes of the induction were comparable with that of RIF. Furthermore, the potency of (+/-)-BENIs was found to be ~10-fold higher than that of (+/-)-ISRAs and (+/-)-FELOs. A similar pattern was observed for induction of PXR-regulated genes including *CYP2A6*, *CYP2B6*, and *CYP3A4* in human hepatocytes by CCBs. There were no differences between the effects of (+)/(-) enantiomers. Thus, the tested dihydropyridines are equipotent activators of human PXR and inducers of PXR-regulated genes, regardless of their optical configuration. On the other hand, the unexpected finding was enantiospecific activation of AhR and induction of *CYP1A* genes by optical isomers of the CCBs under study. Transcriptional activity of AhR in gene reporter assays, expression of CYP1A1 in HepG2 cells, and expression of CYP1A1 and CYP1A2 in human hepatocytes were increased by CCBs as follows (descending order): (-)-BENI > (+)-BENI; (-)-ISRA > (+)-ISRA; (+)-FELO > (-)-FELO. Because there is a common chiral centre in all three dihydropyridine CCBs, the difference between enantiospecific effects of BENI and ISRA in contrast to FELO is probably determined by the specific structure of each derivative. Collectively, the potential of the tested dihydropyridine CCBs to cause induction-based DDIs and other adverse effects is a result of dual activation of AhR and PXR. Activation of AhR and induction of *CYP1A* genes were found to be enantioselective, whereas activation of PXR and induction of genes *CYP2* and/or *CYP3* took place regardless of optical configuration.

Overall, in the present thesis I provide the first evidence of enantiospecific effects of two groups of clinically used chiral drugs – statins and dihydropyridine CCBs – on PXR and AhR, respectively. Taking into account the broad spectrum of processes mediated by enantiospecific activation of these xenoreceptors (induction of CYPs and other XMEs, consequent DDIs, toxicity, and others), the data presented here may have clinical and toxicological implications.

7 CONCLUSION

The present thesis deals with the enantiospecific effects of cholesterol-lowering drugs (statins) and antihypertensive drugs (dihydropyridine CCBs) on the transcriptional activities of human xenoreceptors and on the expression of drug-metabolizing cytochrome P450s. The major findings are

(i) AT, FLU, and ROS enantiospecifically activate PXR and induce genes *CYP2A6*, *CYP2B6*, and *CYP3A4*.

(ii) BENI, FELO, and ISRA enantiospecifically activate AhR and induce genes *CYP1A1* and *CYP1A2*.

(iii) BENI, FELO, and ISRA non-enantiospecifically activate PXR and induce genes *CYP2A6*, *CYP2B6*, and *CYP3A4*.

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9 CURRICULUM VITAE

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Publications

Novotna A., Kamenickova A., Pecova M., **Korhonova M.**, Bartonkova I., Dvorak Z. (2013): Profiling of enantiopure drugs towards aryl hydrocarbon (AhR), glucocorticoid (GR) and pregnane X (PXR) receptors in human reporter cell lines. *Chem-Biol Interact* 208: 64-76. [**IF_{2012} - 2.967**]. Contributions to the published work: performed data analysis, conducted experiments.

Novotna A., Srovnalova A., Svecarova M., **Korhonova M.**, Bartonkova I., Dvorak Z. (2014): Differential effects of omeprazole and lansoprazole enantiomers on aryl hydrocarbon receptor in human hepatocytes and cell lines. *PLoS One*, 9: 1-8. [**IF_{2012} – 3.730**]. Contributions to the published work: performed data analysis, conducted experiments.

Novotna A., **Korhonova M.**, Bartonkova I., Sashilov A.A., Denison M.S., Bogdanova K., Kolar M., Dvorak Z.: (2014) Enantiospecific effects of ketoconazole on aryl hydrocarbon receptor. *PLoS One*, 9. 101832 [**IF_{2013} – 3.534**]. Contributions to the published work: performed data analysis, conducted experiments.

Novotna A., Krasulova K., Bartonkova I., **Korhonova M.**, Bachleda P., Anzenbacher P., Dvorak Z. (2014): Dual effects of ketoconazole cis-enantiomers on CYP3A4 in human hepatocytes and HepG2 cells. *PLoS One*. 9. 111286 [**IF_{2013} – 3.534**]. Contributions to the published work: performed data analysis, conducted experiments.

Korhonova M., Doricakova A., Dvorak Z. (2015): Optical Isomers of Atorvastatin, Rosuvastatin and Fluvastatin Enantiospecifically Activate Pregnane X Receptor PXR and Induce CYP2A6, CYP2B6 and CYP3A4 in Human Hepatocytes. *PLoS One* 10(9): e0137720. [**IF_{2014} – 3.234**]. Contributions to the published work: participated in research design, performed data analysis, conducted experiments, wrote or contributed to the manuscript.

Stepankova M., Krasulova K., Doricakova A., Kurka O., Anzenbacher P., Dvorak Z. (2016): Optical isomers of dihydropyridine calcium channel blockers display enantiospecific effects on the expression and enzyme activities of human xenobiotics-metabolizing cytochromes P450. *Toxicol Lett* 262: 173–186. [**IF_{2015} - 3.522**]. Contributions to the published work: participated in research design, performed data analysis, conducted experiments, wrote or contributed to the manuscript.

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Conference reports

Korhonova M., Novotna A., Bartonkova I., Dvorak Z.: Enantiospecific effects of lansoprazole and omeprazole on the expression of human cytochrome P450 1A (CYP1A) via aryl hydrocarbon receptor (AhR). *20th International Symposium on Microsomes and Drug Oxidations*; Stuttgart, Germany; 18-22 May 2014; p. 140.

Korhonova M., Dvorak Z.: Stereoselective effects of statins on xenobiotic-metabolizing pathways. *17th European Congress of Endocrinology 2015*; Dublin, Ireland; 16-20 May 2015; e-poster 149.

Stepankova M., Doricakova A., Dvorak Z.: Optical isomers of statins enantiospecifically activate pregnane X receptor PXR and induce CYP2A6, CYP2B6 and CYP3A4 in human hepatocytes. *18th European Congress of Endocrinology 2016*; Munich, Germany; 28-31 May 2016 e-poster 774

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APPENDIX I.

Korhonova M., Doricakova A., Dvorak Z. (2015): Optical isomers of atorvastatin, rosuvastatin and fluvastatin enantiospecifically activate pregnane X receptor PXR and induce CYP2A6, CYP2B6 and CYP3A4 in human hepatocytes. *PLoS One* 10(9): e0137720. [**IF_{2014} - 3,234**]

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RESEARCH ARTICLE

Optical Isomers of Atorvastatin, Rosuvastatin and Fluvastatin Enantiospecifically Activate Pregnane X Receptor PXR and Induce CYP2A6, CYP2B6 and CYP3A4 in Human Hepatocytes

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Abstract

Atorvastatin, fluvastatin and rosuvastatin are drugs used for treatment of hypercholesterolemia. They cause numerous drug-drug interactions by inhibiting and inducing drug-metabolizing cytochromes P450. These three statins exist in four optical forms, but they are currently used as enantiopure drugs, i.e., only one single enantiomer. There are numerous evidences that efficacy, adverse effects and toxicity of drugs may be enantiospecific. Therefore, we investigated the effects of optical isomers of atorvastatin, fluvastatin and rosuvastatin on the expression of drug-metabolizing P450s in primary human hepatocytes, using western blots and RT-PCR for measurement of proteins and mRNAs, respectively. The activity of P450 transcriptional regulators, including pregnane X receptor (PXR), aryl hydrocarbon receptor (AhR) and glucocorticoid receptor (GR), was assessed by gene reporter assays and EMSA. Transcriptional activity of AhR was not influenced by any statin tested. Basal transcriptional activity of GR was not affected by tested statins, but dexamethasone-inducible activity of GR was dose-dependently and enantioselectively inhibited by fluvastatin. Basal and ligand-inducible transcriptional activity of PXR was dose-dependently influenced by all tested statins, and the potency and efficacy between individual optical isomers varied depending on statin and optical isomer. The expression of CYP1A1 and CYP1A2 in human hepatocytes was not influenced by tested statins. All statins induced CYP2A6, CYP2B6 and CYP3A4, and the effects on CYP2C9 were rather modulatory. The effects varied between statins and enantiomers and induction potency decreased in order: atorvastatin (RR>RS = SR>SS) > fluvastatin (SR>RS = SS>RR) >> rosuvastatin (only RS active). The data presented here might be of toxicological and clinical importance.

Introduction

Statins are a class of drugs used for the treatment of hypercholesterolemia, a major risk factor for the development of atherosclerotic disease. Statins lower the level of plasma low-density lipoprotein LDL cholesterol by inhibiting 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, the enzyme playing a central role in the production of cholesterol in the liver.

Structurally, statins are chiral compounds having two asymmetrical centres in the molecule, enabling formation of four different enantiomers: 3R5R, 3R5S, 3S5R and 3S5S (Fig 1). Individual enantiomers of a drug can qualitatively and quantitatively differ in their biological activities, including their pharmacokinetics, pharmacodynamics, toxicokinetics and toxicodynamics. Notoriously known examples of diastereomers with substantially different biologial activities are R/S-thalidomide, R/S-salbutamol, levo/dextro-methorphan and many others [1]. Therefore, enantiopure drugs have been developed and introduced to the therapy. Regarding the most frequently prescribed statins, following enantiopure formulations are used in the clinics: 3R5R-atorvastatin (Lipitor, Pfizer; since Nov 2011 generic), 3R5S-rosuvastatin (Crestor, AstraZeneca; approved 12th Aug 2003) and 3R5S-fluvastatin (Lescol, Novartis; approved 31st Dec 1993; since 2011 generic).

Statins cause severe adverse effects such as asymptomatic elevation in liver enzyme activity, myopathy and increased risk of diabetes [2]. The risk of adverse effects rises with statins being used simultaneously with other drugs, which may cause drug-drug interactions. Pharmacokinetic of statins is complex. Statins are substrates for multiple membrane transporters, including organic anion transporting polypeptide OATP1B1, breast cancer resistance protein BCRP, and multidrug resistance protein 1 MDR1 [3]. Furthermore, statins undergo substantial microsomal metabolism by the enzyme system of cytochromes P450. Atorvastatin is metabolized mainly by CYP3A4, therefore, inhibitors of CYP3A4 such as protease inhibitor nelfinavir [4] or calcium channel blocker mibefradil [5] affect pharmacokinetics of atorvastatin. Fluvastatin is metabolized primarily by CYP2C9 [6]. Hepatic metabolism of rosuvastatin is predominantly mediated by the CYP2C9 enzyme, with little involvement of CYP3A4 [7]. Therefore, less extent of clinically significant pharmacokinetic drug-drug interactions between rosuvastatin/fluvastatin and other drugs as compared to atorvastatin have been observed [8,9]. Some drug-drug interactions are caused by activation or inhibition of major trasncriptional regulators of drug-metabolizing enzymes, in particular, aryl hydrocarbon receptor (AhR), glucocorticoid receptor (GR) and pregnane X receptor (PXR). Consequently, and induction or down-regulation of drug-metabolizing enzymes may occur. There are numerous reports demonstrating the activation of PXR and induction of PXR-regulated genes by statins [10–14]. Taking in account the presence of two chiral centres in molecules of statins, we tested, whether the effects of statins on the expression of drug-metabolizing cytochromes P450 and the activity of their transcriptional regulators are enantiospecific. Indeed, we have recently demonstrated that several clinically used chiral drugs, including antifungal ketoconazole [15,16], anticoagulant warfarin [17], and proton pump inhibitors omeprazole and lansoprazole [18,19] have enantiospecific effects towards transcriptional regulators (PXR, GR, AhR) of drug-metabolizing enzymes.

The aim of the current paper was to examine stereospecific effects of atorvastatin, fluvastatin and rosuvastatin enantiomers on: (i) the expression of drug-metabolizing cytochromes P450 in primary human hepatocytes; (ii) transcriptional activities of master regulators of drug-metabolizing enzymes, i.e. AhR, GR and PXR receptors, using gene reporter assays. We demonstrate that optical isomers of tested statins activate PXR and induce CYP3A4 in human hepatocytes with enantiospecific pattern. The data presented here might be of toxicological and clinical importance.

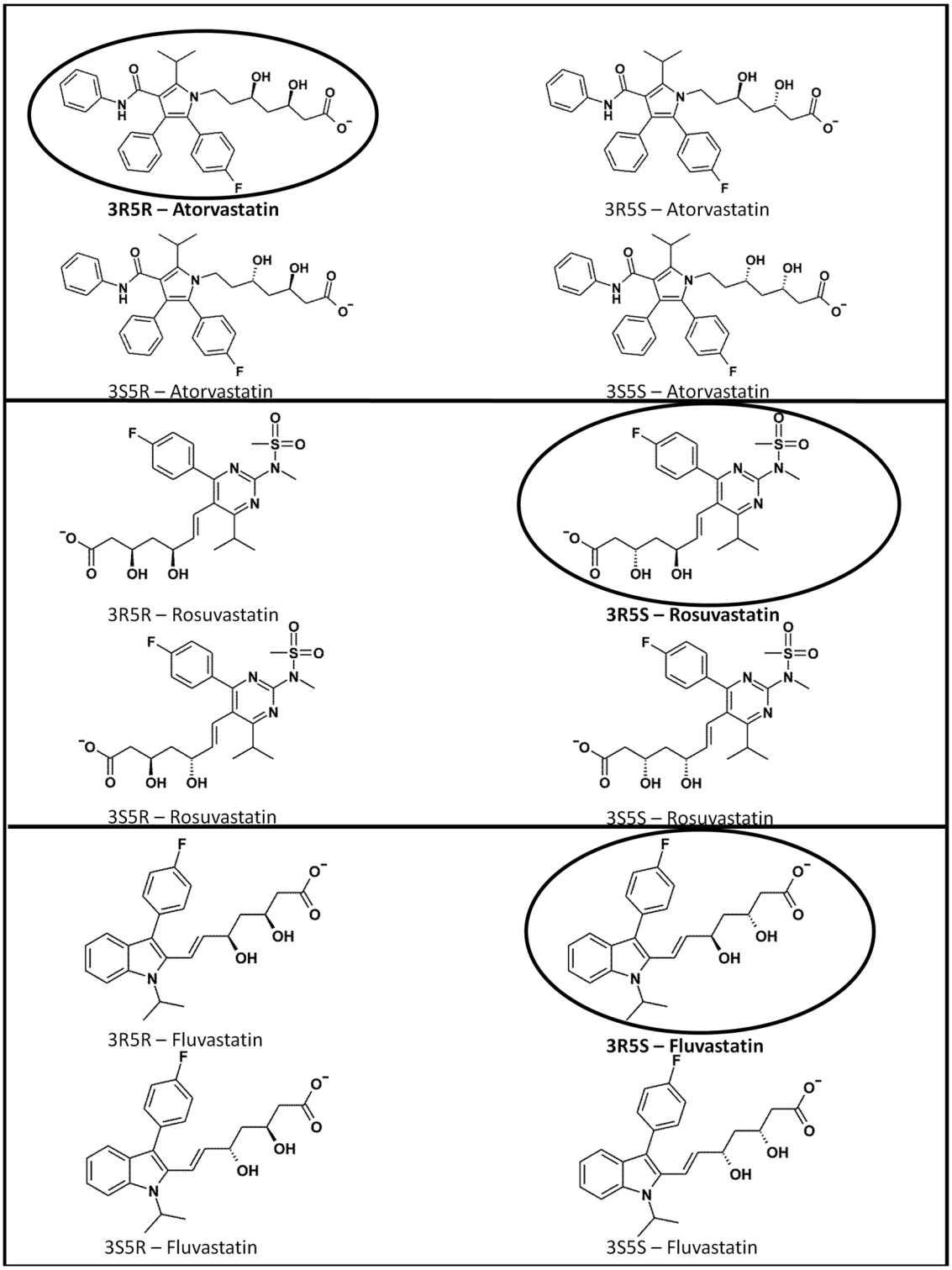

Fig 1. Chemical structures of enantiopure forms of statins. Four individual enantiomers of atorvastatin, rosuvastatin and fluvastatin are shown in the figure. Clinically used enantiopure forms are circled.

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Materials and Methods

Chemicals

Dimethylsulfoxide (DMSO), rifampicin (RIF), dexamethasone (DEX), mifepristone (RU486), resveratrol, hygromycin B and 3R5R-atorvastatin were purchased from Sigma-Aldrich (Prague, Czech Republic). 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was from Ultra Scientific (Rhode Island, USA). 3R5S- atorvastatin, 3S5R- atorvastatin and 3S5S-atorvastatin were purchased from Toronto Research Chemicals Inc. (Toronto, Canada). 3R5R- fluvastatin, 3R5S- fluvastatin, 3S5R- fluvastatin, 3S5S-fluvastatin, 3R5R- rosuvastatin, 3S5R- rosuvastatin and 3S5S-rosuvastatin were from TLC PharmaChem Inc. (Vaughan, Canada). 3R5S-rosuvastatin was purchased from Santa Cruz Biotechnology Inc. (Heidelberg, Germany). Luciferase lysis buffer and FuGENE® HD Transfection Reagent were from Promega (Madison, California, USA). All other chemicals were of the highest quality commercially available.

Cell culture

Human Caucasian colon adenocarcinoma cells LS174T (ECACC No. 87060401) and LS180 (ECACC No. 87021202) were purchased from *European Collection of Cell Cultures* (ECACC). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% of fetal bovine serum, 100 U/ml streptomycin, 100 μg/ml penicillin, 4 mM L-glutamine, 1% non-essential amino acids, and 1 mM sodium pyruvate. Cells were maintained at 37°C and 5% CO_2 in a humidified incubator. Stably transfected gene reporter cell lines AZ-AHR and AZ-GR were as described elsewhere [20,21].

Primary human hepatocytes used in this study were isolated from human liver obtained from three multiorgan donors: HH59 (female; 42 years), HH61 (male; 64 years) and HH63 (male; 68 years). The use of liver cells of donors HH59, HH61 and HH63 was approved by "Ethical committee at the Faculty Hospital Olomouc", and it was in accordance with Transplantation law #285/2002 Sb; "Ethical committee at the Faculty Hospital Olomouc" waived the authors from obtaining consent from the next of kin, regarding human hepatocytes obtained from liver donors HH59, HH61 and HH63. Cells were cultured in serum-free medium. Cultures were maintained at 37°C and 5% CO_2 in a humidified incubator.

Gene reporter assay and cytotoxicity assay

A stably transfected gene reporter cell line AZ-AHR, derived from human hepatoma HepG2 cells transfected with a construct containing several AhR binding sites upstream of a luciferase reporter gene, was used for assessment of AhR transcriptional activity [20]. A stably transfected gene reporter cell line AZ-GR, derived from human cervix carcinoma HeLa cells transfected with a construct containing several GR response elements upstream of a luciferase reporter gene, was used for measurement of GR transcriptional activity [21]. A transiently transfected LS180 human colon adenocarcinoma cells were used for assessment of PXR transcriptional activity. A chimera p3A4-luc reporter construct containing the basal promoter (-362/+53) with proximal PXR response element and the distal xenobiotic responsive enhancer module (-7836/-7208) of the CYP3A4 gene 5´-flanking region inserted to pGL3-Basic reporter vector was used. The reporter plasmid was transiently transfected to LS180 cells by lipofection (FuGENE® HD Transfection Reagent). All cell lines were incubated for 24 h with tested compounds and/or vehicle (DMSO; 0.1% v/v), in the presence (antagonist mode) or absence (agonist mode) of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; 5 nM), rifampicin (RIF; 10 μM) or dexamethasone (DEX; 100 nM). After the treatments, cells were lysed and luciferase activity was measured on Tecan Infinite M200 Pro plate reader (Schoeller Instruments, Prague, Czech

Republic). In parallel, cell viability was determined by conventional MTT test (MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide).

mRNA determination and qRT-PCR

Total RNA was isolated using TRI Reagent® (Molecular Research Center, Ohio, USA). cDNA was synthesized from 1000 ng of total RNA using M-MuLV Reverse Transcriptase (New England Biolabs, Ipswich, Massachusetts, USA) at 42°C for 60 min in the presence of random hexamers (New England Biolabs). qRT-PCR was carried out using LightCycler® 480 Probes Master (Prague, Roche Diagnostic Corporation, Czech Republic) on a Light Cycler® 480 II apparatus (Roche Diagnostic Corporation). CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP3A4 and GAPDH mRNAs were determined as described previously [22]. Measurements were performed in triplicates. Gene expression was normalized to GAPDH as a housekeeping gene.

Simple Western blotting by Sally Sue

Total protein extracts were prepared from cells cultured on 6-well plates. Cells were washed twice with ice-cold PBS and scraped into 1 ml of PBS. The suspension was centrifuged (4500 rpm/5 min/4°C) and the pellet was resuspended in 150 μl of ice-cold lysis buffer (150 mM NaCl; 10 mM Tris pH 7.2; 0.1% (w/v) SDS; anti-protease cocktail, 1% (v/v) Triton X-100; anti-phosphatase cocktail, 1% (v/v) sodium deoxycholate; 5 mM EDTA). The mixture was vortexed and incubated for 10 min on ice and then centrifuged (15000 rpm/13 min/4°C). Supernatant was collected and the protein content was determined by the Bradford reagent.

All reagents used for running the *simple western by Sally Sue*™ were obtained from ProteinSimple (San Jose, California) and prepared according to manufacturer's recommendations (http://www.proteinsimple.com/sally_sue.html). CYP1A1 (goat polyclonal, sc-9828, G-18), CYP1A2 (mouse monoclonal, sc-53614, 3B8C1), CYP2A6 (mouse monoclonal, sc-53615, F16P2D8), CYP2B6 (rabbit polyclonal, sc-67224, H-110), CYP3A4 (mouse monoclonal; sc-53850, HL3) primary antibodies and rabbit anti-goat secondary antibody (sc-2768) were purchased from Santa Cruz Biotechnology Inc. β-actin (mouse monoclonal; 3700S, 8H10D10) primary antibody was from Cell Signalling Technology (Denvers, Massachusetts, USA). CYP2C9 (rabbit polyclonal, AV41809, QC17985) was purchased from Sigma-Aldrich (Prague, Czech Republic). Antibody diluent, goat anti-rabbit secondary antibody, and goat anti-mouse secondary antibody were purchased from ProteinSimple. The capillaries, containing a proprietary UV-activated chemical linked reagent and 384-well plates were obtained from ProteinSimple. All samples and reagents were prepared according to the recommended ProteinSimple manual. Target proteins were identified using primary antibodies and immunoprobed using a horseradish peroxidase-conjugated secondary antibody and chemiluminescent substrate. The resulting chemiluminescent signal was detected and quantified by the Compass Software version 2.6.5.0 (ProteinSimple). For quantitative data analysis, the CYPs signals were normalized to β-actin as a loading control.

Electrophoretic mobility shift assay EMSA

Electrophoretic mobility shift assay was performed in nuclear fractions from LS174T cells using Nuclear extract kit (Active Motif) according to manufacturer's protocol. Consequently, nuclear fractions were incubated for 2 h at 30°C with DMSO (0.1% v/v), RIF (10 μM) and tested compounds at concentration 10 μM. The following double-stranded 5´-biotinylated oligonucleotides containing DR3 motif from the XREM sequence of CYP3A4 gene promoter were used. Gel mobility shift assay was performed using LightShift Chemiluminescent EMSA

Kit (Thermo Scientific, Waltham, MA, USA) as described previously [23,24]. The sequences of DR3 oligonucleotide were: sense 5´-GAATGAACTTGCTGACCCTCT-3´; antisense 5´-AGA GGGTCAGCAAGTTCATTC-3´.

Statistics

Student´s t-test, One-way ANOVA followed by Dunnett's post test as well as calculations of EC_{50} and IC_{50} values were calculated using GraphPad Prism version 6.0 for Windows, GraphPad Software, La Jolla, California, USA.

Results

Cytotoxicity of statin enantiomers in human cancer cell lines

Prior to gene reporter assays, we examined the cytotoxicity of tested compounds in AZ-AHR, AZ-GR and LS180 cell lines. For this purpose, the cells were incubated for 24 h with individual enantiomers of atorvastatin, fluvastatin and rosuvastatin at concentrations ranging from 100 pM to 100 μM. The vehicle was DMSO (0.1% v/v). After the treatment, a conventional MTT test was performed and the values of IC_{50} were calculated. Based on the results from cytotoxicity testing (Fig 2), gene reporter assays were performed in concentrations of tested compounds up to 100 μM, with exception of atorvastatin, where maximal concentration of 10 μM was used for incubations in AZ-AHR and AZ-GR cells.

Effects of statin enantiomers on transcriptional activity of aryl hydrocarbon receptor

Transcriptional activity of AhR was assessed in human gene reporter cell line AZ-AHR incubated for 24 h with tested compounds. An induction of AhR-dependent luciferase activity by model agonist dioxin (TCDD; 5 nM) in eight consecutive passages of AZ-AHR cells varied from 443-fold to 2353-fold (average induction 1107-fold), as compared to DMSO-treated cells. No significant induction of luciferase activity was observed for any atorvastatin enantiomer. 3S5S-rosuvastatin, but not other optical isomers, dose-dependently increased luciferase activity with average EC_{50} value of 17.5 ± 0.4 μM. 3R5R- fluvastatin, 3S5R- fluvastatin and 3S5S- fluvastatin slightly increased luciferase activity with average EC_{50} values of 22.0 ± 13.4 μM, 14.4 ± 4.2 μM and 14.7 ± 0.9 μM, respectively. However, the efficacy of 3S5S-rosuvastatin was about 0.1% of induction attained by TCDD. Similarly, optical isomers of fluvastatin activated AhR with efficacy about 0.01% (Fig 3). TCDD-inducible transcriptional activity of AhR was dose-dependently inhibited by 3R5S- rosuvastatin and 3R5S-fluvastatin. The decrease of TCDD-inducible luciferase activity correlated with decrease of AZ-AHR cells viability (Fig 2), therefore, the observed effect was rather due to the partial cytotoxicity of the compounds than due to antagonism of AhR. All other forms of rosuvastatin, fluvastatin and all atorvastatin enantiomers did not antagonize AhR. Overall, the gene reporter assays in AZ-AHR cells imply zero clinical or toxicological potential of statin enantiomers in terms of AhR activation.

Effects of statin enantiomers on transcriptional activity of glucocorticoid receptor

Transcriptional activity of GR was assessed in human gene reporter cell line AZ-GR incubated for 24 h with tested compounds. An induction of GR-dependent luciferase activity by model agonist dexamethasone (DEX; 100 nM) in eight consecutive passages of AZ-GR cells varied from 9-fold to 63-fold (average induction 32-fold), as compared to DMSO-treated cells. None of the tested statins induced GR-dependent luciferase activity. Dexamethasone-inducible

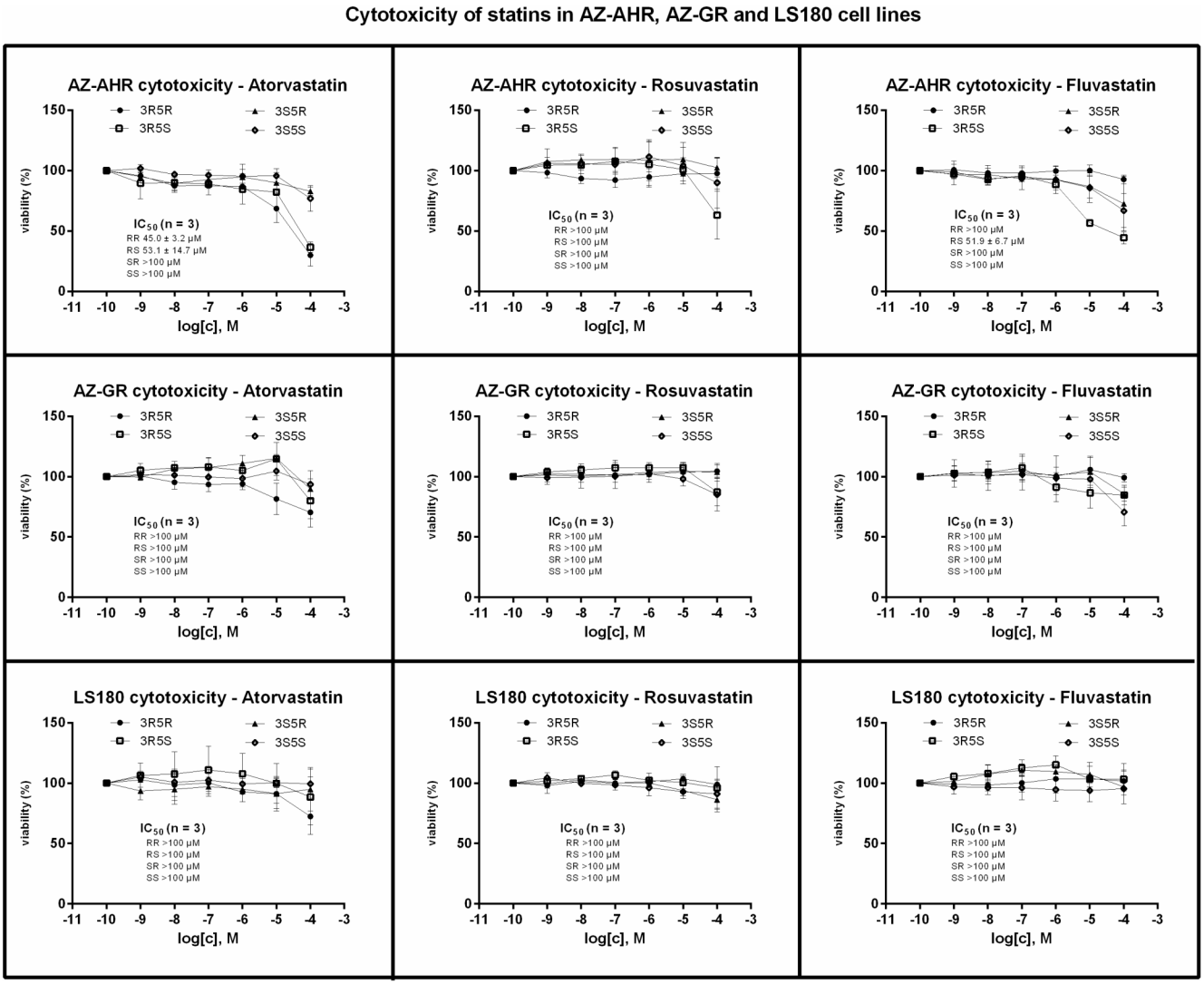

Fig 2. Cytotoxicity of statin enantiomers in human cancer cell lines. AZ-AHR, AZ-GR and LS180 cells were seeded in 96-well plates, stabilized for 16 h and then incubated for 24 h with enantiopure forms of atorvastatin, rosuvastatin and fluvastatin in concentration ranging from 10^{-10} M to 10^{-4} M. The vehicle was dimethylsulfoxide (DMSO; 0.1% v/v). After the treatment, a conventional MTT test was performed and absorbance was measured at 540 nm. Treatments were performed in triplicates. The data are the mean ± SD from experiments performed in three consecutive passages of cells and are expressed as percentage of viability of control cells. The values of IC_{50} were calculated where appropriate and they are indicated in plots. Student´s t-test, One-way ANOVA followed by Dunnett's post test and IC_{50} values were calculated using GraphPad Prism.

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transcriptional activity of GR was decreased by 3R5R-atorvastatin (10 μM) and 3R5S-rosuvastatin (100 μM), but the decrease was not dose-dependent and occurred probably due to the cytotoxicity of tested compounds. On the other hand, 3S5S- fluvastatin, 3R5R- fluvastatin, 3R5S- fluvastatin, but not 3S5R- fluvastatin, dose-dependently antagonized GR and there were significant differences between fluvastatin optical isomers (Fig 4).

Effects of statin enantiomers on transcriptional activity of pregnane X receptor

Transcriptional activity of PXR was tested in human colon adenocarcinoma cells LS180 transiently transfected with p3A4-luc reporter construct, incubated for 24 h with tested

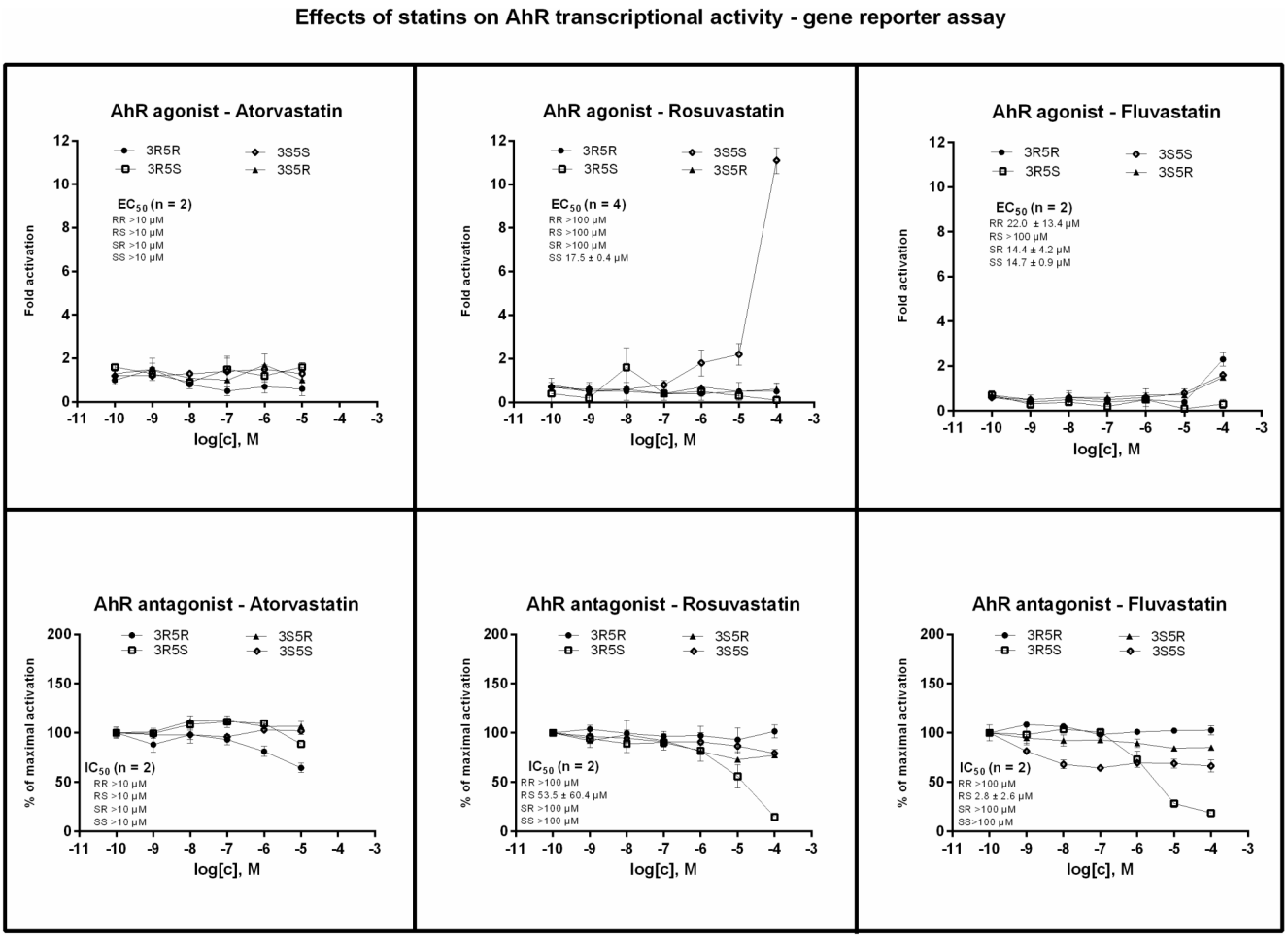

Fig 3. Effects of statin enantiomers on trancriptional activity of human aryl hydrocarbon receptor. AZ-AHR cells were seeded in 96-well plates and stabilized for 16 h and then incubated for 24 h with enantiopure forms of atorvastatin, rosuvastatin and fluvastatin in concentration ranging from 10^{-10} M to 10^{-4} M in the absence (agonist mode–upper panels) or presence (antagonist mode–lower panels) of dioxin (TCDD; 5 nM). The vehicle was DMSO (0.1% v/v). After the treatments, cells were lysed and luciferase activity was measured. Treatments were performed in triplicates. Data are expressed as a fold induction of luciferase activity over control cells (agonist mode) or as a percentage of maximal activation attained by TCDD (antagonist mode). The values of EC_{50} and IC_{50} from *n* independent cell passages were calculated where appropriate and the average values are indicated in plots. Representative gene reporter assays are shown. Student´s t-test, One-way ANOVA followed by Dunnett's post test and EC_{50}/IC_{50} values were calculated using GraphPad Prism.

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compounds. An induction of PXR-dependent luciferase activity by model agonist rifampicin (RIF; 10 μM) in three consecutive passages varied from 8-fold to 13-fold (average induction 10-fold), as compared to DMSO-treated cells. Transcriptional activity of PXR was dose-dependently induced by all tested statins, and the potency and efficacy between individual optical isomers varied substantially. Ligand-inducible transcriptional activity of PXR was influenced by all tested statins, again differentially, depending on statin and optical isomer:

FLUVASTATIN: The potencies of optical isomers of fluvastatin towards PXR were comparable and half maximal effective concentrations EC_{50} ranged from 8.7 μM to 15.4 μM. The efficacies of fluvastatin enantiomers in 100 μM concentrations slightly varied with average inductions of luciferase activity approx. 5-fold (for 3R5R-fluvastatin and 3S5R-fluvastatin) and 3-fold (for 3R5S-fluvastatin and 3S5S-fluvastatin) (Fig 5; upper-right panel). Combined treatments of LS180 cells with PXR agonist rifampicin and enantiomers of fluvastatin revealed inversed U-shaped curves. Dose-dependent augmentation of rifampicin-inducible luciferase

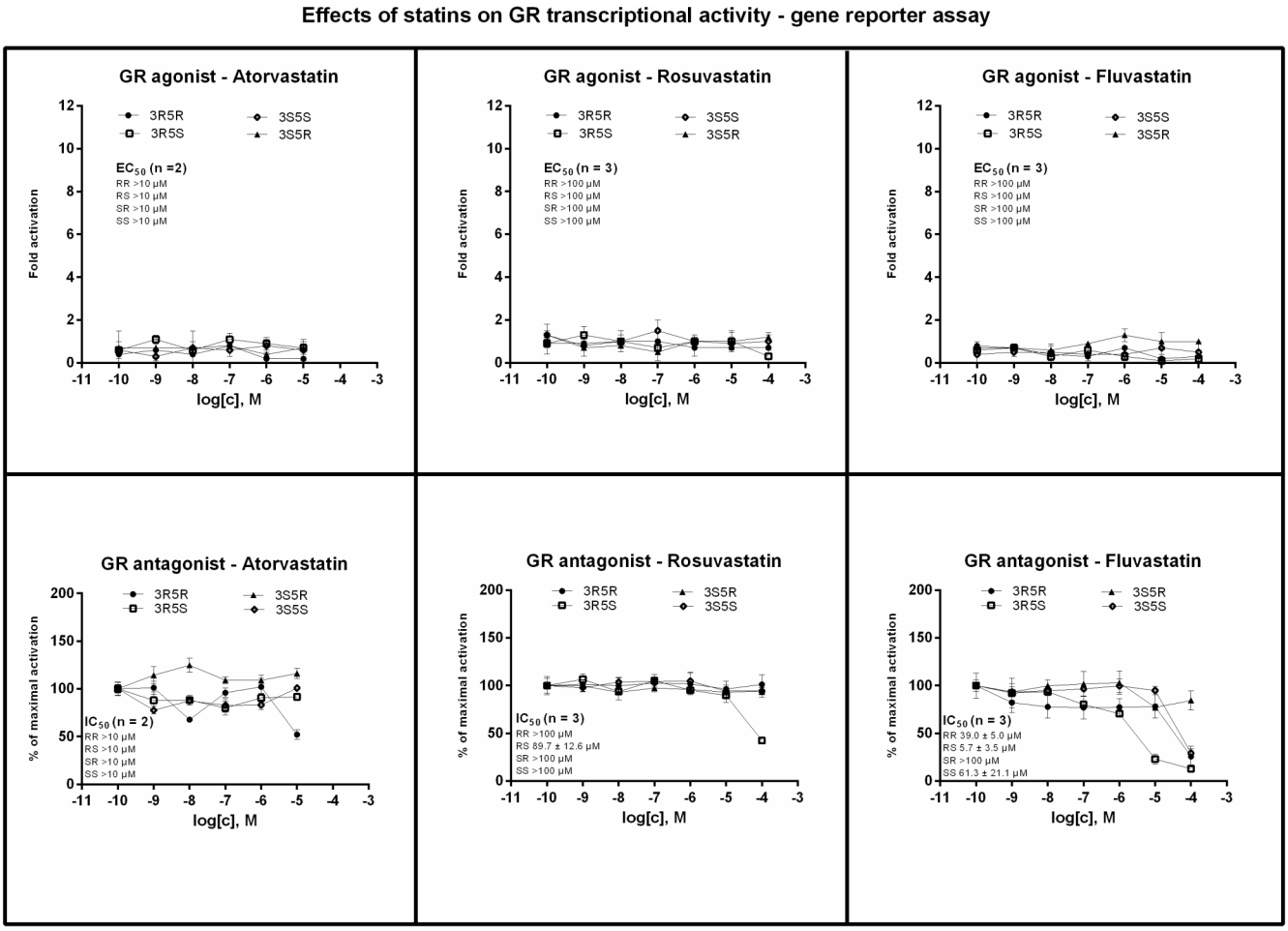

Fig 4. Effects of statin enantiomers on transcriptional activity of human glucocorticoid receptor. AZ-GR cells were seeded in 96-well plates and stabilized for 16 h and then incubated for 24 h with enantiopure forms of atorvastatin, rosuvastatin and fluvastatin in concentration ranging from 10^{-10} M to and 10^{-4} M in the absence (agonist mode–upper panels) or presence (antagonist mode–lower panels) of dexamethasone (DEX; 100 nM). The vehicle was DMSO (0.1% v/v). After the treatments, cells were lysed and luciferase activity was measured. Treatments were performed in triplicates. Data are expressed as a fold induction of luciferase activity over control cells (agonist mode) or as a percentage of maximal activation attained by DEX (antagonist mode). The values of EC_{50} and IC_{50} from *n* independent cell passages were calculated where appropriate and the average values are indicated in plots. Representative gene reporter assays are shown. Student´s t-test, One-way ANOVA followed by Dunnett's post test and EC_{50}/IC_{50} values were calculated using GraphPad Prism.

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activity (150% of rifampicin value) was observed for all statins, regardless optical configuration, in concentrations up to 10^{-6} M, followed by drop of luciferase activity (85% of rifampicin value) at concentrations of statins of 10^{-4} M (Fig 5; lower-right panel). *ROSUVASTATIN*: The efficacies of rosuvastatin enantiomers in 100 μM concentrations were similar and varied around 3-fold induction. The potencies of rosuvastatin optical isomers substantially differed, with following EC_{50} values: 3S5S (1.2 μM) > 3R5R (5.8 μM) > 3R5S (11.9 μM) > 3S5R (15.6 μM) (Fig 5; upper-middle panel). Rifampicin inducible PXR transcriptional activity was not influenced by 3S5R-rosuvastatin, whereas 3R5S-rosuvastatin displayed inverse U-shaped curve, similarly as fluvastatin enantiomers. In contrast, 3R5R-rosuvastatin and 3S5S-rosuvastatin in combination with rifampicin revealed U-shaped curve with minimum at concentrations range 10^{-8} M- 10^{-6} M (60% of rifampicin value) (Fig 5; lower-middle panel). *ATORVASTATIN*: The efficacies of atorvastatin 3R5R- atorvastatin, 3R5S- atorvastatin and 3S5R- atorvastatin in 100 μM concentrations slightly varied with average inductions of luciferase activity

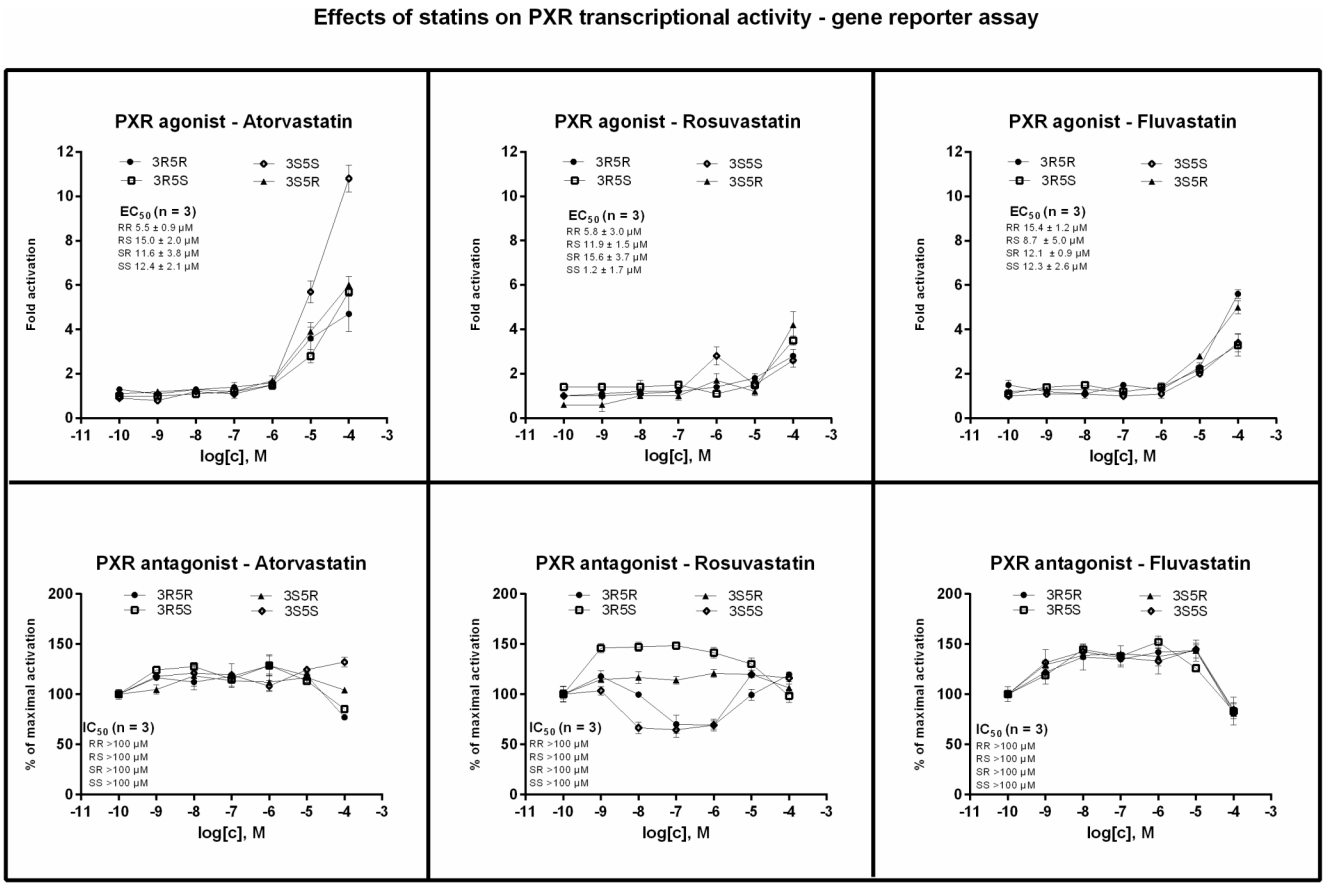

Fig 5. Effects of statin enantiomers on transcriptional activity of human pregnane X receptor. LS180 cells, transiently transfected with p3A4-luc reporter, were seeded in 96-well plates and stabilized for 16 h and then incubated for 24 h with enantiopure forms of atorvastatin, rosuvastatin and fluvastatin in concentration ranging from 10^{-10} M to 10^{-4} M in the absence (agonist mode–upper line) or presence (antagonist mode–lower line) of rifampicin (RIF; 10 μM). The vehicle was DMSO (0.1% v/v). After the treatments, cells were lysed and luciferase activity was measured. Treatments were performed in triplicates. Data are expressed as a fold induction of luciferase activity over control cells (agonist mode) or as a percentage of maximal activation attained by RIF (antagonist mode). The values of EC_{50} and IC_{50} from *n* independent cell passages were calculated where appropriate and the average values are indicated in plots. Representative gene reporter assays are shown. Student´s t-test, One-way ANOVA followed by Dunnett's post test and EC_{50}/IC_{50} values were calculated using GraphPad Prism.

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approx. 5-fold. Contrary, the efficacy of 3S5S-atorvastatin (100 μM) was much higher, reaching an induction 11-fold. The half maximal effective concentrations EC_{50} for 3S5S-atorvastatin, 3R5S- atorvastatin and 3S5R- atorvastatin ranged from 11.6 μM to 15.0 μM. Interestingly, the potency of clinically used 3R5R-atorvastatin was significantly different from other optical isomers, with EC_{50} of 5.5 μM (Fig 5; upper-left panel). Ligand-inducible transcriptional activity of PXR was not affected by any optical isomer of atorvastatin (Fig 5; lower-left panel). Slight decrease in luciferase activity at 100 μM concentrations of statin corresponds to descrease in cell viability (Fig 2; lower-left panel).

Effects of statin enantiomers on the expression of drug-metabolizing cytochromes P450 in primary human hepatocytes

We examined a capability of statin enantiomers to induce transcriptionally regulated drug-metabolizing cytochromes P450 in three human hepatocytes cultures (HH59, HH61, HH63). Hepatocytes were treated for 24 h (for determination of mRNA) or 48 h (for determination of

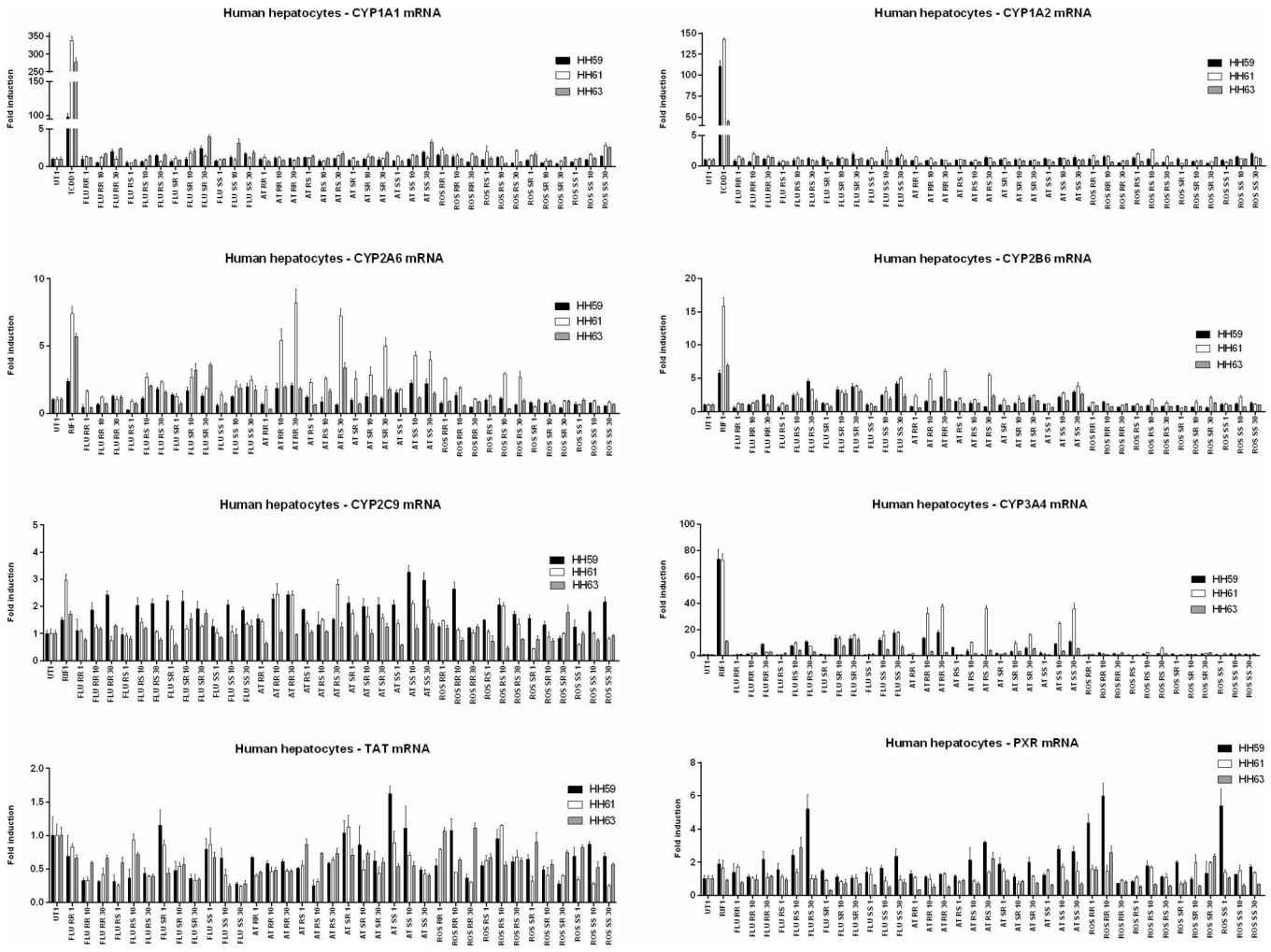

Fig 6. Effects of statin enantiomers on the expression of drug-metabolizing cytochromes P450, PXR and tyrosin aminotransferase TAT at mRNA level in primary human hepatocytes. Primary human hepatocytes from three different donors (HH59, HH61, HH63) were used. Cells were incubated for 24 h with vehicle (DMSO; 0.1% v/v), dioxin (TCDD; 5 nM), rifampicin (RIF; 10 μM) and individual enantiomers of statins (1 μM, 10 μM, 30 μM). Bar graphs of RT-PCR analyses of CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP3A4, PXR and TAT mRNAs are shown. The data are the mean ± SD from triplicate measurements and are expressed as a fold induction over vehicle-treated cells. The data were normalized to GAPDH mRNA levels. Student´s t-test and One-way ANOVA followed by Dunnett's post test were calculated using GraphPad Prism.

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proteins) with optical isomers of tested statins (1 μM, 10 μM, 30 μM), dioxin (TCDD; 5 nM), rifampicin (RIF; 10 μM) and vehicle (DMSO; 0.1% v/v).

CYP1A1 and CYP1A2. Dioxin strongly induced CYP1A1 and CYP1A2 mRNAs in all human hepatocytes cultures after 24 h of incubation, and the magnitudes of induction in cultures HH59, HH61 and HH63 for CYP1A1/CYP1A2 mRNAs were 98-fold/110-fold, 339-fold/143-fold, and 278-fold/45-fold, respectively. None of the statins tested did significantly induce CYP1A1 or CYP1A2 mRNA in any human hepatocyte culture (Fig 6). Dioxin strongly induced CYP1A1 and CYP1A2 proteins in all human hepatocytes cultures after 48 h of incubation, and the magnitudes of induction in cultures HH59, HH61 and HH63 for CYP1A1/CYP1A2 proteins were 35-fold/19-fold, 220-fold/83-fold, and 35-fold/50-fold, respectively. In line with data at mRNAs level, we did not observe significant induction of CYP1A1 or CYP1A2 protein by any tested statin (Fig 7). Since CYP1A1 and CYP1A2 are dominantly regulated by AhR, the

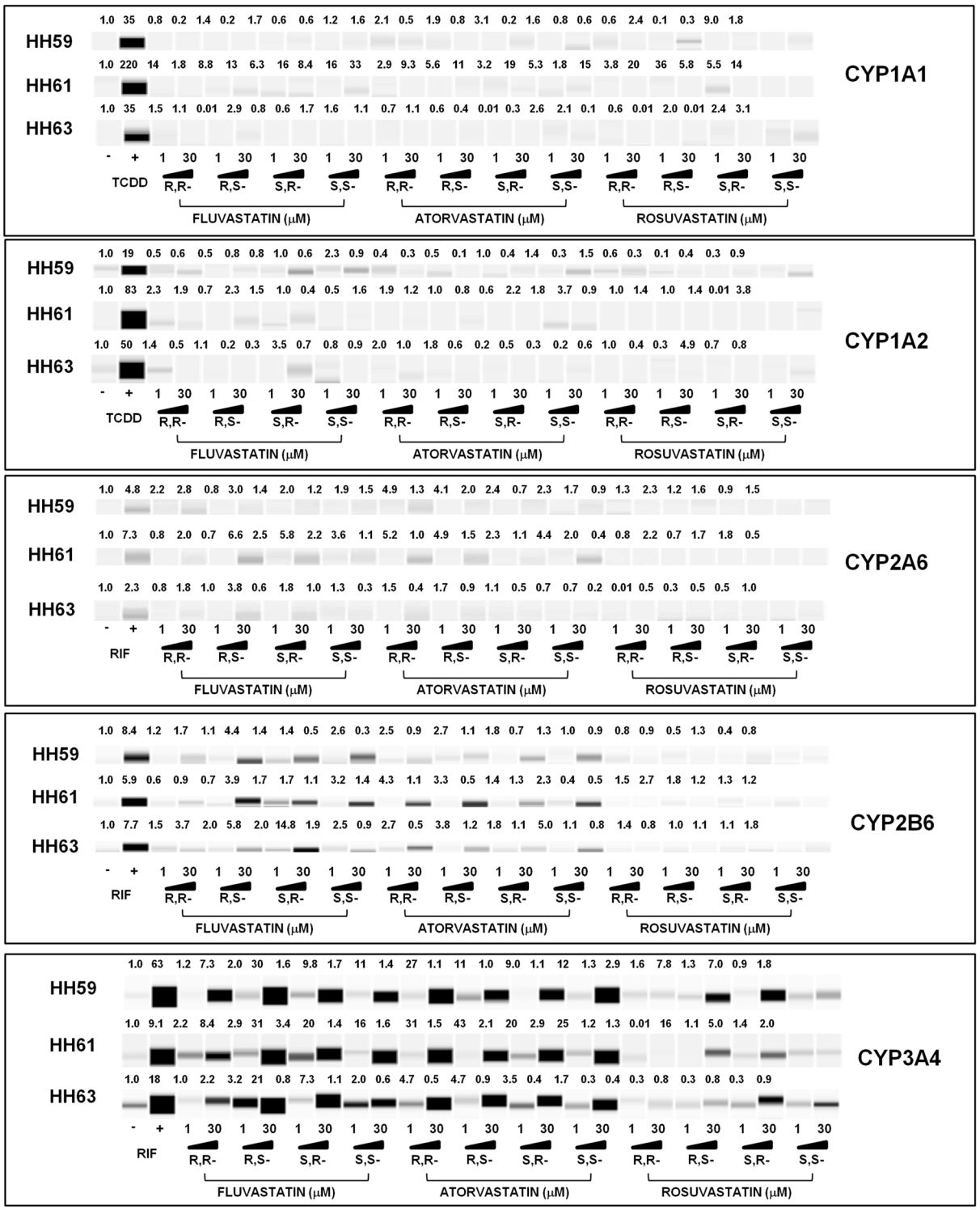

Fig 7. Effects of statin enantiomers on the expression of drug-metabolizing cytochromes P450 at protein level in primary human hepatocytes. Primary human hepatocytes from three different donors (HH59, HH61 and HH63) were used. Cells were incubated for 48 h with vehicle (DMSO; 0.1% v/v), dioxin (TCDD; 5 nM), rifampicin (RIF; 10 μM) and individual enantiomers of statins (1 μM, 30 μM). Simple Western blots of CYP1A1, CYP1A2, CYP2A6, CYP2B6 and CYP3A4 are shown. The data are expressed as a fold induction over vehicle-treated cells and normalized to β-actin levels. *Note*: *analyses of 780 samples are contained in a figure*.

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effects of statins on CYP1A1 and CYP1A2 expression are consistent with their effects on AhR in gene reporter assays (Fig 3).

CYP2A6. Induction of CYP2A6 mRNA by rifampicin after 24 h of incubation was 2.4-fold, 5.2-fold and 7.1-fold in human hepatocytes cultures HH59, HH61 and HH63, respectively. Induction of CYP2A6 protein by rifampicin after 48 h of incubation was 4.8-fold, 7.3-fold and 2.3-fold in human hepatocytes cultures HH59, HH61 and HH63, respectively. Rosuvastatin enantiomers did not significantly induce the expression of CYP2A6 mRNA and protein, with exception of weak increase of CYP2A6 mRNA in culture HH61by 3R5S-rosuvastatin (10 μM; 30 μM). Induction profiles of CYP2A6 by fluvastatin enantiomers differed among individual cultures, which could be caused by interindivual variability of the donors. Generaly, 3S5S-fluvastatin, 3R5S-fluvastatin, 3S5R-fluvastatin, but not 3R5R-fluvastatin, weakly induced CYP2A6 mRNA and protein. Induction of CYP2A6 mRNA by atorvastatin enantiomers was higher than those by fluvastatin and rosuvastatin in all human hepatocytes cultures, and in some cases even higher that that by rifampicin, implying again interindividual variability between human hepatocytes donors. The magnitude of CYP2A6 induction by atorvastatin enantiomers increased in order: 3R5R > 3R5S = 3S5R > 3S5S (Figs 6 and 7).

CYP2B6. Inductions of CYP2B6 mRNA/protein by rifampicin after 24 h/48 h of incubation were 5.8-fold/8.4-fold, 16-fold/5.9-fold and 6.9-fold/7.7-fold in human hepatocytes cultures HH59, HH61 and HH63, respectively. The induction profiles of CYP2B6 by tested statins displayed similar patter as those for CYP2A6. Rosuvastatin did induce neither CYP2B6 mRNA nor CYP2B6 protein in any human hepatocyte culture. We found moderate, dose-dependent induction of CYP2B6 by 3S5S-fluvastatin, 3R5S-fluvastatin, 3S5R-fluvastatin, but not 3R5R-fluvastatin, in all three hepatocytes cultures. Atorvastatin was the strongest inducer of CYP2B6, as compared to rosuvastatin and fluvastatin. Dose-dependent induction of CYP2B6 mRNA and protein by atorvastatin optical isomers increased as follows:
3R5R > 3R5S = 3S5S > 3S5R (Figs 6 and 7).

CYP2C9. Induction of CYP2C9 mRNA by rifampicin after 24 h of incubation in three human hepatocytes cultures varied from 1.5-fold to 3-fold. We did not evaluate induction of CYP2C9 protein, because commercial CYP2C9 antibodies were not compatible with SallySue Simple Western System used for analyses. The effects of optical isomers of tested statins on CYP2C9 expression were rather positive modulatory, displaying weak inductions with similar patterns as those for CYP2A6 and CYP2B6 (Figs 6 and 7).

CYP3A4. Inductions of CYP3A4 mRNA/protein by rifampicin after 24 h/48 h of incubation were 59-fold/63-fold, 61-fold/9-fold and 11-fold/18-fold in human hepatocytes cultures HH59, HH61 and HH63, respectively. Rosuvastatin did not induce CYP3A4 mRNA, but 3S5R-rosuvastatin and 3R5S-rosuvastatin increased CYP3A4 protein in two human hepatocyte cultures. Fluvastatin induced dose-dependently CYP3A4 mRNA and protein in all human hepatocytes cultures. The effects of 3S5S-fluvastatin, 3R5S-fluvastatin and 3S5R-fluvastatin were nearly equipotent, while 3R5R-fluvastatin was much weaker inducer of CYP3A4 as compared to remaining enantiomers. All optical isomers of atorvastatin strongly and dose-dependently induced CYP3A4 mRNA and protein in all human hepatocytes cultures. The magnitude of CYP3A4 induction differed for individual enantiomers as follows:
3R5R > 3R5S = 3S5S > 3S5R (Figs 6 and 7).

Effects of statin enantiomers on the expression of PXR and tyrosin aminotransferase TAT mRNAs in primary human hepatocytes

Glucocorticoid receptor GR plays central role in transcriptional regulation of drug-metabolizing enzymes by multiple mechanisms, therefore, we also analyzed the expression of tyrosine

aminotransferase TAT (exclusive GR-target gene) and PXR (non-exclusive GR-target gene) in primary human hepatocytes. Primary human hepatocytes are routinely cultured in the presence of dexamethasone in concentration, which fully activates GR. Therefore, the TAT gene is induced under these conditions, and we evaluated either inhibitory or synergistic effects of statins on TAT expression. We observed down-regulation of TAT by some of the compounds tested, however, the effects lacked dose-response pattern and they were not systematic between human hepatocytes cultures. The most frequent and pronounced were the effects by fluvastatin enantiomers (Fig 6), which is consistent with antagonism of GR by these compounds (Fig 4). Similarly, tested statins had mild modulatory effects on the expression of PXR mRNA, however, the effects varied between hepatocytes cultures and were not dose dependent (Fig 6).

Binding of PXR to DNA—electrophoretic mobility shift assay

We tested whether the effects of statins on PXR-CYP3A4 signaling pathway involves also changes in the formation of PXR/RXRα-DNA complex. PXR-mediated gene activation requires direct binding of the PXR-RXRα heterodimeric complex to the response elements in the gene promoter. Maximal induction of CYP3A4 gene expression apparently requires an additional DR3 nuclear receptor-binding element 1(dNR1; -7733/-7719) in a distal xenobiotic responsive enhancer module [25,26]. Nuclear extracts from LS174T cells treated by DMSO (0.1% v/v), RIF (10 μM) and individual enantiomers of atorvastatin, rosuvastatin and fluvastatin at concentration 10 μM were incubated with biotin-labeled double-stranded oligonucleotide corresponding to the DR3 PXR response element in the CYP3A4 promoter and resolved on non-denaturing gel. The specificity of PXR-RXRα binding was confirmed by competition with non-labeled double-stranded DR3 oligonucleotide. Positive control RIF strongly stimulated a formation of PXR/RXRα-DNA-binding complex (Fig 8A). All tested compouds increased binding of PXR/RXRα to the DR3 module in comparison with vehicle. In many samples, the intensity of bands was comparable with that by positive control rifampicin. Given that this

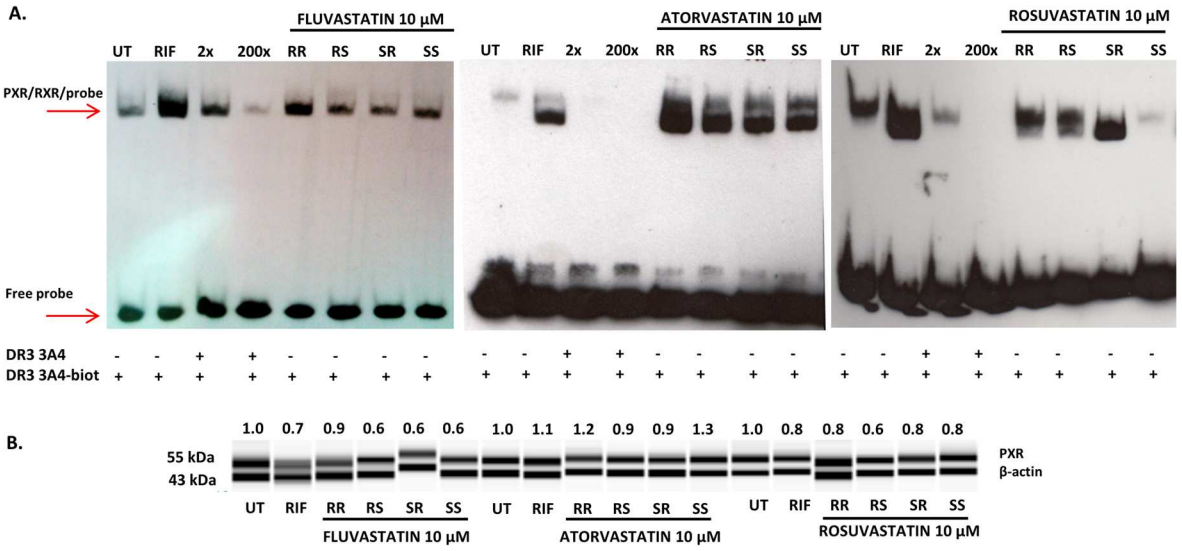

Fig 8. Effect of statin enantiomers on the binding of PXR/RXR complex to the DR3 motif of human CYP3A4 gene promoter. Nuclear fractions of LS174T cells from three independent cell passages were incubated for 2 h at 30°C with DMSO (0.1% v/v), RIF (10 μM) and individual enantiomers of atorvastatin, rosuvastatin and fluvastatin at concentration 10 μM. Treated nuclear extracts were incubated with a biotin-labeled CYP3A4-DR3 probe and electrophoresed on 5% polyacrylamide gel as described under "Materials and methods section." **A.** The complex formation of CYP3A4 DR3 response element with PXR-RXRα heterodimer. **B.** Simple western blot showing equal expression levels of PXR nuclear extracts used for EMSA (normalized to β-actin levels).

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method is semi-quantitative, we do not attempt to compare the intensity of individual bands between enantiomers. Immunoblot analysis confirmed that equal amounts of PXR proteins were used in the gel shift assay (Fig 8B).

Discussion

In the current paper, we investigated enantioselective effects of three, massively used, chiral statins on the expression of inducible drug-metabolizing cytochromes P450 in primary human hepatocytes and on activity of major drug-metabolizing pathways transcriptional regulators. We describe here, for the first time, that atorvastatin, rosuvastatin and fluvastatin enantiospecifically induce CYP2B6, CYP2A6 and CYP3A4 in human hepatocytes, and also that they enantiospecifically influence transcriptional activity of PXR and GR. The discovery of statins was a breakpoint in pharmacotherapy of hypercholesterolemia, therefore, they are also considered as blockbuster drugs. However, numerous drug-drug interactions were reported in patients used statins simultaneously with other drugs. The mechanistic bases for these interactions involve mainly either inhibition or induction of drug-metabolizing cytochromes P450 by statins. The examples of drugs, the metabolism of which is influenced by inhibition of P450 catalytic activity by statins are antihypertensive losartan [27], antidiabetic rapaglinide [28], calcium channel blockers nifedipine [29] and verapamil [30] etc. For instance, fluvastatin was described as a potent inhibitor of CYP2C9 human recombinant enzyme [31], as well as in human liver microsomes and human hepatocytes [32]. Enantiospecific pattern of CYP2C9 inhibition by fluvastatin was described, when K_i values were approx. four to five times higher for (-)3S,5R-fluvastatin as compared to (+)3R,5S-fluvastatin [33]. The differences in pharmacokinetic of fluvastatin enantiomers were also observed in humans, when AUC value for (-)3S,5R-fluvastatin was 1.8 times higher than that for (+)3R,5S-fluvastatin [34,35]. Induction of drug-metabolizing CYPs is also a frequent cause for drug-drug interactions. There are numerous reports demonstrating the activation of PXR and induction of PXR-regulated drug-metabolizing cytochromes P450 by statins [10–14].

The statins investigated in the current paper are clinically used as pure enantiomers, i.e. 3R5R-atorvastatin, 3R5S-rosuvastatin and 3R5S-fluvastatin. Since these statins were introduced in a clinical practice directly as enantiopure drugs, the data on induction of drug-metabolizing P450s by optical isomers by tested statins are missing. The rational for use of pure enantiomers is increasing therapeutic efficacy and/or diminishing adverse effects and toxicity of the drug. This concept, unlike in case of statins, led to introduction of enantiopure drugs, which were originally used as racemates. The examples are omeprazole/esomeprazole, citalopram/escitalopram, modafinil/armodafinil, cetirizine/levocetirizin and many others. We have recently described that several clinically used chiral drugs, including ketoconazole [15,16], warfarin [17], omeprazole and lansoprazole [18,19] have enantiospecific effects on PXR, GR and/or AhR and the expression of drug-metabolizing P450s. Taken together, the aim of the current work was to investigate, whether the effects of optical isomers of clinically used chiral statins may induce drug-metabolizing P450s in stereospecific manner, and to compare the effects of clinically used enantiomers with remaining optical isomers of the statins.

None of the tested statins and their optical isomers induced CYP1A1 and CYP1A2 in primary human hepatocytes. Consistently, transcriptional activity of AhR was not influenced by enantiomers of atorvastatin, fluvastatin and rosuvastatin, as revealed by gene reporter assays.

All optical isomers of all tested statins caused formation of PXR-DNA complex in three independent EMSA experiments, but quantitative profiles between enantiomers were not reproducible. Xenobiotics-inducible cytochromes P450, belonging to families CYP2 and CYP3, were differentially induced by all statins. The least active were optical isomers of rosuvastatin,

which only moderately activated PXR in gene reporter assays (about 2–4 fold at 100 μM), without significant differences between enantiomers. Rosuvastatin did not induce CYP2/CYP3 genes, with exception of significant induction of CYP3A4 protein, but not CYP3A4 mRNA, by 3R5S-rosuvastatin and 3S5R-rosuvastatin in all human hepatocytes cultures. Enantiomer 3R5S-rosuvastatin also induced CYP2A6 mRNA. Since clinically used optical isomer of rosuvastatin in 3R5S form, the induction of CYP2A6 and CYP3A4 is unfavorable. The induction profiles of CYP2A6, CYP2B6 and CYP3A4 by atorvastatin enantiomers were similar, and the potency of individual optical isomers decreased in order RR>RS = SR>SS. Clinically used atorvastatin is 3R5R form, hence, it is again unfavorable situation with regard to P450 induction-based interactions. Half maximal effective concentration EC_{50} in PXR-gene reporter assays was the lowest for 3R5R-atorvastatin (about 50% of other atorvastatin enantiomers), which is consistent with the fact that CYP2/3 families are dominantly regulated by PXR. On the other hand, the magnitude of luciferase induction was the highest for 3S5S-atorvastatin, which was the weakest inducer of CYP2/3 genes.

Optical isomers of fluvastatin RR, RS and SS displayed dose-dependent antagonistic activity against GR, which is a master regulator of xenobiotic metabolic pathways by multiple mechanisms, including the regulation of PXR expression. Antiglucocorticoid activity of 3R5R-fluvastatin and 3S5S-fluvastatin was also confirmed in human hepatocytes, where we observed down-regulation of TAT mRNA by these two compounds. Therefore, the induction of P450s by fluvastatin comprises both agonist effects on PXR and antagonist effects on GR. The magnitude of induction of CYP2A6, CYP2B6 and CYP3A4 was much weaker by 3R5R-fluvastatin as compared to other three enantiomers.

Besides GR and PXR, important transcriptional regulators of CYP2 and CYP3 genes are nuclear receptors, steroid receptors and also xenoreceptor CAR (Constitutive Androstane Receptor). It was demonstrated that several clinically important statins are activators of FXR (Farnesoid X Receptor) and CAR [11,36]. Indeed, there is mutual cross-talk between PXR and CAR, in terms of sharing response elements, co-activators, target genes and ligands [37]. Therefore, it is likely that tested statins may have enantiospecific effects also against CAR.

A myriad of QSAR (Quantitative Structure-Activity Relationship) studies was performed with clinically used statins both, in the phase of their development and in the research on newly synthesized HMG-CoA inhibitors [38]. On the other hand, no systematic attempts have been made regarding QSAR of stains and their off-targets, e.g. PXR, GR or CAR.

Overall, the current study is the first report on enantioselective effects of statins on the expression of xenobiotic-metabolizing human enzymes. The data contained in the manuscript show that the potential for drug-drug interactions involving induction of P450s is higher for clinically used optical isomers of rosuvastatin, atorvastatin and fluvastatin, as compared to their respective enantiomers, which are not in therapeutic use.

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Author Contributions

Conceived and designed the experiments: ZD. Performed the experiments: MK AD. Analyzed the data: MK AD ZD. Contributed reagents/materials/analysis tools: ZD. Wrote the paper: MK ZD.

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APPENDIX II.

Stepankova M., Krasulova K., Doricakova A., Kurka O., Anzenbacher P., Dvorak Z. (2016): Optical isomers of dihydropyridine calcium channel blockers display enantiospecific effects on the expression and enzyme activities of human xenobiotics-metabolizing cytochromes P450. *Toxicol Lett* 2016, 262: 173-186. [**IF_{2015} - 3,522**]

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Optical isomers of dihydropyridine calcium channel blockers display enantiospecific effects on the expression and enzyme activities of human xenobiotics-metabolizing cytochromes P450

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HIGHLIGHTS

- Dihydropyridine calcium channel blockers (CCBs) are chiral drugs.
- We examined effects of CCBs on human xenobiotics-metabolizing P450 s.
- CCBs have stereospecifically inhibited human CYP catalytic activities.
- CCBs activated of AhR and PXR xenoreceptors; the former stereospecifically.
- CCBs induced CYP1A1/2, CYP2A6, CYP2B6 and CYP3A4 in human hepatocytes.

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ABSTRACT

Dihydropyridine calcium channel blockers (CCBs) are used as anti-hypertensives and in the treatment of angina pectoris. Structurally, CCBs have at least one chiral center in the molecule, thereby existing in two or more different enantiomers. In the current paper we examined effects of benidipine, felodipine and isradipine enantiomers on the expression and enzyme activities of human xenobiotics-metabolizing cytochromes P450.

All CCBs dose-dependently activated aryl hydrocarbon receptor (AhR) and pregnane X receptor (PXR), as revealed by gene reporter assays. Activation of AhR, but not PXR, was enantiospecific. Consistently, CCBs induced CYP1A1 and CYP1A2 mRNAs, but not protein, in human hepatocytes and HepG2 cells, with following pattern: benidipine (−)>(+), isradipine (−)>(+) and felodipine (+)>(−). All CCBs induced CYP2A6, CYP2B6 and CYP3A4 mRNA and protein in human hepatocytes, and there were not differences between the enantiomers. All CCBs transformed AhR in its DNA-binding form, as revealed by electromobility shift assay. Tested CCBs inhibited enzyme activities of CYP3A4 (benidipine (+)>(−); felodipine (−)>(+); isradipine (−)–(+)) and CYP2C9 (benidipine (−)>(+); felodipine (+)>(−); isradipine (−)>(+)). The data presented here might be of toxicological and clinical importance.

1. Introduction

Dihydropyridine calcium channel blockers (CCBs), such as benidipine, clevidipine, felodipine, isradipine, nifedipine, nitrendipine and many others "-dipines", reduce systemic vascular resistance and arterial pressure, and they are used as anti-hypertensives and in the treatment of angina pectoris. All CCBs

Abbreviations: AhR, Aryl Hydrocarbon Receptor; CCBs, calcium channel blockers; CYP, cytochrome P450; DEX, dexamethasone; DMSO, dimethysulfoxide; GR, Glucocorticoid Receptor; HLMs, human liver microsomes; PXR, pregnane X receptor; RIF, rifampicin; TCDD, 2,3,7,8 tetrachlorodibenzo-*p*-dioxin.

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bind to α_{1c} subunit of the L-type calcium channel, which is the main pore-forming unit of the channel (Hockerman et al., 1997). The L-type calcium channels facilitate calcium influx in response to membrane depolarization and regulate intracellular processes such as contraction, secretion, neurotransmission, and gene expression in many different cell types. Blockade of these channels in vascular tissues results in a decrease in smooth-muscle and myocardial contractility and reduction of blood pressure (Abernethy and Schwartz, 1999). Structurally, dihydropyridine CCBs are chiral compounds having at least one asymmetrical center in the molecule, thereby existing in two or more different enantiomers. Individual enantiomers of a drug can qualitatively and quantitatively differ in their biological activities, including their pharmacokinetics, pharmacodynamics, toxicokinetics and toxicodynamics. Notoriously known examples of stereoisomers with considerably different biological activities are R/S-thalidomide, R/S-salbutamol, levo/dextro-methorphan and many others (Smith, 2009). Therefore, enantiopure drugs have been developed and introduced to the pharmacotherapy.

Benidipine has two chiral atoms in the molecule allowing formation of four enantiomers; (+)-α-; (−)-α-; (+)-β- and (−)-β-isomer. Therapeutically used formulation is a racemate of alpha isomers (approved in China 2008, sold as Coniel – Kyowa Hakko Kirin Co., Ltd.; Caritec – Stancare, Ranbaxy Laboratories Ltd.). The hypotensive activity of (+)-α-benidipine was 30 to 100 times stronger than that of (−)-α-benidipine in spontaneously hypertensive rats (Muto et al., 1988). Benidipine was reported as an inhibitor of CYP3A4, CYP1A1, CYP2C9, CYP2C19 and CYP2D6 in human liver microsomes (HLMs) (Katoh et al., 2000). Due to the presence of one chiral carbon in the structure of felodipine and isradipine, they exist in two forms, i.e. (+)(*R*)-felodipine/(−)(*S*)-felodipine and (+)(*S*)-isradipine/(−)(*R*)-isradipine. Both drugs are used as racemates (Plendil, AstraZeneca, approved 25th July 1991, since 2004 generic; Dynacirc, SmithKline Beecham, approved 20th December 1990, since 2006 generic). *In vitro* and *in vivo* studies demonstrated that (−)(*S*)-felodipine is more potent than (+)(*R*)-felodipine (Eltze et al., 1990). Pharmacological activity of (+)(*S*)-isradipine is 150 times higher as compared to (−)(*R*)-isradipine (Ruegg and Hof, 1990). Felodipine inhibited CYP3A4 and CYP2C9 activities in HLMs (Ma et al., 2000). On the other hand, there is a little information about inhibitory potency of isradipine, except of weak inhibition of CYP3A4 activity (Wang et al., 1999).

Many CCBs cause substantial adverse effects including tachycardia, orthostatic hypotension, fluid retention, headache, fatigue, vertigo, muscle cramps and dizziness. The risk of adverse effects rises with dihydropyridines being used simultaneously with other drugs. Dihydropyridines are metabolized to less active metabolites mainly by cytochrome P450 CYP3A and to a lesser extent by other P450 s (Guengerich et al., 1991; Yoon et al., 2007). Therefore, interactions with CYP3A inhibitors or inducers may occur (Holtbecker et al., 1996; Jalava et al., 1997; Lown et al., 1997). Drug-drug interactions may be caused by activation of major transcriptional regulators of drug-metabolizing enzymes, in particular, aryl hydrocarbon receptor (AhR), glucocorticoid receptor (GR) and pregnane X receptor (PXR). Activation of PXR and induction of PXR-regulated genes by dihydropyridines (nifedipine, nicardipine, isradipine, clevidipine) was described (Drocourt et al., 2001; Zhang et al., 2006).

Collectively, CCBs are used as racemic mixtures, but they exert adverse effects and they are activators of PXR and inducers of P450s. On the other hand, there are substantial differences between therapeutic effects of enantiopure CCBs. In addition, we have recently demonstrated that clinically used chiral drugs, including cholesterol-lowering statins (Korhonova et al., 2015), antifungal ketoconazole (Novotna et al., 2014a; Novotna et al., 2014b) and proton pump inhibitors omeprazole and lansoprazole (Novotna and Dvorak 2014; Novotna et al., 2014c) have enantiospecific effects on the expression and activities of drug-metabolizing P450s.

Therefore, the aim of the current paper was to examine stereospecific effects of benidipine, felodipine and isradipine enantiomers on: (i) the expression of major drug-metabolizing P450 s in primary human hepatocytes and HepG2 cells; (ii) transcriptional activities of AhR, GR and PXR receptors, using gene reporter assays and EMSA; (iii) the catalytic activities of drug-metabolizing P450 s in human liver microsomes.

2. Materials and methods

2.1. Chemicals

Dimethylsulfoxide (DMSO), rifampicin (RIF), dexamethasone (DEX), coumarin, testosterone, diclofenac, bufuralol, chlorzoxazone, resorufin, 7-hydroxycoumarin, 7-hydroxy-4-(trifluoromethyl)coumarin, 1′-hydroxymidazolam, 1′hydroxybufuralol and hygromycin B were purchased from Sigma-Aldrich (Prague, Czech Republic). 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was from Ultra Scientific (Rhode Island, USA). Ethoxyresorufin, 7-ethoxy-4-(trifluoromethyl)coumarin and 4′-hydroxydiclofenac were purchased from Fluka (Buchs, Switzerland). Midazolam was purchased from Abcam (Cambridge, UK) and 6β-hydroxytestosteron was purchased from Ultrafine (Manchester, UK) and paclitaxel from Chemos CZ (Prague, Czech Republic). The 6-hydroxypaclitaxel and S-mephenytoin were purchased from SantaCruz Biotechnology Inc. (Heidelberg, Germany), (*S*)-4-hydroxy mephenytoin was bought from Toronto Research Chemicals Inc. (Toronto, Canada). Luciferase lysis buffer and FuGENE® HD Transfection Reagent were from Promega (Madison, California, USA). Human liver microsomes were obtained from Xenotech (Lenexa, Kansas, USA). Details of the CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4/5 enzymatic activities of the mixture can be accessed from the Xenotech Web site (www.xenotechllc.com). All other chemicals were of the highest quality commercially available.

2.2. Preparation of enantiopure dihydropyridines

Benidipine, felodipine and isradipine enantiomers were isolated from their respective racemates (Sigma-Aldrich, St. Louis, USA) by chiral semipreparative chromatography using Knauer Smartline HPLC system (Knauer, Berlin, Germany). Lux Cellulose-1 column (250 × 10 mm, dp = 5 μm, Phenomenex, Torrance, USA) and methanol as mobile phase (MP) was used for the separation of benidipine enantiomers. Lux Cellulose-3 (250 × 4.6 mm, dp = 5 μm, Phenomenex) and water:isopropanol 3:2 (v/v) as MP was used for felodipine enantiomers. Lux Cellulose-3 column (250 × 10 mm, dp = 5 μm, Phenomenex) and hexane:isopropanol 9:1 (v/v) as MP was selected for isradipine chiral separation. Manual collection of fractions was performed and collected fractions were dried using either stream of nitrogen (benidipine and isradipine enantiomers) or by lyophilization (felodipine enantiomers). For molecular mass confirmation and chiral purity control, LC/MS^2 analysis using Acquity UPLC/Q-TOF Premier (Waters, Milford, USA) and respective chiral columns was performed. Measured *m*/*z* values of molecular ions ($[M+H]^+$) were 506.2269, 384.0794 and 372.1547 for benidipine, felodipine and isradipine enantiomers, respectively (with deviations from theoretical values −4.3, +6.5 and −3.2 ppm, respectively). A previously described method (Kang et al., 2005) was used for the confirmation of absolute configuration of individual enantiomers of benidipine (based on elution order of particular enantiomers). Supelco Chirobiotic V2 column (100 × 4.6 mm,

dp = 5 μm) was used for the comparative analysis. Mobile phase consisted of 0.01% (v/v) acetic acid and 0.0001% (v/v) triethylamine in methanol. Absolute configuration of felodipine and isradipine enantiomers was determined using Chiralyser MP optical rotation detector (Knauer). Automatic symmetry calibration was performed before each measurement.

Following chiral purity was achieved: 98.5% and 98.5% for (+)- and (−)-benidine, 99.5% and 97.0% for (+)- and (−)-felodipine and 99.6% and 97.5% for (+)- and (−)-isradipine, respectively (Fig. 1).

2.3. Cell cultures

Human Caucasian colon adenocarcinoma cells LS180 (ECACC No. 87021202) and Human Caucasian hepatocellular carcinoma cells HepG2 (ECACC No. 85011430) were purchased from *European Collection of Cell Cultures* (ECACC). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% of fetal bovine serum, 100 U/ml streptomycin, 100 μg/ml penicillin, 4 mM L-glutamine, 1% non-essential amino acids, and 1 mM sodium pyruvate. Cells were maintained at 37 °C and 5% CO_2 in a humidified incubator. Stably transfected gene reporter cell lines AZ-AHR and AZ-GR were as described elsewhere (Novotna et al., 2011, 2012). Primary human hepatocytes used in this study were isolated from human liver obtained from three multiorgan donors: HH64 (male; 73 years), HH65 (male; 34 years) and HH66 (male; 65 years). The tissue acquisition protocol was in accordance with the requirements stated by the local ethical commission in the Czech Republic. Human hepatocytes were isolated and primary cultures prepared and cultured as described (Ferrini et al., 1997).

2.4. Gene reporter assay and cytotoxicity assay

Transcriptional activity of AhR and GR was studied in stably transfected gene reporter cell lines AZ-AHR and AZ-GR, respectively. Human colon adenocarcinoma cells LS180, transiently transfected with a chimera p3A4-luc reporter construct by lipofection (FuGENE® HD Transfection Reagent), were used for assessment of PXR transcriptional activity (Pavek et al., 2010). Cells were incubated for 24 h with tested compounds and/or vehicle (DMSO; 0.1% v/v), in the presence (antagonist mode) or absence (agonist mode) of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; 5 nM), rifampicin (RIF; 10 μM) or dexamethasone (DEX; 100 nM). After the treatments, cells were lysed and luciferase activity was measured on Tecan Infinite M200 Pro plate reader (Schoeller Instruments, Prague, Czech Republic). In parallel, cell viability was determined by conventional MTT test.

2.5. mRNA determination and qRT-PCR

Total RNA was isolated using TRI Reagent® (Molecular Research Center, Ohio, USA). cDNA was synthesized from 1000 ng of total RNA using M-MuLV Reverse Transcriptase (New England Biolabs, Ipswich, Massachusetts, USA) at 42 °C for 60 min in the presence of random hexamers (New England Biolabs). qRT-PCR was carried out using LightCycler® 480 Probes Master (Prague, Roche Diagnostic Corporation, Czech Republic) on a Light Cycler® 480 II apparatus (Roche Diagnostic Corporation). CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP3A4 and GAPDH mRNAs were determined as described previously (Vrzal et al., 2013). Measurements were performed in triplicates. Gene expression was normalized to GAPDH as a housekeeping gene.

2.6. Simple western blotting by Sally Sue™

Total protein extracts were prepared as described previously (Korhonova et al., 2015). All reagents used for running the *simple western by Sally Sue*™ were obtained from ProteinSimple (San Jose, California) and prepared according to manufacturer's recommendations (http://www.proteinsimple.com/sally_sue.html). CYP1A1 (goat polyclonal, sc-9828, G-18), CYP1A2 (mouse monoclonal, sc-53614, 3B8C1), CYP2A6 (mouse monoclonal, sc-53615, F16P2D8), CYP2B6 (rabbit polyclonal, sc-67224, H-110), CYP3A4 (mouse

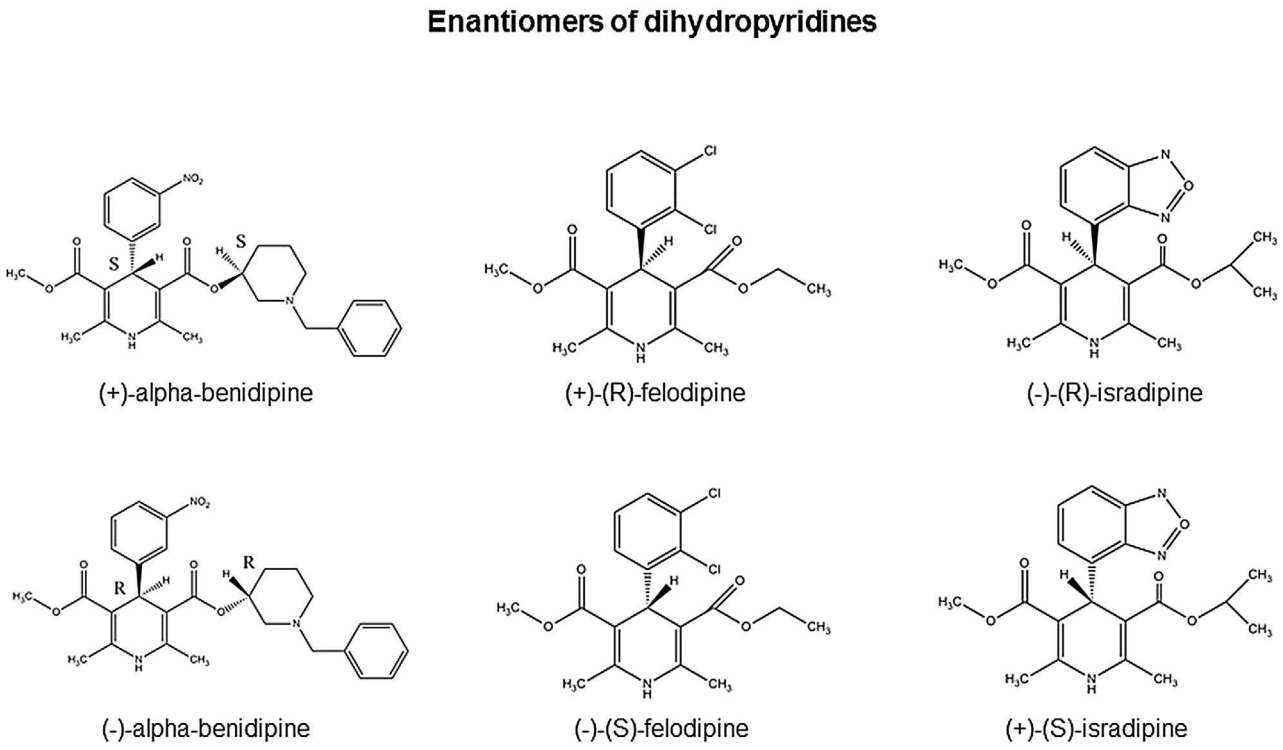

Fig. 1. Chemical structures of chiral dihydropyridines.

monoclonal; sc-53850, HL3) primary antibodies and rabbit anti-goat secondary antibody (sc-2768) were purchased from Santa Cruz Biotechnology Inc. β-actin (mouse monoclonal; 3700S, 8H10D10) primary antibody was from Cell Signalling Technology (Denvers, Massachusetts, USA). Antibody diluent, goat anti-rabbit secondary antibody, and goat anti-mouse secondary antibody were purchased from ProteinSimple. The capillaries, containing a proprietary UV-activated chemical linked reagent and 384-well plates were obtained from ProteinSimple. All samples and reagents were prepared according to the recommended ProteinSimple manual. Target proteins were identified using primary antibodies and immunoprobed using a horseradish peroxidase-conjugated secondary antibody and chemiluminescent substrate. The resulting chemiluminescent signal was detected and quantified by the Compass Software version 2.6.5.0 (ProteinSimple). For quantitative data analysis, the CYPs signals were normalized to β-actin as a loading control.

2.7. Electrophoretic mobility shift assay (EMSA)

MCF-7 cells at 100% confluence were incubated for 2 h with vehicle as a negative control (UT; 0.1% DMSO v/v), TCDD (5 nM) and studied compounds at the concentrations 10 μM. Consequently, nuclear fractions were isolated using nuclear extract kit (Active Motif) according to manufacturer's protocol. The following double-stranded 5′-biotinylated oligonucleotides containing specific DNA-binding sequence for AhR corresponding to the 27-bp protein binding site of DRE 3 were used (Denison et al., 1988). Gel mobility shift assay was performed as follows: nuclear protein (15 μg) from each sample was pre-incubated in binding buffer (final: 10 mM Tris, 50 mM KCl, 1 mM DTT; pH 7.5) along with final concentrations of 2.5% glycerol, 0.05% NP-40, ddH_2O and nonspecific competitor Poly (dI.dC) before adding the biotin-labeled probe (10 fmol/μL). Unlabeled probe (2 pmol/μL) was added to reaction mixture for competitive experiment. The complete binding reaction was incubated at room temperature for 20 min. Finally 5 μL of 5 × loading buffer with bromophenol blue was added before loading in the non-denaturing 5% polyacrylamide gel for electrophoretic separation. The protein-DNA complexes were electroblotted to positively charged nylon membrane. Thereafter, transferred DNA was cross-linked using a UV-light cross-linker instrument. Biotin-labeled DNA was detected using a streptavidin-horseradish peroxidase conjugate and chemiluminescent substrate contained in Light- Shift Chemiluminescent EMSA Kit (Thermo Scientific, Waltham, MA, USA). The sequences of DRE oligonucleotides were as follows:

sense 5′ - GATCCGGCTCTTCTCACGCAACTCCGAGCTCA - 3′;
antisense 5′ - GATCTGAGCTCGGAGTTGCGTGAGAAGAGCCG - 3′

2.8. Enzyme kinetic

The enzyme activities of the individual CYP forms were determined according to established protocols (Phillips and Shephard, 2006). The CYP1A2 assay was based on 7-ethoxyresorufin *O*-deethylation, the CYP2A6 assay on coumarin 7-hydroxylation, the CYP2B6 on 7-ethoxy-4-(trifluoromethyl)coumarin 7-deethylation, activity of the CYP2C9 was based on diclofenac 4′-hydroxylation, of the CYP2C8 enzyme on the paclitaxel 6-hydroxylation, CYP2C19 assay was based on S-mephenytoin 4′-hydroxylation, of the CYP2D6 on bufuralol 1′-hydroxylation; CYP3A4 activity was determined by testosterone 6β-hydroxylation and midazolam 1′-hydroxylation and CYP2E1 activity was assessed by chlorzoxazone 6-hydroxylation. Monitoring of the metabolites formed from specific substrates was done by HPLC using the Prominence system (Shimadzu, Kyoto, Japan) equipped with a LiChroCART 250-4 LiChrospher 100 RP-18 column or Chromolith®HighResolution RP-18 endcapped column (Merck, Darmstadt, Germany) and UV or fluorescence detection. S-mephenytoin 4′-hydroxylation was determined by QTRAP 5500, AB Sciex (Framingham, MA, USA) and SPE-based RapidFire 330 technology (Agilent, Santa Clara, CA, USA).

The preliminary experiments to determine Michaelis constant (K_m) and limiting velocity (V_{max}) of individual CYP forms were performed. The incubation conditions were specific for each CYP form and they were within the linear range for the V_{max} of the reaction (time of incubation, substrate concentration corresponding to K_m and amount of HLMs). The reaction conditions of the inhibition studies were identical to those for the determination of individual CYP activities. The amount of HLMs (expressed as the amount of CYP in pmol and concentration of HLMs protein in mg/ml in the reaction vessel) in the reaction was as follows: 100 pmol for CYP3A4 testosterone assay, 12.56 pmol for CYP3A4 midazolam assay, 160 pmol for CYP2E1, 70 pmol for CYP2D6, 13 pmol for CYP2C19, 35 pmol for CYP2C9, 60 pmol for CYP2C8, 35 pmol for CYP2B6, 35 pmol for CYP2A6 and 35 pmol for CYP1A2. The reaction mixtures were buffered with 100 mM K/PO4 (pH 7,4) or 50 mM K/PO_4 (pH 7,4) and contained a NADPH-generating system consisting of the isocitrate dehydrogenase, $NADP^+$, isocitric acid and $MgSO_4$. Concentration of DMSO was below 0.1% to avoid enzyme inhibition by higher concentrations of organic solvents. The incubations were carried out in two independent measurements in triplicates at 37 °C. The apparent K_i values were determined by additional measurements using substrate concentration corresponding to 1/2 K_m, K_m and 2 K_m in the case of inhibition (where $IC_{50} < 10$ μM). Inhibition of CYP activities was evaluated by plotting the remaining activity against the inhibitor concentration by GraphPad Prism (La Jolla, California, USA). The values of IC_{50} were obtained by the analysis of the plot of the logarithm of the inhibitor concentration versus the percentage of activity remaining after inhibition using Sigma Plot 12 scientific graphing software (SPSS, Chicago, Illinois, USA). Throughout the paper, the apparent Ki values are presented. The inhibition data were fit to different models of enzyme inhibition type (uncompetitive, mixed-model, competitive, non-competitive) by nonlinear regression analysis also with GraphPad Prism. Prism fit the data according to equations stated in (Copeland, 2000). The appropriate type of model to be used for each data set was selected on the basis of visual inspection of the Lineweaver-Burk, Dixon, and Scatchard plots.

2.9. Statistics

Student's *t*-test, One-way ANOVA followed by Dunnett's post test as well as calculations of EC_{50} and IC_{50} values were calculated using GraphPad Prism version 6.0 for Windows, GraphPad Software, La Jolla, California, USA. Stereoselective differences between individual enantiomers of the same drug in the inhibitory effect were analysed with Statistica 12 software (StatSoft, Prague, Czech Republic). The Shapiro-Wilks test was used as a test of normality. The *t*-test was used for the parametric data; the Mann-Whitney test was used for the nonparametric data.

3. Results

3.1. Cytotoxicity of dihydropyridine enantiomers in human cancer cell lines

Prior to the gene reporter assays, we examined the cytotoxicity of tested compounds in AZ-AHR, AZ-GR and LS180 cell lines. The cells were incubated for 24 h with individual (+) and (−) enantiomers of benidipine, felodipine and isradipine at concentrations ranging from 100 pM to 50 μM. The vehicle was DMSO (0.1% v/v). After the treatment, a conventional MTT test was

performed and the values of IC_{50} were calculated, where appropriate. Based on the results from cytotoxicity testing (Figs. 2–4 – upper panels), gene reporter assays were performed in concentrations of tested compounds up to 50 μM.

3.2. Effects of dihydropyridine enantiomers on transcriptional activity of aryl hydrocarbon receptor

Transcriptional activity of AhR was assessed in human gene reporter cell line AZ-AHR incubated for 24 h with tested compounds. An induction of AhR-dependent luciferase activity by model agonist dioxin (TCDD; 5 nM) in two consecutive passages of AZ-AHR cells was 652-fold and 717-fold, as compared to DMSO-treated cells. (−)-benidipine dose-dependently increased luciferase activity (EC_{50} 11.7 μM), while no significant induction of luciferase activity was observed for (+)-benidipine. Dose-dependent activation of AhR was caused by (+)-felodipine, (−)-felodipine, (+)-isradipine and (−)-isradipine, with average EC_{50} values 6.5 μM, 12.1 μM, 11.3 μM and 11.4 μM, respectively. Whereas the potencies of (+/−)felodipines and (+/−)-isradipines were similar to each other, the efficacies of (+)-felodipine and (+)-isradipine were much higher as compared to their (−) counter-parts (Fig. 2). Combined treatments of AZ-AHR cells with AhR agonist TCDD (5 nM) and enantiomers of felodipine and isradipine revealed inversed U-shaped curves. Dose-dependent augmentation of TCDD-inducible luciferase activity was observed regardless optical configuration, in concentrations up to 1 μM, followed by a decrease of luciferase activity with rising concentrations of dihydropyridines up to 50 μM. Unlike (+)-benidipine, (−)-benidipine did not augment TCDD-induced luciferase activity. The decrease in luciferase activity by higher concentrations of (+/−)-benidipines was also caused partly by their cytotoxicity (Fig. 2). Overall, tested dihydropyridines dose-dependently and *enantio*-specifically activated AhR. Both the potencies and efficacies of the compounds were much lower as compared to TCDD.

3.3. Effects of dihydropyridine enantiomers on transcriptional activity of glucocorticoid receptor

Transcriptional activity of GR was assessed in human gene reporter cell line AZ-GR incubated for 24 h with tested compounds. An induction of GR-dependent luciferase activity by model agonist dexamethasone (DEX; 100 nM) in two consecutive passages of AZ-GR cells was 19-fold and 31-fold, as compared to DMSO-treated cells. Whereas none of the tested dihydropyridines induced GR-dependent luciferase activity, they all dose-dependently

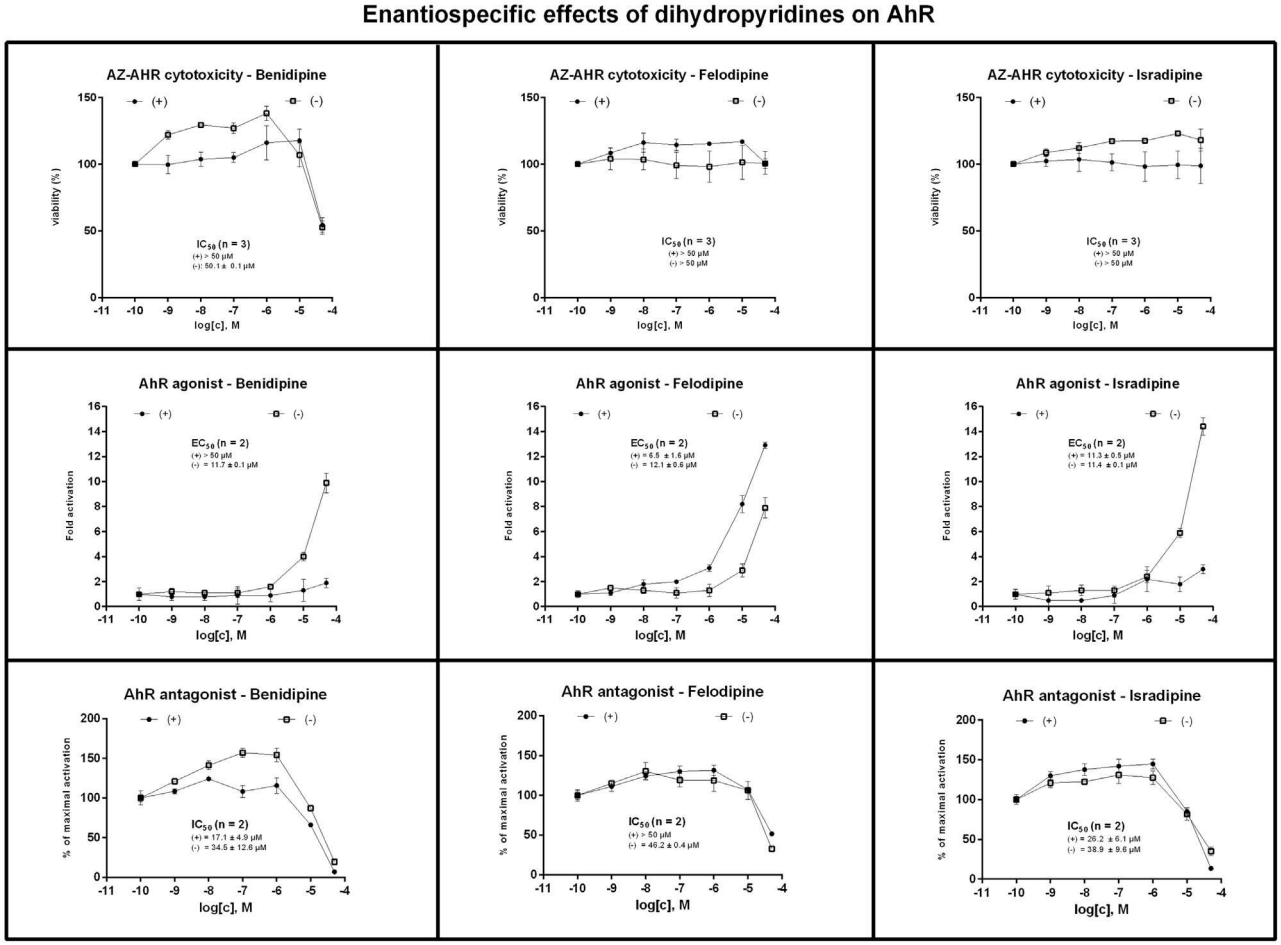

Fig. 2. Effects of dihydropyridine enantiomers on transcriptional activity of human aryl hydrocarbon receptor. AZ-AHR cells were seeded in 96-well plates, stabilized for 16 h, and then incubated for 24 h with (+)benidipine, (−)benidipine, (+)felodipine, (−)felodipine, (+)isradipine, (−)isradipine and vehicle (DMSO; 0.1% v/v), in the presence or in the absence of dioxin (TCDD; 5 nM). Treatments were performed in triplicates. *Upper panels:* A conventional MTT test was performed and absorbance was measured at 540 nm. The data are the mean ± SD from experiments performed in three consecutive passages of cells and are expressed as a percentage of viability of control cells. The values of IC_{50} were calculated where appropriate and they are indicated in plots. *Middle panels:* Agonist mode, i.e. incubations in the absence of TCDD. Cells were lysed and luciferase activity was measured. Data are expressed as a fold induction of luciferase activity over control cells. Half-maximal effective concentrations EC_{50} were calculated where appropriate and the average values are indicated in plots. *Lower panels:* Antagonist mode, i.e. incubations in the presence of TCDD. Half-maximal inhibitory concentrations IC_{50} were calculated where appropriate and the average values are indicated in plots. Data are expressed as a percentage of maximal activation attained by TCDD.

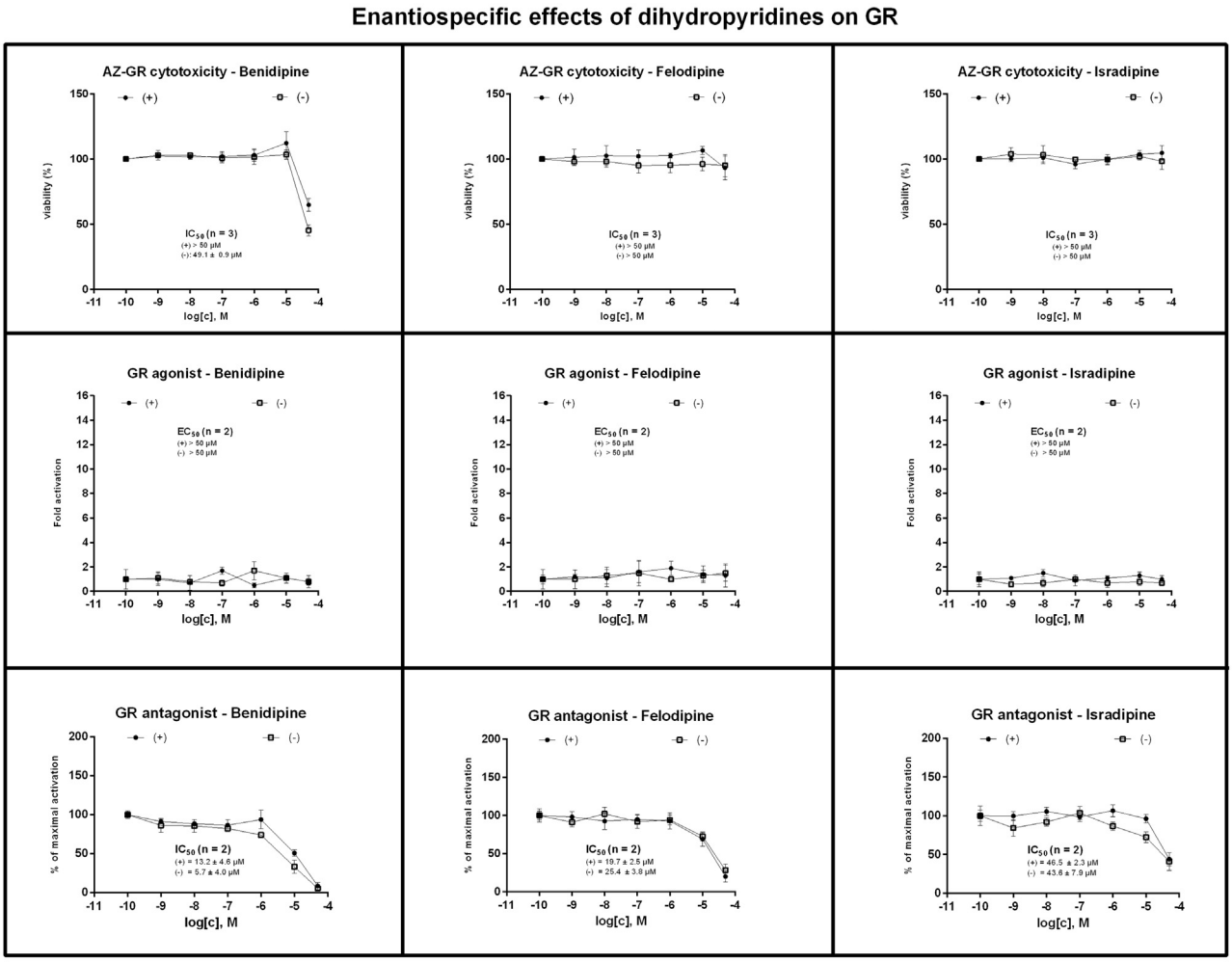

Fig. 3. Effects of dihydropyridine enantiomers on transcriptional activity of human glucocorticoid receptor. AZ-GR cells were seeded in 96-well plates, stabilized for 16 h, and then incubated for 24 h with (+)benidipine, (−)benidipine, (+)felodipine, (−)felodipine, (+)isradipine, (−)isradipine and vehicle (DMSO; 0.1% v/v), in the presence or in the absence of dexamethasone (DEX; 100 nM). Treatments were performed in triplicates. *Upper panels:* A conventional MTT test was performed and absorbance was measured at 540 nm. The data are the mean ± SD from experiments performed in three consecutive passages of cells and are expressed as a percentage of viability of control cells. The values of IC_{50} were calculated where appropriate and they are indicated in plots. *Middle panels:* Agonist mode, i.e. incubations in the absence of dexamethasone. Cells were lysed and luciferase activity was measured. Data are expressed as a fold induction of luciferase activity over control cells. *Lower panels:* Antagonist mode, i.e. incubations in the presence of dexamethasone. Half-maximal inhibitory concentrations IC_{50} were calculated where appropriate and the average values are indicated in plots. Data are expressed as a percentage of maximal activation attained by DEX.

decreased dexamethasone-inducible transcriptional activity of GR. However, there were no significant differences between optical isomers of individual dihydropyridines, and cytotoxic effects of (+/−)-benidipines should be considered (Fig. 3).

3.4. Effects of dihydropyridine enantiomers on transcriptional activity of pregnane X receptor

Transcriptional activity of PXR was tested in human colon adenocarcinoma cells LS180 transiently transfected with p3A4-luc reporter construct, incubated for 24 h with tested compounds. An induction of luciferase activity by model PXR agonist rifampicin (RIF; 10 μM) in two consecutive passages was 9-fold and 10-fold, as compared to DMSO-treated cells. Transcriptional activity of PXR was dose-dependently induced by all tested dihydropyridines, regardless their optical configuration, up to 10 μM concentration, followed by a drop of luciferase activity at 50 μM concentration. The efficacies of individual compounds were similar and magnitudes of the inductions varied between 47% to 80% of that by RIF. Half maximal effective concentrations (EC_{50}) of (+/−)-isradipines were 1.2 μM and 0.4 μM, respectively. There was not a difference between potency of (+/−)-felodipines, displaying EC_{50} of 0.6 μM and 0.7 μM, respectively. Interestingly, (+/−)-benidipines were about ten times more potent as compared to other two dihydropyridines, and EC_{50} of (+)-benidipine and (−)-benidipine was 0.06 μM and 0.09 μM, respectively. Rifampicin-inducible transcriptional activity of PXR was decreased by all tested dihydropyridines with no significant differences between individual enantiomers. The most pronounced decrease of basal and ligand-inducible luciferase was by 50 μM benidipines, which was probably due to their cytotoxicity (Fig. 4).

3.5. Effects of dihydropyridine enantiomers on the expression of CYP1A1 and CYP3A4 mRNAs and proteins in human cancer cell line HepG2

Since we observed activation of AhR and PXR by dihydropyridines in gene reporter assays, we tested whether these compounds also induce CYP1A1 and CYP3A4, which are target genes for AhR and PXR, respectively. Therefore, we incubated HepG2 cells with tested compounds (1 μM and 10 μM) and model inducers for 24 h (mRNAs analyses) and 48 h (proteins analyses). In

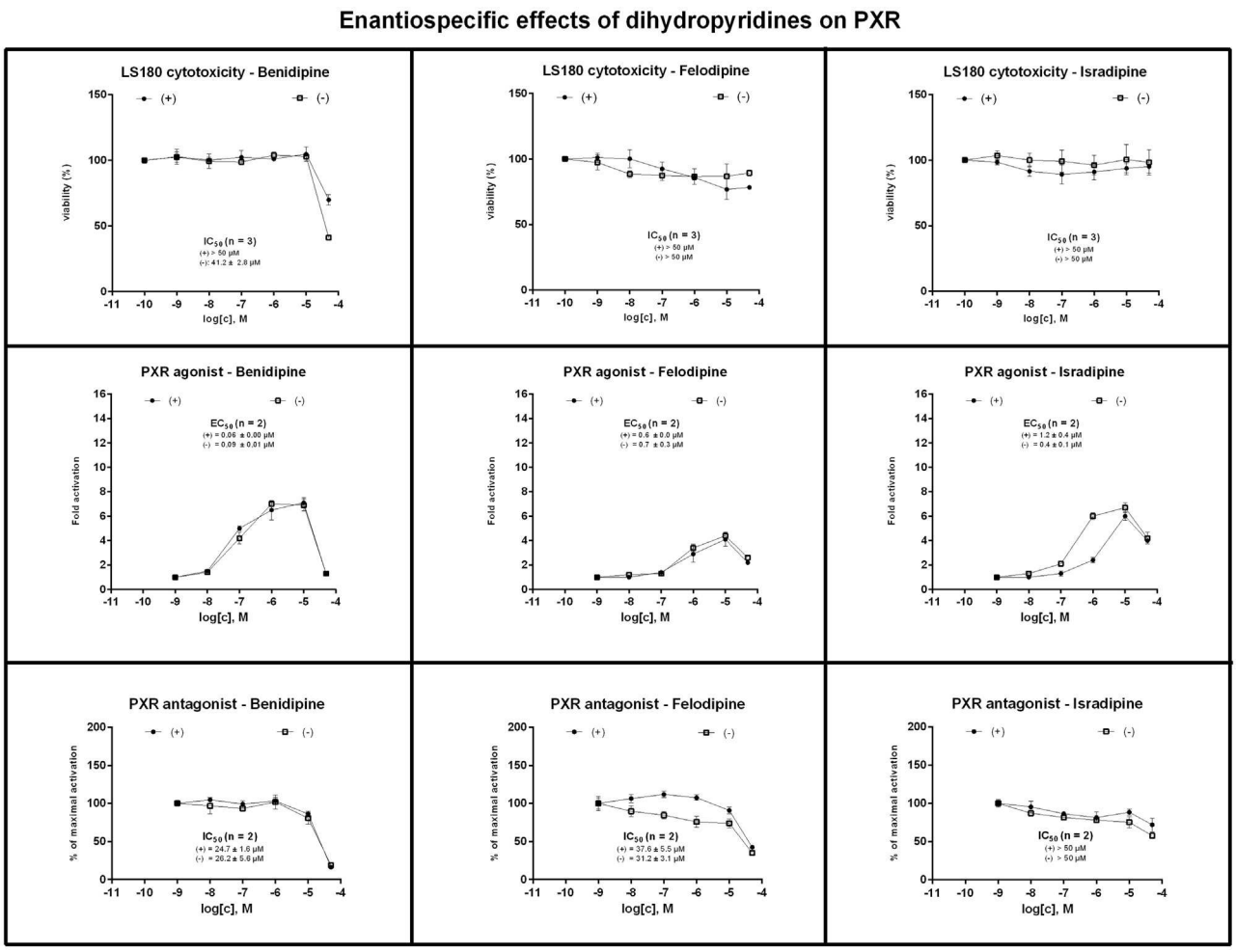

Fig. 4. Effects of dihydropyridine enantiomers on transcriptional activity of human pregnane X receptor. LS180 cells transiently transfected with p3A4-luc reporter were seeded in 96-well plates, stabilized for 16 h, and then incubated for 24 h with (+)benidipine, (−)benidipine, (+)felodipine, (−)felodipine, (+)isradipine, (−)isradipine and vehicle (DMSO; 0.1% v/v), in the presence or in the absence of rifampicin (RIF; 10 μM). Treatments were performed in triplicates. *Upper panels:* A conventional MTT test was performed and absorbance was measured at 540 nm. The data are the mean ± SD from experiments performed in three consecutive passages of cells and are expressed as a percentage of viability of control cells. The values of IC_{50} were calculated where appropriate and they are indicated in plots. *Middle panels:* Agonist mode, i.e. incubations in the absence of rifampicin. Cells were lysed and luciferase activity was measured. Data are expressed as a fold induction of luciferase activity over control cells. Half-maximal effective concentrations EC_{50} were calculated where appropriate and the average values are indicated in plots. *Lower panels:* Antagonist mode, i.e. incubations in the presence of rifampicin. Half-maximal inhibitory concentrations IC_{50} were calculated where appropriate and the average values are indicated in plots. Data are expressed as a percentage of maximal activation attained by RIF.

three consecutive passages of HepG2 cells, rifampicin weakly induced (approx. 1.5-fold) CYP3A4 mRNA, but not protein. Out of compounds tested, only (+)-benidipine slightly induced CYP3A4 mRNA, while CYP3A4 protein was not induced by any dihydropyridine. The results are consistent with gene reporter assays, where benidipine was identified as the most potent PXR activator. Average inductions of CYP1A1 mRNA and protein in three consecutive passages of HepG2 cells incubated with TCDD were 325-fold and 21-fold, respectively. All tested dihydropyridines induced dose-dependently CYP1A1 mRNA. Enentiospecific patterns of induction were consistent with those from gene reporter assays in AZ-AHR cells, i.e. (−)-benidipine > (+)-benidipine); (−)-isradipine > (+)-isradipine); (+)-felodipine > (−)-felodipine). Significant induction of CYP1A1 protein was attained only by 10 μM (−)-benidipine (Fig. 5).

3.6. Effects of dihydropyridine enantiomers on the expression of drug-metabolizing cytochromes P450 in primary human hepatocytes

We examined a capability of dihydropyridine enantiomers to induce transcriptionally regulated drug-metabolizing cytochromes P450 in three human hepatocytes cultures (HH64, HH65, HH66). Hepatocytes were incubated for 24 h (for determination of mRNAs) and 48 h (for determination of proteins) with optical isomers of dihydropyridines, dioxin, rifampicin and vehicle (Figs. 5 and 6).

3.6.1. CYP1A1 and CYP1A2

Dioxin strongly induced CYP1A1 and CYP1A2 mRNAs and proteins in all human hepatocytes cultures. The induction profiles of CYP1A1 and CYP1A2 mRNAs by dihydropyridines displayed similar pattern; with exception of (+)-felodipine, all compounds weakly and dose-dependently induced CYP1A genes with following magnitude: (−)-isradipine > (−)-benidipine > (+)-isradipine ∼ (+)-benidipine ∼ (−)-felodipine. The data are mostly consistent with CYP1A1 mRNA induction in HepG2 cells and AhR in gene reporter assays. Inter-individual variability between hepatocytes from different donors and metabolic competence of human hepatocytes may account for few inconsistencies. Dihydropyridine enantiomers did not significantly induce the expression of CYP1A1 or CYP1A2 protein, with exception of weak increase of CYP1A1 protein in culture HH66 by felodipine enantiomers.

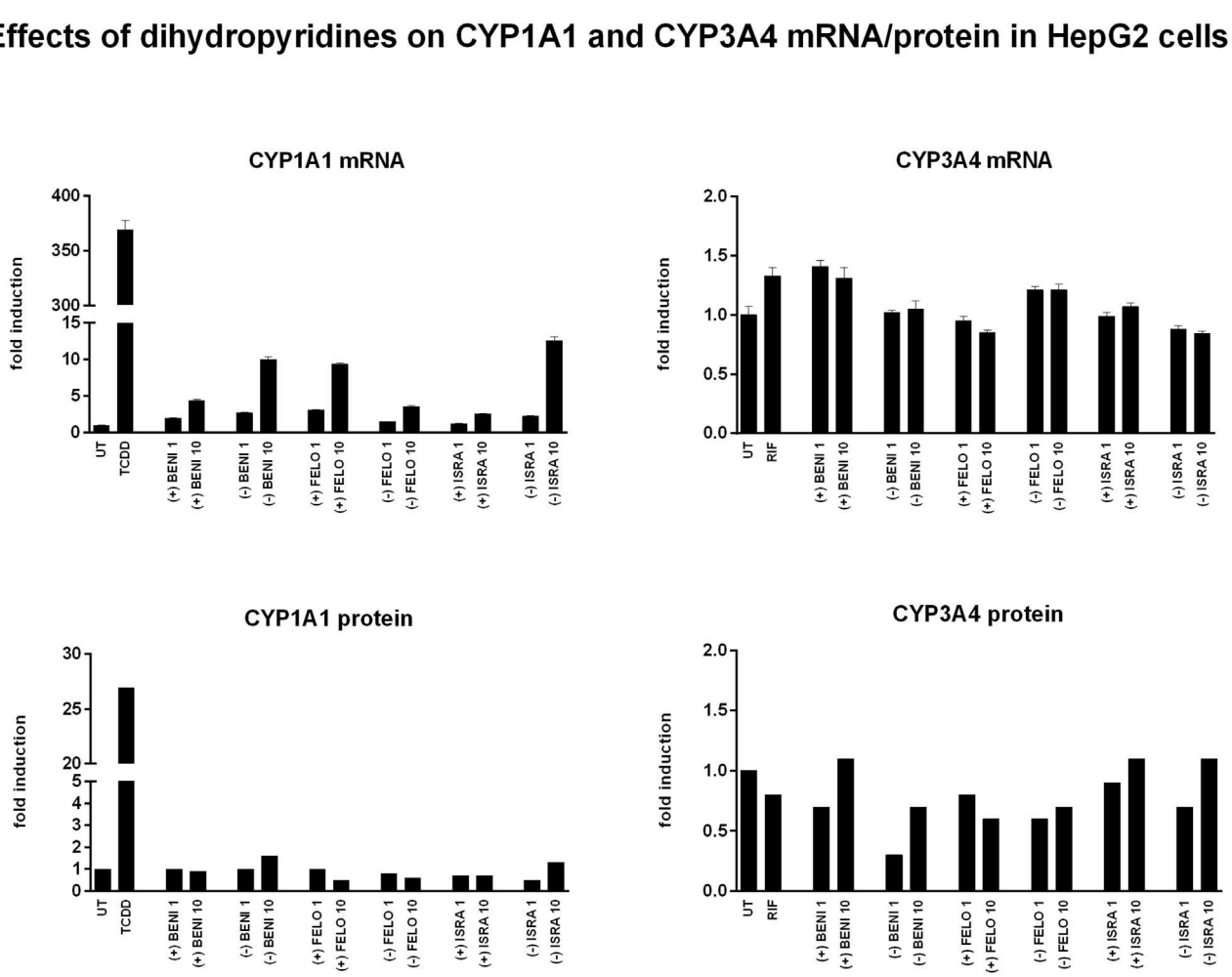

Fig. 5. Effect of dihydropyridine enantiomers on CYP1A1 and CYP3A4 mRNAs and proteins levels in HepG2 cells. HepG2 cells were seeded in 6-well plates and stabilized for 16 h. All experiments were performed in three consecutive cell passages. Cells were incubated for 24 h (mRNA) or 48 h (protein) with (+)benidipine, (−)benidipine, (+) felodipine, (−)felodipine, (+)isradipine, (−)isradipine and vehicle (DMSO; 0.1% v/v), in the presence or in the absence of rifampicin (RIF; 10 μM) and dioxin (TCDD; 5 nM). RT-PCR analyses (mRNAs) and western blots (proteins) of CYP1A1 and CYP3A4 are shown. Data are the mean ± SD from triplicate measurements (samples), each performed in three independent cell passages, and are expressed as fold induction over vehicle-treated cells. The data for mRNAs and proteins were normalized to GAPDH mRNA or β-actin levels, respectively.

3.6.2. CYP2A6

Inductions of CYP2A6 mRNA/protein by rifampicin after 24 h/48 h of incubation were 5.2-fold/1.5-fold and 13-fold/6.5-fold in human hepatocytes cultures HH64 and HH65, respectively. Since the induction of CYP2A6 mRNA by rifampicin after 24 h was weak (1.6-fold), we did not analyze CYP2A6 protein in culture HH66. Induction profiles of CYP2A6 mRNA and protein by dihydropyridine enantiomers differed between individual cultures, probably due to the inter-individual variability of the donors. Generally, the effects on the expression of CYP2A6 mRNA/protein by felodipine enantiomers were equipotent. Stronger induction of CYP2A6 mRNA was observed for (+)-isradipine, however the data were not consistent with its effect on CYP2A6 protein. The induction profiles of CYP2A6 by benidipine enantiomers varied between hepatocyte cultures and were not dose-dependent.

3.6.3. CYP2B6

Inductions of CYP2B6 mRNA/protein by rifampicin after 24 h/48 h of incubation were 20-fold/5.8-fold, 25-fold/3.3-fold and 5.8fold/3.3-fold in human hepatocytes cultures HH64, HH65 and HH66, respectively. All dihydropyridines dose-dependently induced CYP2B6 mRNA in all hepatocytes cultures, and their effects were nearly equipotent and they were not enantiospecific. Similar induction profiles were observed for CYP2B6 protein.

3.6.4. CYP2C9

Induction of CYP2C9 mRNA by rifampicin after 24 h of incubation in three human hepatocytes cultures varied from 0.9-fold to 2.3-fold. Given low induction of CYP2C9 mRNA, we did not evaluate induction of CYP2C9 protein. The effects of optical isomers of tested dihydropyridines on CYP2C9 mRNA expression were rather positive modulatory.

3.6.5. CYP3A4

Inductions of CYP3A4 mRNA/protein by rifampicin after 24 h/48 h of incubation were 59-fold/4.4-fold, 81-fold/36-fold and 6.5-fold/7.1-fold in human hepatocytes cultures HH64, HH65 and HH66, respectively. All dihydropyridines dose-dependently induced CYP3A4 mRNA in all hepatocytes cultures, and their effects were not enantiospecific. The magnitude of CYP3A4 induction was: (+/−)-benidipine ∼ (+)-isradipine > (+/−)-felodipine ∼ (−)-isradipine. We observed only weak inductions of CYP3A4 at protein level, with exception of strong increase of CYP3A4 mRNA by benidipine enantiomers in hepatocyte culture HH65.

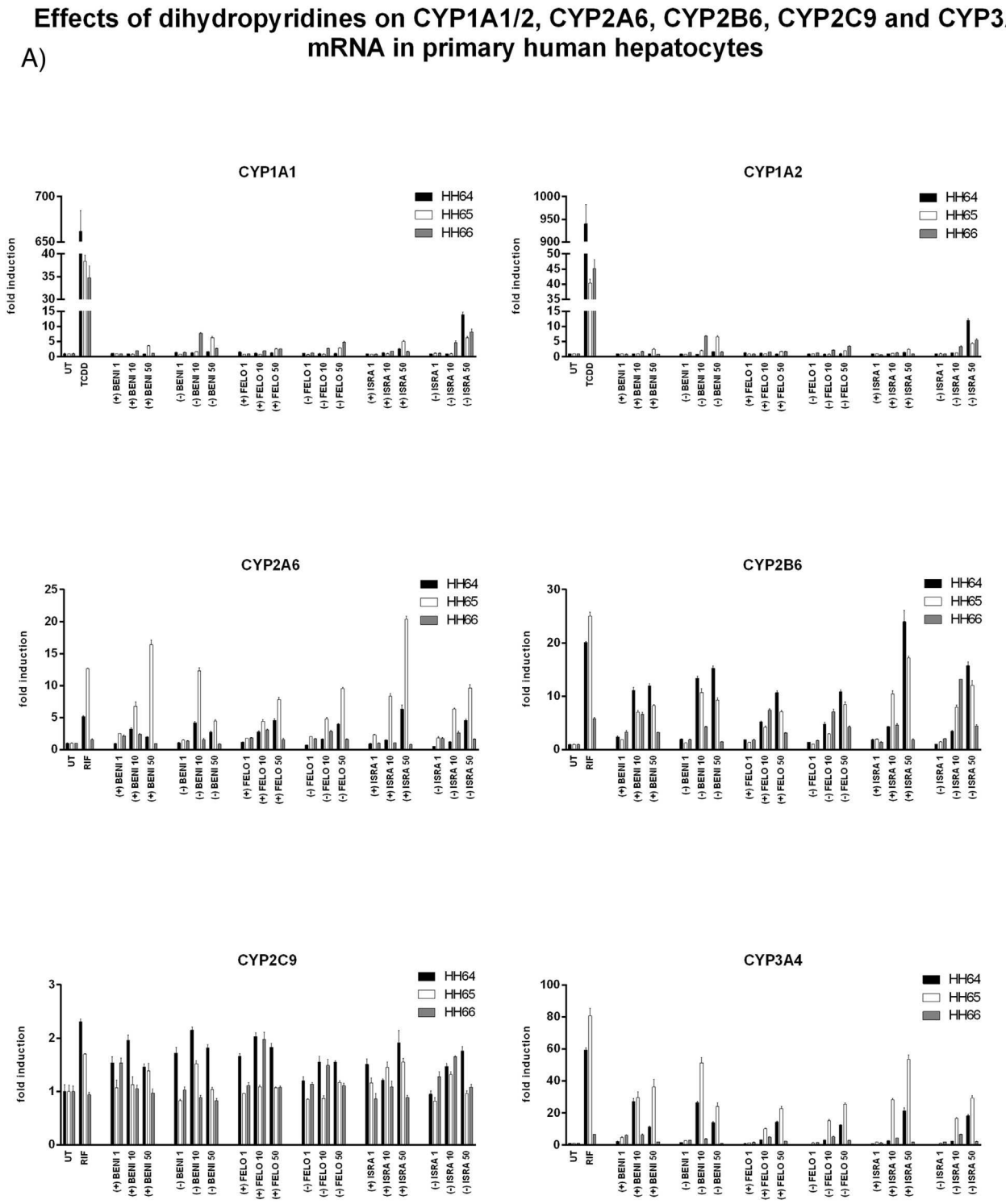

Fig. 6. Effects of dihydropyridine enantiomers on the expression of drug-metabolizing cytochromes P450 in primary human hepatocytes. Primary human hepatocytes from three different donors (HH64, HH65 and HH66) were incubated for 24 h (mRNA) and 48 h (proteins) with (+)benidipine, (−)benidipine, (+)felodipine, (−)felodipine, (+) isradipine, (−)isradipine, dioxin (TCDD; 5 nM), rifampicin (RIF; 10 μM) and vehicle (DMSO; 0.1% v/v). *Panel A:* RT-PCR analyses of CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C9 and CYP3A4 mRNAs are shown in bar graphs. The data are the mean ± SD from triplicate measurements and are expressed as a fold induction over vehicle-treated cells. The data were normalized to GAPDH mRNA levels. *Panel B:* Simple Western analyses of CYP1A1, CYP1A2, CYP2A6, CYP2B6 and CYP3A4 proteins are shown in bar graphs. The data are expressed as a fold induction over vehicle-treated cells and normalized to β-actin levels.

3.7. Electrophoretic mobility shift assay EMSA

We examined whether benidipines, felodipines and isradipines are able to transform human AhR to its DNA-binding form. We incubated human breast cancer cells MCF7 with tested compounds and we performed electrophoretic mobility shift assay (EMSA) in nuclear extracts. All tested compounds induced binding of AhR to DNA, and the effects were enantiospecific as follows: (+)-benidipine > (−)-benidipine; (−)-felodipine > (+)-felodipine; (+)-isradipine > (−)-isradipine (Fig. 7). Surprisingly, semi-quantitative

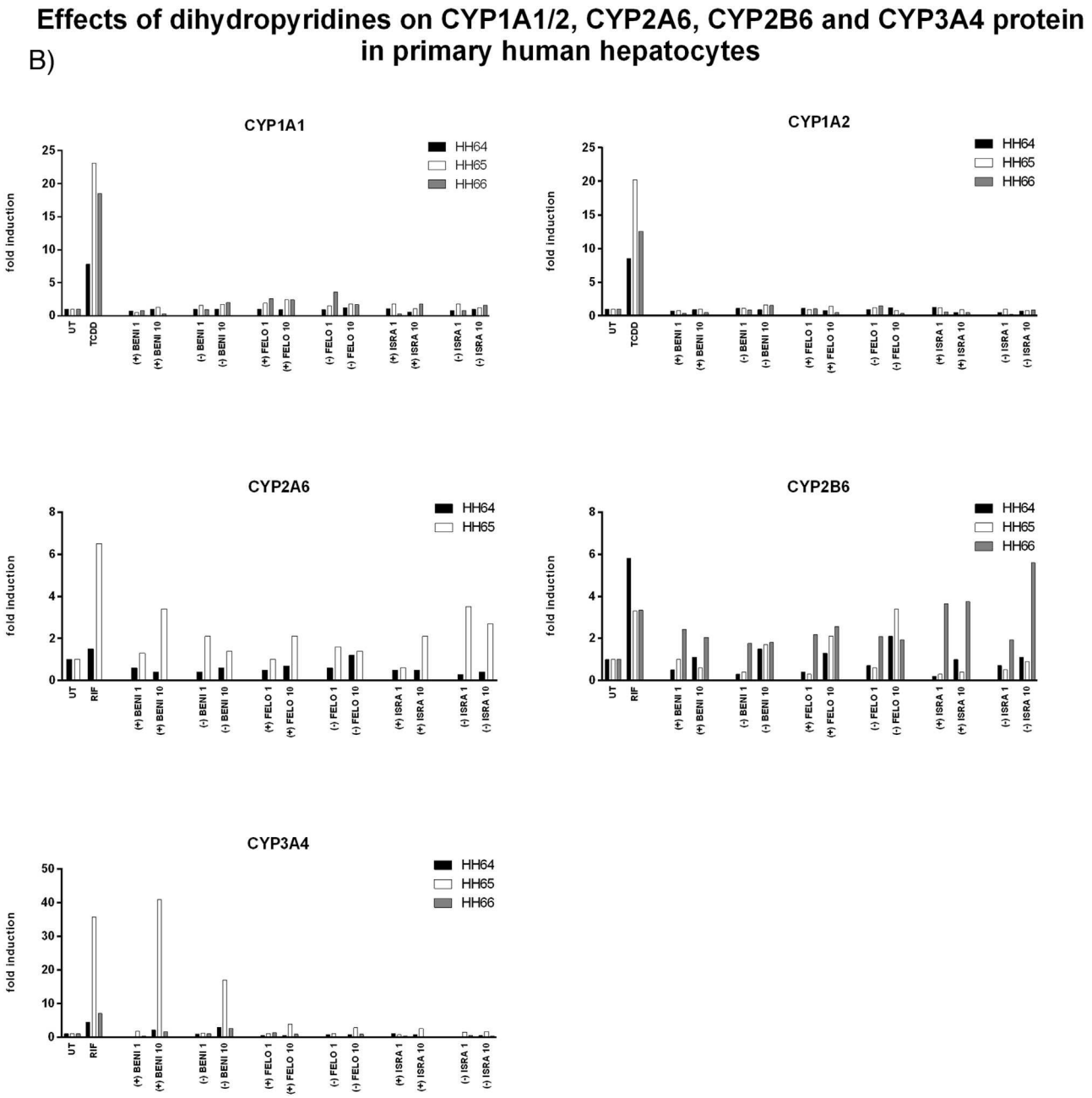

Fig. 6. (Continued)

enantiospecific effects of dihydropyridines in EMSA were inversely correlated with their effects on CYP1A mRNA expression and AhR transcriptional activity.

3.8. Effects of dihydropyridine enantiomers on the catalytic activities of cytochromes P450

The inhibitory effects (IC_{50}) of dihydropyridine enantiomers on the activities of drug-metabolizing CYPs using ten probe drugs were tested in HLMs (Table 1; Fig. 8). In cases where studied compound showed $IC_{50} < 10\,\mu M$, additional experiments were performed and mechanism of inhibition and K_i values were determined (Table 2). In detail:

Benidipine enantiomers strongly inhibited CYP3A4, CYP2C9, CYP2C19 and CYP2D6. The enzyme activities of CYP1A2, CYP2A6, CYP2B6, CYP2C8 and CYP2E1 were inhibited faintly, with IC_{50} exceeding 50 μM. Further we examined mechanism of CYP inhibition by benidipines. Two pre-incubation times (3 min and 30 min) with two different probes (testosterone and midazolam) were applied to evaluate inhibition of CYP3A4. The 3 min and 30-min pre-incubations revealed competitive and non-competitive inhibition for both CYP3A4 probes, respectively. The inhibition of CYP3A4 was stereospecific, where (+)- benidipine was stronger inhibitor as compared to (−)-benidipine. In 3-min pre-incubations, the K_i values for (+)-benidipine/(−)-benidipine were 8.42 μM/23.14 μM and 2.91 μM/7.89 μM, in testosterone and midazolam assay, respectively. In 30-min pre-incubations, the K_i values for (+)-benidipine/(−)-benidipine were 2.53 μM/3.04 μM and 4.75 μM/5.27 μM, in testosterone and midazolam assay, respectively. In contrast, (−)-benidipine (K_i = 6.84 μM) was stronger

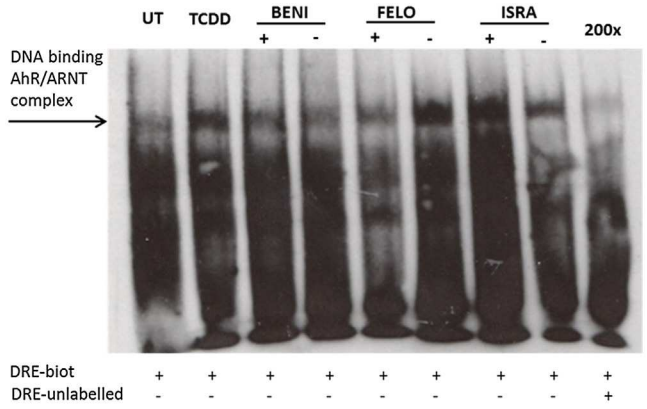

Fig. 7. Electrophoretic mobility shift assay EMSA. MCF-7 cells at 100% confluence were incubated for 2 h with vehicle as a negative control (UT; 0.1% DMSO v/v), TCDD (5 nM) and studied compounds ((+)benidipine, (−)benidipine, (+)felodipine, (−)felodipine, (+)isradipine, (−)isradipine) at the concentrations 10 μM. Nuclear extracts were incubated with a biotin-labeled probe containing AhR binding site and electrophoresed on 5% polyacrylamide gel as described under "Materials and methods section." Experiments were performed in three independent cell passages.

inhibitor of CYP2C9 as compared to (+)-benidipine (K_i = 20.17 μM). There was no significant difference in inhibition of CYP2C19 by (+)-benidipine (K_i = 10.22 μM) and the (−)-benidipine (K_i = 8.35 μM). The inhibition of CYP2D6 was moderate and enantiospecific, with K_i for (+)-benidipine and (−)-benidipine 66.66 μM and 60.91 μM, respectively. The mechanism of CYP2C9, CYP2C19 and CYP2D6 by benidipines was non-competitive.

Felodipine enantiomers strongly inhibited CYP3A4 and CYP2C19. The catalytic activities of CYP2A6, CYP2C8, CYP2C9 were affected rather moderately, but with enantiospecific pattern. The inhibition of CYP2C8 by (+)-felodipine (IC_{50} = 11.77 μM) was approx. 4-fold stronger as compared to (−)-felodipine (IC_{50} = 45.05 μM). In contrast, (+)-felodipine (IC_{50} > 50 μM) was weaker inhibitor of CYP2C9 as compared to (−)-felodipine (IC_{50} = 36.48 μM). (−)-felodipine was stronger inhibitor of CYP2A6 than (+)-felodipine, with IC_{50} of 18.52 and 26.26 μM, respectively. The inhibition of CYP1A2, CYP2B6, CYP2D6 and CYP2E1 was very weak with IC_{50} > 50 μM. More detailed kinetic experiments revealed that (−)-felodipine is stronger inhibitor of CYP3A4 than (+)-felodipine, in all experimental set ups. In 3-min pre-incubations, the K_i values for (+)-felodipine/(−)-felodipine were 28.66 μM/17.47 μM and 28.90 μM/15.99 μM, in testosterone and midazolam assay, respectively. For both substrates, inhibitory mechanism was competitive. In 30-min pre-incubations, the K_i values for (+)-felodipine/(−)-felodipine were 26.39 μM/12.29 μM and 8.06 μM/5.81 μM, in testosterone and midazolam assay, respectively. For both substrates, inhibitory mechanism was non-competitive. Regarding CYP2C19, (+)-felodipine (K_i = 1.74 μM) inhibited its activity 2-fold more than (−)-felodipine (K_i = 3.53 μM). The mechanisms of CYP2C19 inhibition by (+)-felodipine and (−)-felodipine were mixed and non-competitive, respectively.

Isradipine enantiomers were potent inhibitors of CYP3A4 and CYP2C19, while their effects on catalytic activities of CYP1A2, CYP2A6, CYP2B6, CYP2C9 CYP2D6 and CYP2E1 were negligible (IC_{50} > 50 μM). The data for CYP2C8 are not available due to the interference between metabolite 6-hydroxy-paclitaxel and isradipine. In 3-min pre-incubations, the K_i values for CYP3A4 *vs* (+)-isradipine/(−)-isradipine were 11.09 μM/8.37 μM and 7.25 μM/6.43 μM, in testosterone and midazolam assay, respectively. The mechanisms of inhibition were uncompetitive and competitive for testosterone and midazolam, respectively. In 30-min pre-incubations, the K_i values for CYP3A4 *vs* (+)-isradipine/(−)-isradipine were 25.79 μM/24.45 μM and 4.59 μM/7.68 μM, in testosterone and midazolam assay, respectively. Testosterone and midazolam assay was inhibited by uncompetitive and non-competitive mechanisms, respectively. In both incubation periods, there was no significant difference in the inhibition potencies of both isradipine enantiomers. The activity of CYP2C19 was inhibited enantiospecifically and uncompetitively, with K_i values of 3.73 μM and 6.36 μM for (+)-isradipine and (−)-isradipine, respectively.

4. Discussion

In the current paper we examined effects of benidipine, felodipine and isradipine enantiomers on the expression and enzyme activities of human xenobiotics-metabolizing cytochromes P450.

Two isolated studies described the activation of PXR by dihydropyridine CCBs, including nifedipine, nicardipine, isradipine and clevidipine (Drocourt et al., 2001; Zhang et al., 2006). Even

Table 1
Evaluation of studied calcium channels blockers as inhibitors of CYPs in HLMs. Table presents IC_{50} values of enantiomers of individual studied drugs for the inhibition of CYPs in HLMs. Bold numbers present stronger inhibitor among two enantiomers.

IC_{50} [μM]		CYP3A4[a]		CYP3A4[b]		CYP1A2	CYP2A6
		TST	MDZ	TST	MDZ		
BENI	(+)-	**9.15 ± 1.28**	**10.34 ± 3.32**	**2.49 ± 0.29**	**2.12 ± 1.26**	>50 μM	>50 μM
	(−)-	25.37 ± 4.69	20.87 ± 5.64	3.23 ± 0.46	6.40 ± 2.16	>50 μM	>50 μM
FELO	(+)-	31.02 ± 1.19	28.42 ± 3.70	26.4 ± 0.69	8.41 ± 3.64	>50 μM	26.26 ± 1.10
	(−)-	**17.68 ± 1.22**	**15.99 ± 1.97**	**12.3 ± 1.75**	5.70 ± 0.76	>50 μM	**18.52 ± 1.23**
ISRA	(+)-	22.19 ± 2.45	29.76 ± 3.55	22.83 ± 1.19	8.36 ± 1.15	>50 μM	>50 μM
	(−)-	23.11 ± 3.87	20.11 ± 6.21	28.19 ± 1.24	8.78 ± 1.26	>50 μM	>50 μM

IC_{50} [μM]		CYP2B6	CYP2C8	CYP2C9	CYP2C19	CYP2D6	CYP2E1
BENI	(+)-	>50 μM	>50 μM	25.53 ± 2.34	24.87 ± 1.73	16.1 ± 1.41	>50 μM
	(−)-	>50 μM	>50 μM	**8.43 ± 1.32**	26.38 ± 1.89	**12.5 ± 1.19**	>50 μM
FELO	(+)-	>50 μM	11.77 ± 0.17	>50 μM	**1.93 ± 0.38**	>50 μM	>50 μM
	(−)-	>50 μM	45.05 ± 2.81	36.48 ± 2.42	4.41 ± 0.15	>50 μM	>50 μM
ISRA	(+)-	>50 μM	°No data	>50 μM	**6.55 ± 1.29**	>50 μM	>50 μM
	(−)-	>50 μM	°No data	>50 μM	10.66 ± 1.45	>50 μM	>50 μM

[A] . . . 3 min preincubation time; [B] . . . 30 min preincubation time; TST· · · testosterone assay; MDZ· · · midazolam assay ° . . . due to interference in paclitaxel assay, not assessed.

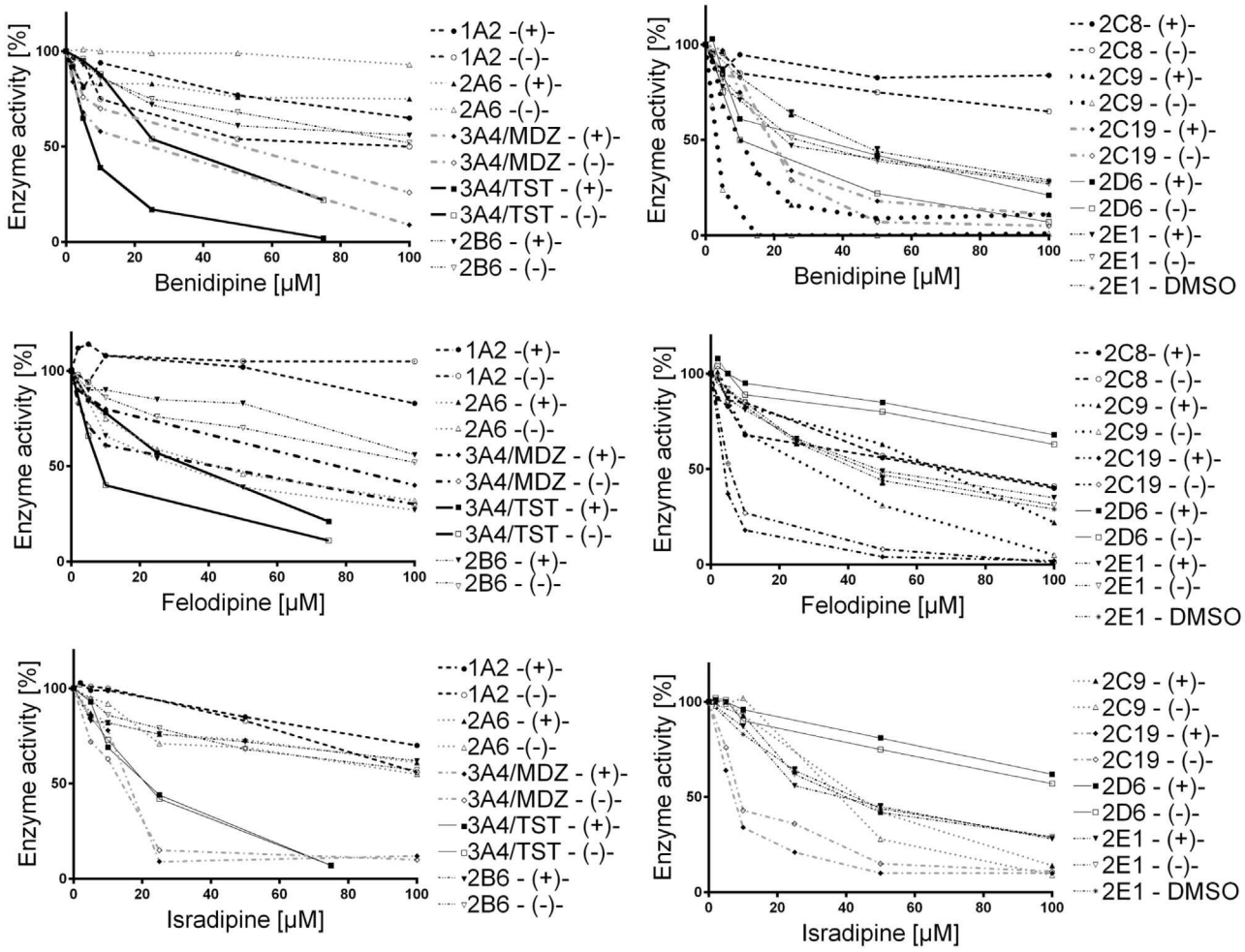

Fig. 8. Effects of dihydropyridine enantiomers on the catalytic activities of cytochromes P450. Same line pattern was used for individual activity and for both enantiomers labeled by different symbol. The inhibition activities are expressed as the mean from two independent experiments performed in triplicates. Data are showed as a per cent activity remaining relative to the control (100%, without studied compound).

though over 40 different dihydropyridine CCBs exist, only four representatives were yet tested for interactions with PXR-CYP3A4 signaling pathway. Most likely, the activation of PXR and induction of CYP3A4 is common property of dihydropyridine CCBs, therefore, an extensive study including all the existing derivatives is not needed. On the other hand, dihydropyridine CCBs contain in their structure at least one chiral center, but the effects of pure optical isomers were not investigated so far. We have selected four frequently used dihydropyridine CCBs, and employing preparative chromatography, we isolated pure (+)/(−) enantiomers of benidipine, felodipine, isradipine and nisoldipine. The latter CCB was excluded from testing, because it was unstable in aqueous milieu. We observed that all CCBs activate PXR with similar efficacies; the magnitudes of the inductions were comparable with that by RIF. Interestingly, the potencies of (+/−)-benidipines were about ten times higher as compared to those of (+/−)-isradipines and (+/−)-felodipines. Consistently, CCBs induced PXR-regulated genes including CYP2A6, CYP2B6 and CYP3A4 in human hepatocytes, and there were not differences between the effects of (+)/(−) enantiomers. Collectively, individual enantiomers of benidipine and felodipine are equipotent activators of human PXR and inducers of PXR-regulated genes, regardless their optical configuration. In gene reporter assays, (−)-isradipine was three times more potent activator of PXR as compared to (+)-isradipine, but the effects of both enantiomers on the expression of CYP3A4 did not differ.

Unexpected and interesting finding of the current study was enantiospecific activation of AhR and induction of CYP1A genes by optical isomers of tested CCBs. Transcriptional activity of AhR in gene reporter assays, expression of CYP1A1 in HepG2 cells and expression of CYP1A1/CYP1A2 in human hepatocytes were increased by CCBs with following pattern: benidipine (−)>(+), isradipine (−)>(+), felodipine (+)>(−). Since there is common chiral center in all three dihydropyridine CCBs, the difference between (+)/(−) isomers of benidipine and isradipine ((−)>(+)) in contrast to felodipine ((+)>(−)) is probably determined by particular structure of each derivative. Overall, the finding that CCBs activate AhR implies additional potential to elicit drug–drug interactions, food-drug interactions or endocrine disruption.

Dihydropyridine CCBs are CYP3A4 inhibitors (Katoh et al., 2000; Ma et al., 2000; Wang et al., 1999), which we also observed in the current study. The 3-min pre-incubations revealed competitive mechanism of inhibition, which is in line with the fact that CCBs are also substrates of CYP3A4, hence, they compete with a model substrate for binding to the active site of an enzyme. However, 30-min pre-incubations revealed different modes of inhibition, indicating that longer time of pre-incubation allows binding to a different part of the enzyme molecule. (+)-benidipine was stronger inhibitor of CYP3A4 as compared to (−)-benidipine. Taking in account that plasma concentration of benidipine reaches up to 0.1 μM (Shimada et al., 1996) and that K_i values for CYP3A4 are cca 2 μM, interaction of benidipine with other CYP3A4 substrates may

Table 2
The characterisation of potentially strong inhibitors. Table provides the K_i values and mechanism of inhibition of individual enantiomers of testes drugs. Bold numbers represents statistically significantly more potent enantiomers.

CYP	(+)-BENI		(−)-BENI	
	K_i [μM]	mechanism	Ki [μM]	mechanism
3A4 ^{TSTA}	**8.42 ± 1.29** **	comp	23.14 ± 5.89	comp
3A4 ^{MDZA}	**2.91 ± 1.07** *	comp	7.89 ± 3.15	comp
3A4 ^{TSTB}	**2.53 ± 0.07** *	noncom	3.04 ± 0.12	noncom
3A4 ^{MDZB}	**4.75 ± 0.25***	noncom	5.27 ± 0.16	noncom
2C9	20.17 ± 2.31	noncom	**6.84 ± 1.11** *	noncom
2C19	10.22 ± 2.37	noncom	8.35 ± 1.17	noncom
2D6	66.66 ± 3.87	noncom	**60.91 ± 1.37** *	noncom

CYP	(+)-FELO		(−)-FELO	
	K_i [μM]	mechanism	Ki [μM]	mechanism
3A4 ^{TSTA}	28.66 ± 2.82	comp	**17.47 ± 3.05** **	comp
3A4 ^{MDZA}	28.90 ± 3.52	comp	**15.99 ± 3.11** **	comp
3A4 ^{TSTB}	26.39 ± 4.02	uncom	**12.29 ± 1.68** *	uncom
3A4 ^{MDZB}	8.06 ± 0.96	noncom	5.81 ± 0.37	noncom
2C19	**1.74 ± 0.59** *	mixed	3.53 ± 1.79	noncom

CYP	(+)-ISRA		(−)-ISRA	
	K_i [μM]	mechanism	Ki [μM]	mechanism
3A4 ^{TSTA}	11.09 ± 1.08	uncom	8.37 ± 1.07	uncom
3A4 ^{MDZA}	7.25 ± 1.78	comp	6.43 ± 1.06	comp
3A4 ^{TSTB}	25.79 ± 4.04	uncom	24.45 ± 6.49	uncom
3A4 ^{MDZB}	4.59 ± 0.69	noncom	7.68 ± 0.85	noncom
2C19	**3.73 ± 0.49** *	uncom	6.36 ± 0.73	uncom

^{A} . . . 3 min preincubation time; ^{B} . . . 30 min preincubation time; MDZ· · · midazolam assay; TST· · · testosterone assay; * . . . $P < 0.05$; ** . . . $P < 0.005$; comp . . . competitive; noncom . . . noncompetitive;, uncom . . . uncompetitive.

occur. Benidipine possesses a polar nitro group and bulky substituent in its structure, which may be a reason why it binds to CYP3A4 that has a spacious and flexible active site (Hendrychova et al., 2011). (−)-felodipine, the pharmacologically more active form (*eutomer*), had higher inhibition effect towards CYP3A4 than (+)-felodipine (*dystomer*). Racemic felodipine was identified as a weak *in vivo* CYP3A4 inhibitor (Snyder et al., 2014). In addition, *in vivo* interactions between dihydropiridine CCBs and statins, resulting in adverse effects, were described (Wang et al., 2016). Isradipine inhibited CYP3A4 regardless optical configuration of the drug. Since K_i values for isradipines are rather low, the possibility drug–drug interactions due to the inhibition of CYP3A4 should be taken into account.

The activity of CYP2C19 was inhibited by all studied CCBs. We found that benidipine was the weakest inhibitor, its effects of CYP2C19 were not enantiospecific, and the inhibition constants were consistent with that of racemic mixture (Katoh et al., 2000). Strong and enantiospecific inhibition of CYP2C19 was observed for felodipine and isradipine, when (+) enantiomers were much more potent inhibitors of CYP2C19 as compared to their (−) counterparts. While others identified felodipine as a weak *in vivo* inhibitor of the CYP2D6 (Snyder et al., 2014), we did not find significant inhibition of CYP2D6 by felodipines in HLMs. Collectively, the potential use of (−)-felodipine (*eutomer*) is disputable, because it is stronger inhibitor of CYP3A4, but weaker inhibitor of CYP2C19, as compared to (+)-felodipine. Benidipines were weak and equipotent inhibitors of CYP2D6. On the other hand, they were also noncompetitive, but enantiospecific inhibitors of CYP2C9.

In conclusion, dihydropyridine CCBs may cause drug–drug interactions involving both inhibition and induction of xenobiotic-metabolizing enzymes. We demonstrate that dihydropyridine CCBs have stereospecific inhibitory potencies against human CYP catalytic activities. Thereby, drug–drug interactions caused by inhibition of CYP enzymes by studied compounds might arise, in particular in combination with CYP3A4 or CYP2C19 substrates. Dihydropyridine CCBs are activators of AhR and PXR xenoreceptors, the former being activated stereospecifically. Hence, induction of AhR- and PXR-regulated CYPs enzymes by CCBs should be also considered in terms of drug–drug interactions.

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PALACKÝ UNIVERSITY OLOMOUC

Faculty of Science

Department of Cell Biology and Genetics

ENANTIOSPECIFIC INTERACTIONS BETWEEN CLINICALLY USED CHIRAL DRUGS AND REGULATORY PATHWAYS OF HUMAN CYTOCHROME P450s

Ph.D. Thesis Summary

P1527 Biology

Mgr. Martina Štěpánková

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The Ph.D. thesis is based on my own research carried out within framework of Ph.D. study program P1527 Biology in the Department of Cell Biology and Genetics, Faculty of Science, Palacký University, Olomouc, from September 2013 to June 2017.

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Summary

The pharmaceutical industry produces many chiral drugs with an emphasis on the development and introduction of more enantiopure drugs for pharmacotherapy. Numerous studies have revealed that individual enantiomers of a chiral drug can qualitatively and quantitatively differ in their biological activities, including pharmacokinetics, pharmacodynamics, toxicokinetics, and toxicodynamics. For this reason, elimination of the inactive isomer in some cases can be advantageous, because it can reduce adverse effects of the drug. In other cases, however, the racemate is more beneficial. Therefore, research on enantiospecific interactions between individual optical isomers of chiral drugs and drug-metabolizing enzymes is crucial for an effective and safe treatment. I have studied single isomers of clinically used drugs prescribed for hypertension (benidipine, felodipine, and isradipine) and hypercholesterolaemia (atorvastatin, fluvastatin, and rosuvastatin) and their influence on the activity of cytochrome P450 (CYP) transcriptional regulators, including pregnane X receptor (PXR), aryl hydrocarbon receptor (AhR) and glucocorticoid receptor (GR) by the means of gene reporter assays and an electrophoretic mobility shift assay (EMSA). Moreover, effects of the tested stereoisomers on the expression of drug-metabolizing P450s were evaluated using Simple western blotting and quantitative PCR (qPCR) for quantification of proteins and mRNAs, respectively. Overall, I showed that atorvastatin, fluvastatin and rosuvastatin enantiospecifically activate PXR and induce genes *CYP2A6, CYP2B6* and *CYP3A4*. The calcium channel blockers benidipine, felodipine and isradipine enantiospecifically activated AhR and induced genes *CYP1A1* and *CYP1A2*, while they non-enantiospecifically activated PXR and induced genes *CYP2A6, CYP2B6,* and *CYP3A4*. The data obtained may have toxicological and clinical implications.

Souhrn

Farmaceutický průmysl produkuje velké množství chirálních léčiv s důrazem na rozvoj a využití enantio-čistých léčiv. Četné studie prokázaly, že se účinky jednotlivých enantiomerů kvalitativně i kvantitativně liší na farmakokinetické, farmakodynamické, toxikokinetické a toxikodynamické úrovni. Z tohoto důvodu bývá eliminace neaktivního isomeru v některých případech výhodná, jelikož se zmírní vedlejší účinky léčiva. V jiných případech je však pro požadovaný terapeutický účinek vhodnější racemát. Z toho důvodu hraje studium enantiospecifických interakcí mezi jednotlivými optickými izomery chirálních léčiv a enzymy metabolizující léčiva důležitou roli pro zajištění efektivní a bezpečné léčby. Z tohoto důvodu jsem studovala jednotlivé izomery klinicky používaných léčiv často předepisovaných pro léčbu hypertenze (benidipin, felodipin, and isradipin) a hypercholesterolémie (atorvastatin, fluvastatin, and rosuvastatin) a jejich vliv na aktivitu transkripčních regulátorů cytochromů P450 (CYP), konkrétně pregnanového X receptoru (PXR), aryl uhlovodíkového receptoru (AhR) a glukokortikoidního receptoru (GR), pomocí reportérových esejí a gelové retardační analýzy (EMSA). Dále byly hodnoceny účinky testovaných stereoizomerů na expresi CYP450 enzymů metabolizujících léčiva za použití Simple western blottingu a kvantitativní PCR (qPCR) na úrovni proteinů a mRNA. Celkově jsem došla k zajímavému zjištění, že atorvastatin, fluvastatin a rosuvastatin enantiospecificky aktivují PXR a indukují geny *CYP2A6, CYP2B6* a *CYP3A4*. Dále benidipin, felodipin a isradipin enantiospecificky aktivují AhR a indukují geny *CYP1A1* a *CYP1A2* a zároveň ne-enantiospecificky aktivují PXR a indukují geny *CYP2A6, CYP2B6* a *CYP3A4*. Získaná data mohou mít toxikologický a klinický význam.

Contents

1 AIMS

The main aim of this work was to study the effects of individual optical isomers of chiral drugs on regulatory pathways of human CYPs. Well-known cholesterol-lowering drugs (statins) and calcium channel blockers (CCBs) of the dihydropyridine class were selected for this study. The specific goals were to investigate the effects of pure stereoisomers of CCBs and statins on:

a) Function and transcriptional activities of AhR, GR and PXR in human cancer cell lines and in primary human hepatocytes.

b) The expression and catalytic activities of major drug-metabolizing CYPs in human cancer cell lines and in primary cultures of human hepatocytes.

2 INTRODUCTION

A specific spatial arrangement is required for many biochemical processes including binding of a ligand to its receptor and a substrate–enzyme interaction. Chirality is abundant in living systems; thus, proteins, amino acids, carbohydrates, nucleosides and other natural molecular entities are chiral (asymmetric). Metabolism of drugs and other xenobiotics could also be stereoselective because it is facilitated by many enzymes with potential three-dimensional substrate specificity including cytochrome P450s, flavin-containing monooxygenases, alcohol dehydrogenases, sulphotransferases, glutathione S-transferases, UDP-glucuronosyl-transferases, and many others (Jakoby and Ziegler, 1990).

Cytochrome P450s (CYPs) play a crucial role in phase I of xenobiotic biotransformation. These iron-containing proteins catalyse the monooxygenase reaction involving various endogenous and exogenous substrates. CYP1, CYP2, and CYP3 family members metabolise mainly xenobiotics, and these genes are inducible by endogenous and foreign substances through activation of steroid receptors, nuclear receptors, and xenoreceptors. These receptors are ligand-activated transcription factors and include aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), pregnane X receptor (PXR), glucocorticoid receptor (GR), oestrogen receptor (OeR), vitamin D receptor (VDR), and retinoid X receptor (RXR) (Aranda and Pascual, 2001).

Many currently used drugs are chiral, being marketed as racemic mixtures of stereoisomers. They are enantiomers (non-superimposable mirror images) or diastereomers (not mirror images). In either case, stereoisomers can differ markedly from each other in properties such as bioactivity, pharmacokinetics, and toxicity. Therefore, the United States Food and Drug Administration requires the identification and characterisation of each individual component of racemic drugs (Brocks, 2006). There are numerous reports about chiral drugs with one being a more potent stereoisomer than the other. The examples are atenolol (Stoschitzky *et al.*, 1993), propranolol (Stoschitzky *et al.*, 1989), carvedilol (Bartsch *et al.*, 1990), metoprolol (Wahlund *et al.*, 1990), warfarin (Hewick and McEwen, 1973), omeprazole (Andersson *et al.*, 2001), methadone (Olsen *et al.*, 1977), lansoprazole (Katsuki *et al.*, 1996). As a result, pharmaceutical manufacturers tend to produce single-isomer (enantiopure) drugs to achieve higher therapeutic efficacy and diminish adverse effects and toxicity of the drug. This concept led to the introduction of enantiopure drugs, which until then had been used as racemates. The examples are omeprazole–esomeprazole, citalopram–escitalopram, modafinil–armodafinil, and many other pairs. However, in some cases, the use of a racemate is more suitable than the use of a pure enantiomer because of the complementary effects of stereoisomers (Smith, 2009). Thus, it is worthwhile to explore enantiospecific interactions between individual optical isomers of chiral drugs and drug-metabolizing enzymes, to ensure effective and safe pharmacotherapy.

Monitoring of drug–drug interactions (DDIs) is essential to guarantee drug safety. DDIs are caused by multiple mechanisms, including activation or inhibition of major transcriptional regulators of xenobiotic-metabolizing CYPs, in particular, AhR and PXR. Consequently, induction or down-regulation of CYPs may occur. Indeed, it was recently demonstrated that several clinically used chiral drugs, including ketoconazole (Novotna *et al.*, 2014a; Novotna *et al.*, 2014b), warfarin (Rulcova *et al.*, 2010), omeprazole and lansoprazole (Novotna and Dvorak, 2014; Novotna *et al.*, 2014c) have enantiospecific effects on these xenoreceptors.

3 The chiral compounds tested

HMG-CoA reductase inhibitors also known as statins (e.g. atorvastatin, fluvastatin, lovastatin, pravastatin, rosuvastatin, and simvastatin) inhibit the synthesis of cholesterol, and therefore are widely used in the treatment of hypercholesterolaemia (Goldstein and Brown, 1990). Statins induce regression in vascular atherosclerosis and reduce cardiovascular morbidity and mortality in patients with and without coronary artery disease (Hebert *et al.*, 1997; Maron *et al.*, 2000; Pedersen, 1999). Three statins—atorvastatin, fluvastatin, and rosuvastatin—were chosen for analysis of their stereospecific effects on human CYPs in this study (Figure 1). Structurally, the statins under study are chiral compounds having two asymmetrical centres in the molecule, enabling formation of four diastereomers: 3R5R-, 3R5S-, 3S5R-, and 3S5S-. The following single-isomer formulations of these drugs are used in the clinic: 3R5R-atorvastatin (Lipitor, Pfizer; generic since November 2011), 3R5S-rosuvastatin (Crestor, Astra-Zeneca; approved on 12th August 2003), and 3R5S-fluvastatin (Lescol, Novartis; approved on 31st December 1993; generic since 2011).

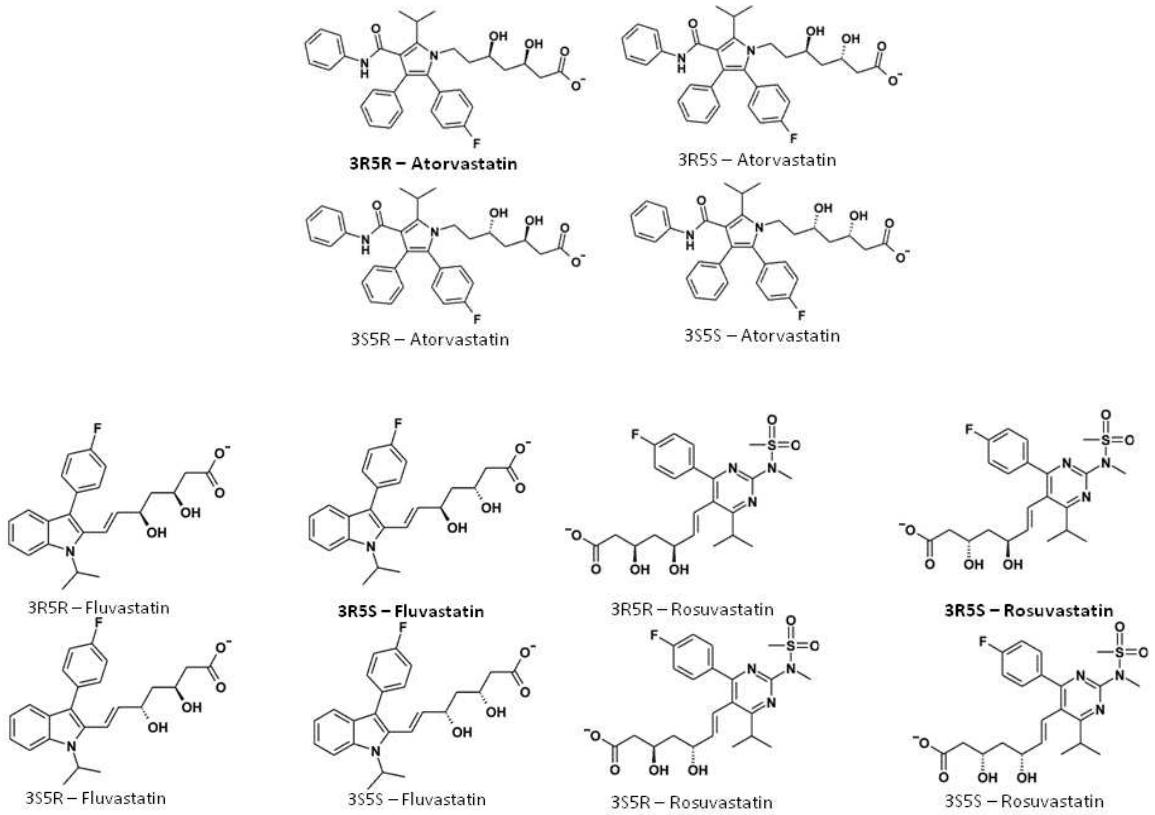

Figure 1: Chemical structures of single-isomer forms of statins. Four individual diastereomers of atorvastatin, rosuvastatin, and fluvastatin are shown. Clinically used single-isomer forms are boldfaced.

The frequent adverse effects associated with statin therapy are relatively mild: headache, rash, and gastrointestinal symptoms. However, statins may cause more serious adverse effects such as asymptomatic elevation in liver transaminases, myopathy, and increased risk of diabetes (Bellosta and Corsini, 2012). The risk of adverse effects increases when statins are used simultaneously with other drugs, which may cause DDIs. Pharmacokinetics of statins is complex. Statins are substrates for multiple membrane transporters (Neuvonen *et al.*, 2006) and undergo substantial microsomal metabolism by CYPs. Atorvastatin is metabolised primarily by CYP3A4; therefore, inhibitors of CYP3A4 may influence the pharmacokinetics of atorvastatin (Hsyu *et al.*, 2001; Prueksaritanont *et al.*, 1999). On the contrary, fluvastatin and rosuvastatin are metabolised primarily by CYP2C9 with little involvement of CYP3A4 in case of rosuvastatin (Olsson *et al.*, 2002; Scripture and Pieper, 2001). Therefore, a lower number of clinically significant pharmacokinetic DDIs between rosuvastatin or fluvastatin and other drugs than with atorvastatin has been reported (Cooper *et al.*, 2002).

The calcium-channel blockers (CCBs) inhibit the flow of extracellular calcium through ion-specific channels in the cell membrane. These channels facilitate calcium influx in response to membrane depolarisation and regulate intracellular processes such as contraction, secretion, neurotransmission, and gene expression in many different cell types. Blockade of these channels in vascular tissues results in a decrease in smooth-muscle and myocardial contractility and a reduction in blood pressure (Abernethy and Schwartz, 1999). Therefore, CCBs are used as anti-hypertensives and in the treatment of angina pectoris. Structurally, dihydropyridine CCBs are chiral compounds having at least one asymmetrical centre in the molecule, thereby existing as two or more optical isomers. Three chiral ‘dipines’—benidipine, felodipine, and isradipine—were chosen here for analysis of stereospecific effects on human CYPs (Figure 2). Benidipine has two chiral atoms in the molecule thus enabling formation of four diastereomers: (+)-α-; (-)-α-; (+)-β-, and (-)-β-isomer. The approved therapeutic formulation is a racemate of α enantiomers (approved in China in 2008, sold as Coniel by Kyowa Hakko Kirin Co., Ltd.; Caritec: Stancare, Ranbaxy Laboratories Ltd.). The potency of (+)-α-benidipine is 30- to 100-fold stronger than that of (-)-α-benidipine in spontaneously hypertensive rats (Muto *et al.*, 1988). Benidipine was described as an inhibitor of CYP3A4, CYP1A1, CYP2C9, CYP2C19, and CYP2D6 in human liver microsomes (Katoh *et al.*, 2000).

In case of felodpipine and isradipine, there is only one chiral carbon present in the structure. Thus, they have two enantiomers: (+)(R)/(-)(S)-felodipine and (+)(S)/(-)(R)-isradipine, respectively. Both drugs are clinically used as racemates (Plendil, AstraZeneca, approved 25th July 1991, generic since 2004; Dynacirc, SmithKline Beecham, approved 20th December 1990, generic since 2006). (-)(S)-felodipine was found to be more potent than (+)(R)-felodipine (Eltze *et al.*, 1990). Pharmacological activity of (+)(S)-isradipine was shown to be 150-fold higher as compared to (−)(R)-isradipine (Ruegg and Hof, 1990).

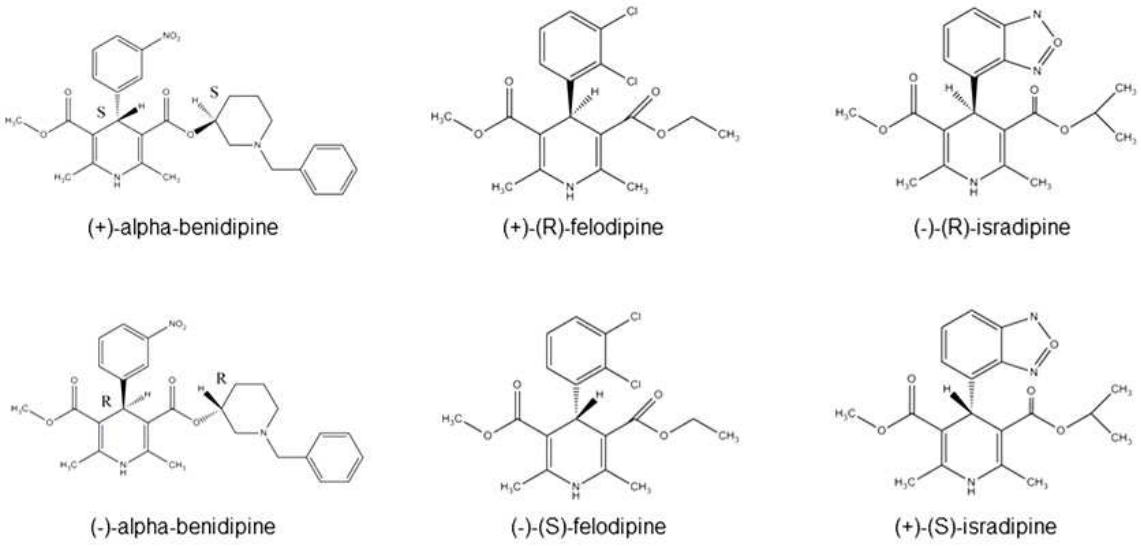

Figure 2: Chemical structures of the chiral dihydropyridines under study.

Many CCBs cause considerable adverse effects, such as tachycardia, orthostatic hypotension, fluid retention, headache, fatigue, vertigo, muscle cramps, and dizziness. Dihydropyridines were reported to be metabolised to less active metabolites mainly by CYP3A and to a lesser extent by other CYPs (Guengerich *et al.*, 1991; Yoon *et al.*, 2007). Thus, interactions with simultaneously administered CYP3A inhibitors or inducers may occur (Holtbecker *et al.*, 1996; Jalava *et al.*, 1997; Lown *et al.*, 1997). DDIs may be caused by activation of major xenoreceptors. Indeed, activation of PXR and induction of PXR-regulated genes by dihydropyridines (nifedipine, nicardipine, isradipine, or clevidipine) have been described (Drocourt *et al.*, 2001; Zhang *et al.*, 2006).

4 MATERIALS AND METHODS

4.1 Biological materials

4.1.1 Human cancer cell lines

Human Caucasian hepatocellular carcinoma cell line HepG2 (ECACC No. 85011430), human Caucasian breast adenocarcinoma cell line MCF-7 (ECACC No. 86012803), human Caucasian colon adenocarcinoma cell lines LS174T (ECACC No. 87060401) and LS180 (ECACC No. 87021202) were purchased from the European Collection of Authenticated Cell Cultures (ECACC). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% of foetal bovine serum, 100 U/mL streptomycin, 100 μg/mL penicillin, 4 mM L-glutamine, 1% of non-essential amino acids, and 1 mM sodium pyruvate. The cells were maintained at 37 °C and 5% CO_2 in a humidified incubator. Cell lines (AZ-AHR and AZ-GR) stably transfected with a gene reporter were described elsewhere (Novotna *et al.*, 2011; Novotna *et al.*, 2012).

4.1.2 Primary human hepatocytes

Hepatocytes were isolated from human liver tissue obtained from six multiorgan donors: HH59 (woman; 42 years), HH61 (man; 64 years), HH63 (man; 68 years), HH64 (man; 73 years), HH65 (man; 34 years), and HH66 (man; 65 years). The tissue acquisition protocol was in accordance with the requirements stated by the local ethics commission in the Czech Republic. The cells were plated in collagen-coated dishes in a hormonally and chemically defined medium (Isom *et al.*, 1985; Pichard-Garcia *et al.*, 2002) consisting of the mixture of William's E and Ham's F-12 media [1:1 (v/v)]. The cultures were maintained at 37 °C and 5% CO_2 in a humidified incubator.

4.2 Compounds and reagents

Dimethylsulphoxide (DMSO), rifampicin (RIF), dexamethasone (DEX), hygromycin B, 3R5R-atorvastatin, foetal bovine serum, DMEM, streptomycin, penicillin, L-glutamine, sodium pyruvate, non-essential amino acids, and TRI Reagent® were purchased from Sigma-Aldrich (Czech Republic). Racemates of felodipine, isradipine, and α-benidipine were also acquired from Sigma-Aldrich, and their respective enantiomers were isolated by chiral semipreparative chromatography

using a Knauer Smartline HPLC system (Germany) as described elsewhere (Stepankova *et al.*, 2016). Following chiral purity was achieved: 98.5%, and 98.5% for (+/-)-benidipines, 99.5% and 97.0% for (+/-)-felodipines and 99.6% and 97.5% for (+/-)-isradipines, respectively. The racemization of individual enantiomers after 48 h in aqueous solution was negative. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) was purchased from Ultra Scientific (USA), whereas 3R5S-, 3S5R-, and 3S5S-atorvastatin from Toronto Research Chemicals Inc. (Canada). 3R5R-, 3R5S-, 3S5R-, 3S5S-fluvastatin, 3R5R-, 3S5R-, and 3S5S-rosuvastatin were acquired from TLC PharmaChem Inc. (Canada). 3R5S-rosuvastatin was purchased from Santa Cruz Biotechnology Inc. (Germany), whereas luciferase lysis buffer and FuGENE® HD Transfection Reagent from Promega (USA). M-MuLV Reverse Transcriptase and random hexamers was purchased from New England Biolabs (USA). Oligonucleotide primers used in qPCR reactions were synthesised by Generi Biotech (Czech Republic). LightCycler® 480 Probes Master was purchased from Roche Diagnostic Corporation (Czech Republic). Reagents for Simple Western blotting by Sally Sue^{TM}, antibody diluent, a goat anti-rabbit IgG antibody, and goat anti-mouse IgG antibody were acquired from ProteinSimple (San Jose, CA). Antibodies against CYP1A1 (goat polyclonal, sc-9828, G-18), CYP1A2 (mouse monoclonal, sc-53614, 3B8C1), CYP2A6 (mouse monoclonal, sc-53615, F16P2D8), CYP2B6 (rabbit polyclonal, sc-67224, H-110), and CYP3A4 (mouse monoclonal; sc-53850, HL3) and a rabbit anti-goat IgG antibody (sc-2768) were purchased from Santa Cruz Biotechnology Inc. (Germany). An anti-β-actin (mouse monoclonal; 3700S, 8H10D10) antibody was acquired from Cell Signaling Technology (USA), and Light Shift Chemiluminescent EMSA Kit from Thermo Scientific (USA). All other reagents were of the highest quality commercially available.

4.3 Methods

4.3.1 Cell viability assay

AZ-AHR, AZ-GR, and LS180 cells were seeded at a density of 25 × 10^4/well (counted on CASY® Cell Counter + Analyzer System, Roche) in a 96-well plate and stabilised for 16 h prior to the treatments. To evaluate the cell damage, the cells were treated for 24 h with various concentrations of the compounds under study, Triton X-100 (2%, v/v), and/or vehicle (DMSO; 0.1%, v/v). After that, the medium was discarded, and the cells were washed with PBS. After addition of 100 µL of

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 0.3 mg/mL), the cells were incubated at 37 °C for 30–40 min. Next, the MTT solution was removed and formazan crystals were dissolved in DMSO. Then, the absorbance was measured at 540 nm using an Infinite M200 (TECAN, Austria). The half-maximal inhibitory concentrations (IC_{50}) were calculated using the data obtained from three independent cell passages.

4.3.2 Gene reporter assay

Transcriptional activities of AhR and GR were studied in stably transfected gene reporter AZ-AHR and AZ-GR cells, respectively. For evaluation of PXR transcriptional activity, LS180 cells were transiently transfected with a chimeric p3A4-luc reporter construct by lipofection (FuGENE® HD Transfection Reagent) as described elsewhere (Pavek *et al.*, 2010). The cells were seeded at density 25×10^4/well in a 96-well plate and stabilised for 16 h prior to the treatments. After that, the cells were treated for 24 h with the tested compounds and/or vehicle (DMSO; 0.1%, v/v), in the presence (antagonist mode) or absence (agonist mode) of TCDD (5 nM), RIF (10 μM), or DEX (100 nM), respectively. After the treatments, the cells were lysed, and luciferase activity was measured on the Infinite M200 (TECAN, Austria).

4.3.3 RNA isolation, reverse transcription, and quantitative PCR (qPCR)

HepG2 cells and primary human hepatocytes were seeded in 6-well plates (1×10^6/well) and stabilised for 16 h prior to the treatments. The cells were then treated for 24 h with the tested compounds, TCDD (5 nM), RIF (10 μM), and/or vehicle (DMSO; 0.1%, v/v). Total RNA was isolated by means of the TRI Reagent®. cDNA was synthesised from 1000 ng of total RNA using M-MuLV Reverse Transcriptase at 42 °C for 60 min in the presence of random hexamers. qPCR was carried out using LightCycler® 480 Probes Master on a Light Cycler® 480 II machine (Roche Diagnostic Corporation). The levels of all mRNAs were determined using primers and Universal Probes Library (UPL; Roche Diagnostic Corporation) probes as described elsewhere (Kubesova *et al.*, 2016). For quantification of mRNA of genes *CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP3A4, TAT, PXR*, and *GAPDH*, the following program was used: an activation step at 95 °C for 10 min was followed by 45 cycles of PCR (denaturation at 95 °C for 10 s and annealing with elongation at 60 °C for 30 s). The measurements were performed in triplicate. Gene expression

was normalised to *GAPDH* as a housekeeping gene. The data were processed according to the delta-delta Ct method.

4.3.4 Simple Western blotting by Sally Sue™

Total protein extracts were prepared from HepG2 cells and primary human hepatocytes cultured in 6-well plates (1 x 10^6 cells/well) and stabilised for 16 h prior to the treatments. After the stabilisation, the cells were incubated for 48 h with the test compounds, TCDD (5 nM), RIF (10 µM), and/or vehicle (DMSO; 0.1%, v/v). For assessment of the expression level of CYP proteins, Simple Western blotting was used according to the ProteinSimple manual (Sally Sue, San Jose, California). Primary antibodies were diluted differently according to the cell type: CYP1A1 (dilutions 1:200 for cell lines and 1:25 for human hepatocytes), CYP1A2 (dilution 1:250), CYP3A4 (dilution 1:10000), β-actin (dilutions 1:1000 for cell lines and 1:100 for human hepatocytes). Target proteins were identified using primary antibodies, a horseradish peroxidase-conjugated secondary antibody, and chemiluminescent substrate. The resulting chemiluminescent signal was detected and quantified in the Compass Software version 2.6.5.0 (ProteinSimple). For quantitative data analysis, the CYP signals were normalised to β-actin as a loading control.

4.3.5 Electrophoretic mobility shift assay (EMSA)

4.3.5.1 Binding of AhR to DNA

MCF-7 cells were seeded in 6-well plates (1 x 10^6 cells/well) and stabilised for 16 h prior to the treatments. The cells were then incubated for 2 h with the compounds under study (10 µM), TCDD (5 nM), and vehicle (DMSO; 0.1%, v/v). Nuclear fractions were isolated using the Nuclear Extract Kit (Active Motif). The following double-stranded 5′-biotinylated oligonucleotides containing a specific DNA-binding sequence for AhR corresponding to the 27-bp protein-binding site of DRE 3 were used:

sense 5′- GATCCGGCTCTTCTCACGCAACTCCGAGCTCA-3′

antisense 5′- GATCTGAGCTCGGAGTTGCGTGAGAAGAGCCG-3′

The EMSA was carried out as follows: nuclear protein (15 µg) from each sample was pre-incubated in binding buffer (final concentrations: 10 mM Tris-HCl, 50 mM KCl, 1 mM DTT; pH 7.5) along with final concentrations of 2.5% glycerol, 0.05% NP-40, double-distilled water (ddH2O), and nonspecific competitor Poly (dI.dC) before addition of the biotin-labelled probe (10 fmol/µL). An unlabelled probe (2 pmol/µL) was added to the reaction mixture for a competitive experiment. The complete binding reaction was incubated at room temperature for 20 min. Finally, 5 µL of 5 x loading buffer with bromophenol blue was added before loading onto a non-denaturing 5% polyacrylamide gel for electrophoretic separation. The protein–DNA complexes were electro-blotted to a positively charged nylon membrane. After that, transferred DNA was cross-linked using a UV-light cross-linker instrument. Biotin-labelled DNA was detected using a streptavidin-horseradish peroxidase conjugate and a chemiluminescent substrate from the Light-Shift Chemiluminescent EMSA Kit (Thermo Scientific, USA).

4.3.5.2 Binding of PXR to DNA

LS174T cells were seeded in 10-mm Petri dishes (1 x 10^7/dish) and stabilised for 16 h. Nuclear fractions were isolated using the Nuclear Extract Kit. Then, nuclear fractions were incubated for 2 h at 30 °C with the tested compounds (10 µM), RIF (10 µM), and vehicle (DMSO; 0.1%, v/v). The following double-stranded 5′-biotinylated oligonucleotides containing the DR3 motif from the XREM sequence of the CYP3A4 gene promoter were used:

sense 5′-GAATGAACTTGCTGACCCTCT-3′

antisense 5′-AGAGGGTCAGCAAGTTCATTC-3′

The EMSA was performed using the LightShift Chemiluminescent EMSA Kit as described above.

4.3.6 Statistical analyses

Data were expressed as mean ± SD. One-way ANOVA followed by Dunnett's multiple-comparison post hoc test as well as calculations of the half-maximal effective concentrations (EC_{50}) and the half maximal inhibitory concentrations (IC_{50}) values were carried out in the GraphPad Prism software (GraphPad Software Inc., San Diego, CA).

5 RESULTS AND DISCUSSION

In the present thesis, I examined the effects of optical isomers of chiral statins and dihydropyridine CCBs on the human xenoreceptor-P450 signalling pathway. *In vitro* models of primary human hepatocyte cultures and human cancer cell lines were used. Experimental approaches included gene reporter assays, qPCR, western blotting, and EMSAs.

5.1 Effects of statin diastereomers on regulatory pathways of human cytochrome P450s

I showed for the first time that atorvastatin, fluvastatin, and rosuvastatin enantiospecifically induce CYP2A6, CYP2B6, and CYP3A4 in human hepatocytes, and enantiospecifically influence transcriptional activities of PXR and GR (Korhoňová *et al.*, 2015). The statins investigated in this study are clinically used as pure enantiomers, i.e. 3R5R-atorvastatin, 3R5S-rosuvastatin, and 3R5S-fluvastatin. These statins were introduced into clinical practice directly as single-isomer drugs, but the data on induction of CYPs by their optical isomers were absent. However, several DDIs, which could be caused by induction of CYPs, were reported in patients using statins simultaneously with other drugs, e.g. anti-hypertensive losartan (Meadowcroft *et al.*, 1999), anti-diabetic repaglinide (Sekhar and Reddy, 2012), and CCBs nifedipine (Lee *et al.*, 2015). Indeed, there are numerous reports indicating activation of PXR and induction of PXR-regulated CYPs by statins (Hoffart *et al.*, 2012; Howe *et al.*, 2011; Kocarek *et al.*, 2002; Plee-Gautier *et al.*, 2012; Yamasaki *et al.*, 2009). Therefore, it was justified to carry out a comparative study of clinically used *versus* other optical isomers of statins regarding their ability to activate xenoreceptors and to induce drug-metabolizing cytochrome P450s.

I found that the analysed statins do not activate AhR and accordingly do not induce CYP1A1 and CYP1A2 in primary human hepatocytes and cancer cell lines (Figures 3-4). In contrast, all the optical isomers of all the tested statins induced formation of the PXR–DNA complex and differently induced *CYP2* and *CYP3* genes (Figures 5-8). Overall, it was demonstrated that the potential of atorvastatin, rosuvastatin, and fluvastatin to cause CYP induction-based DDIs is higher for clinically used optical isomers, than that of their respective diastereomers.

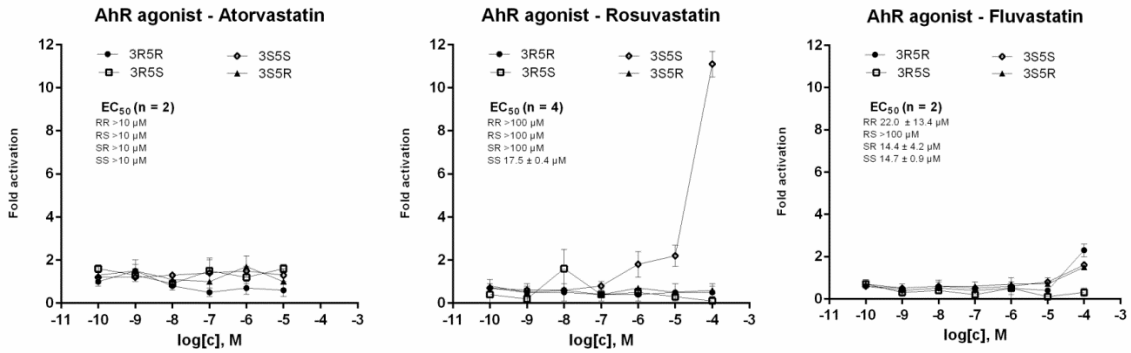

Figure 3: Effects of statin diastereomers on transcriptional activity of human AhR (agonist mode).

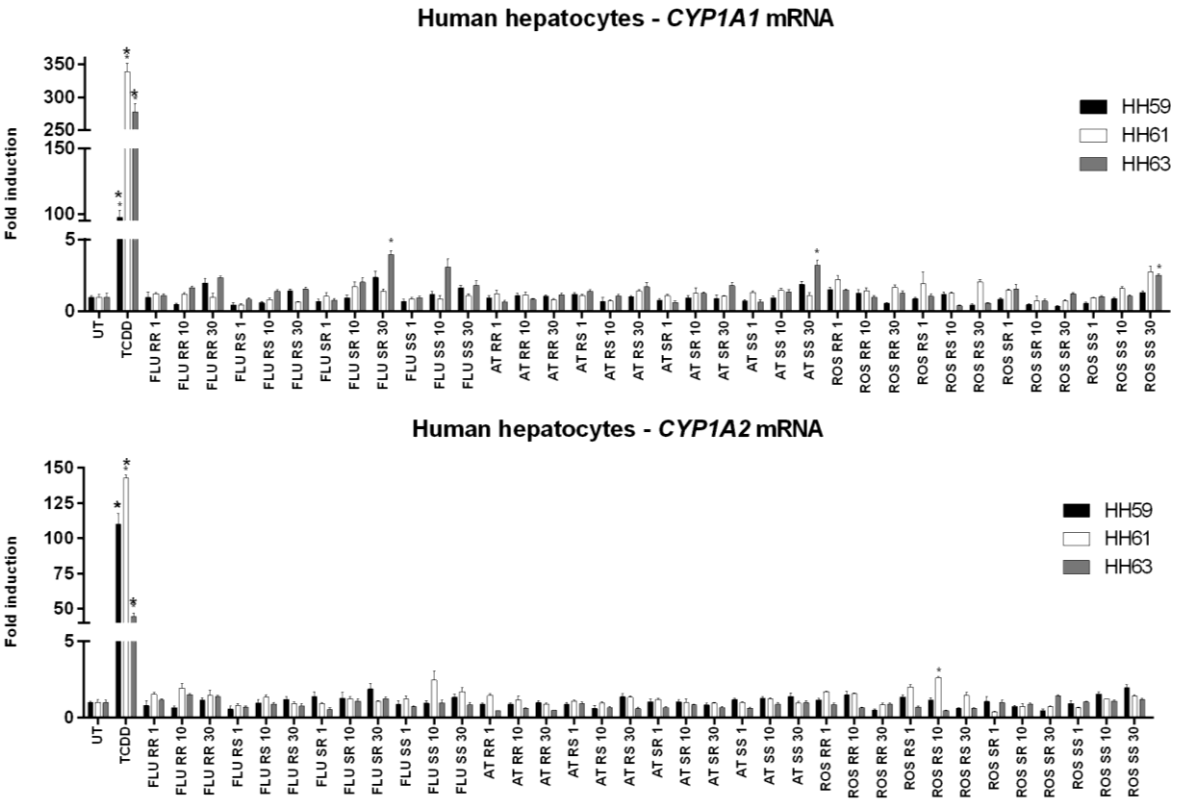

Figure 4: Effects of statin diastereomers on the expression of CYP1A1 and CYP1A2 in primary human hepatocytes. AT = atorvastatin, FLU = fluvastatin, ROS = rosuvastatin.

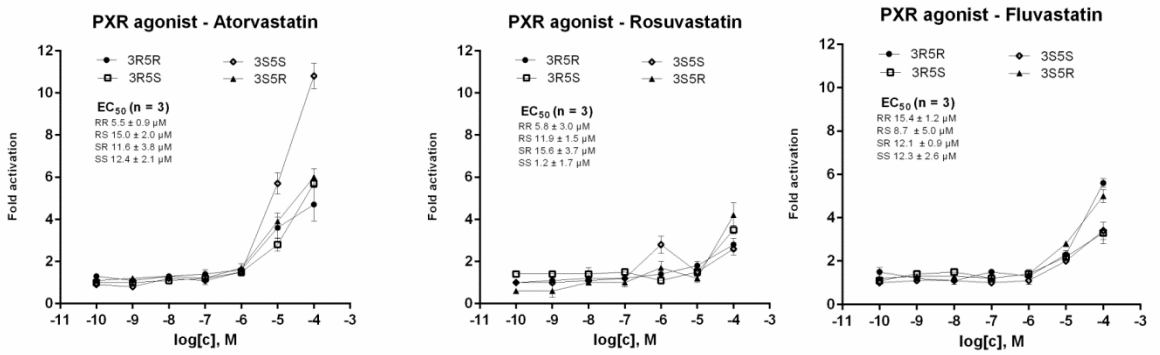

Figure 5: Effects of statin diastereomers on transcriptional activity of human PXR (agonist mode).

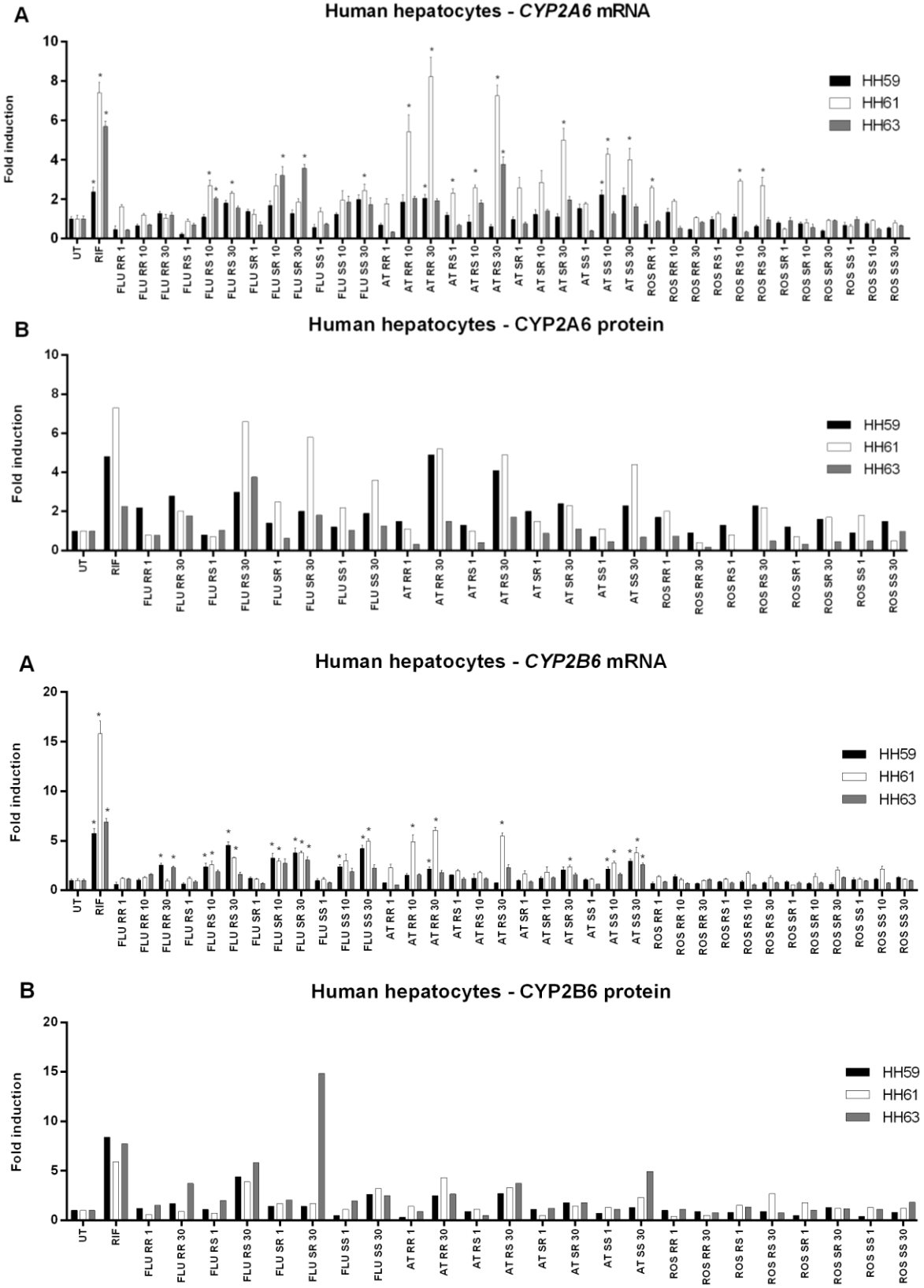

Figure 6: Effects of statin diastereomers on the expression of CYP2A6 and CYP2B6 in primary human hepatocytes. AT = atorvastatin, FLU = fluvastatin, ROS = rosuvastatin.

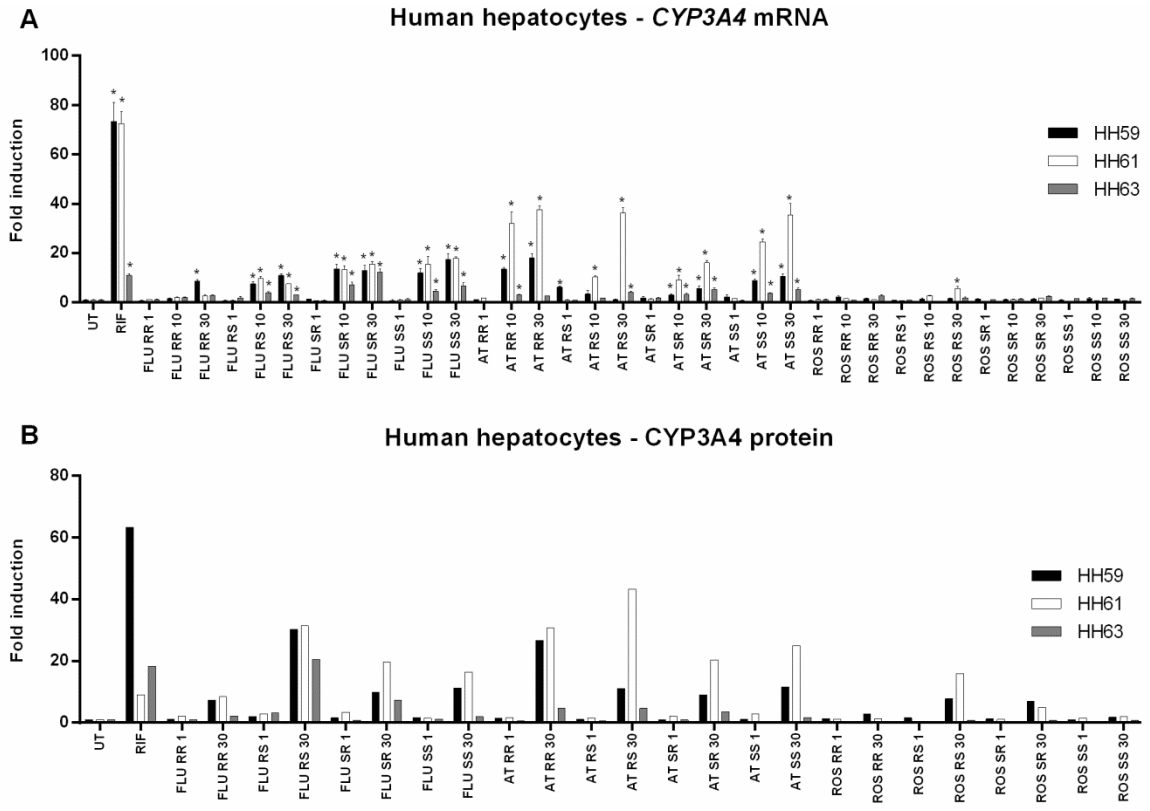

Figure 7: Effects of statin diastereomers on the expression of CYP3A4 in primary human hepatocytes. AT = atorvastatin, FLU = fluvastatin, ROS = rosuvastatin.

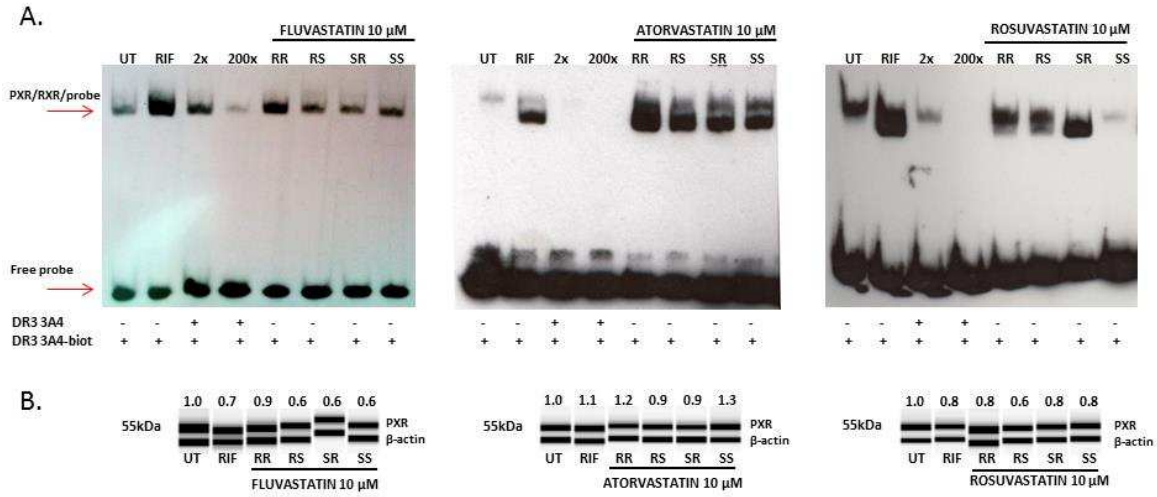

Figure 8: Effects of statin diastereomers on the binding of the PXR–RXR complex to the DR3 motif of the human CYP3A4 gene promoter.

5.2 Effects of dihydropyridine enantiomers on regulatory pathways of human cytochrome P450s

The second achievement of the present thesis is characterisation of the effects of chiral dihydropyridine-type CCBs, including benidipine, felodipine, and isradipine on the expression and enzymatic activities of human CYPs (Stepankova *et al.*, 2016). Despite over 40 different existing dihydropyridine CCBs, only four members have been tested so far for interactions with the PXR–CYP3A4 signalling pathway, including nifedipine, nicardipine, isradipine, and clevidipine (Drocourt *et al.*, 2001; Zhang *et al.*, 2006). Presumably, the activation of PXR and induction of CYP3A4 seems to be a general feature applicable to other dihydropyridine CCBs; therefore, an extensive study including all the existing derivatives is not necessary. On the contrary, dihydropyridine CCBs possess at least one chiral centre in their molecule; consequently, the research into enantiospecific effects of dihydropyridine CCBs on xenoreceptors and cytochrome P450s is worthwhile.

I found that all tested CCBs activate PXR with similar efficacy and the magnitudes of the induction were comparable with that of RIF. Furthermore, the potency of (+/-)-benidipines was found to be ~10-fold higher than that of (+/-)-isradipines and (+/-)-felodipines (Figure 9). A similar pattern was observed for induction of PXR-regulated genes including *CYP2A6*, *CYP2B6*, and *CYP3A4* in human hepatocytes by CCBs (Figures 10-11). There were no differences between the effects of (+)/(-) enantiomers. Thus, the tested dihydropyridines are equipotent activators of human PXR and inducers of PXR-regulated genes, regardless of their optical configuration.

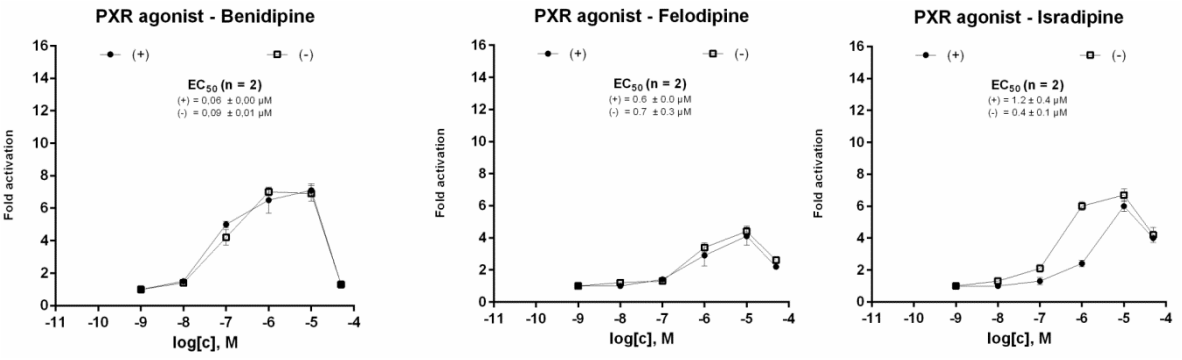

Figure 9: Effects of dihydropyridine enantiomers on transcriptional activity of human PXR (agonist mode).

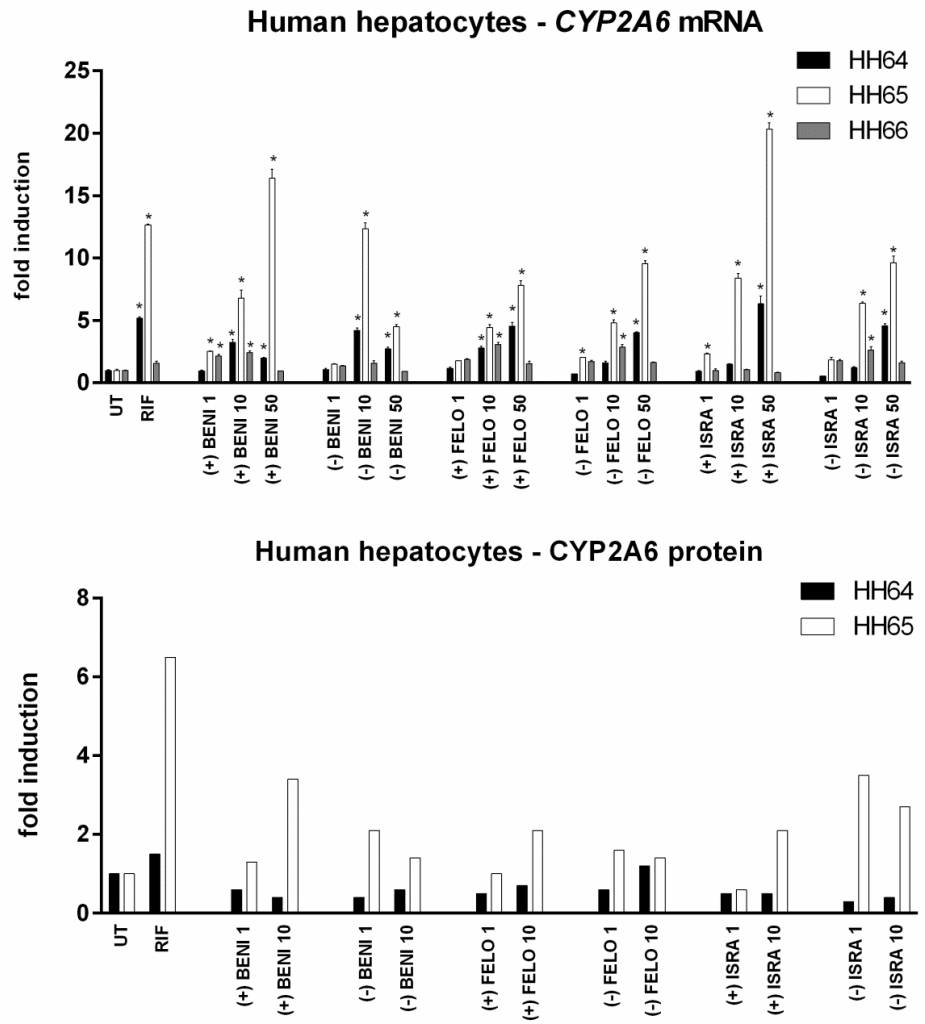

Figure 10: Effects of dihydropyridine enantiomers on the expression of CYP2A6 in primary human hepatocytes. BENI = benidipine, FELO = felodipine, ISRA = isradipine.

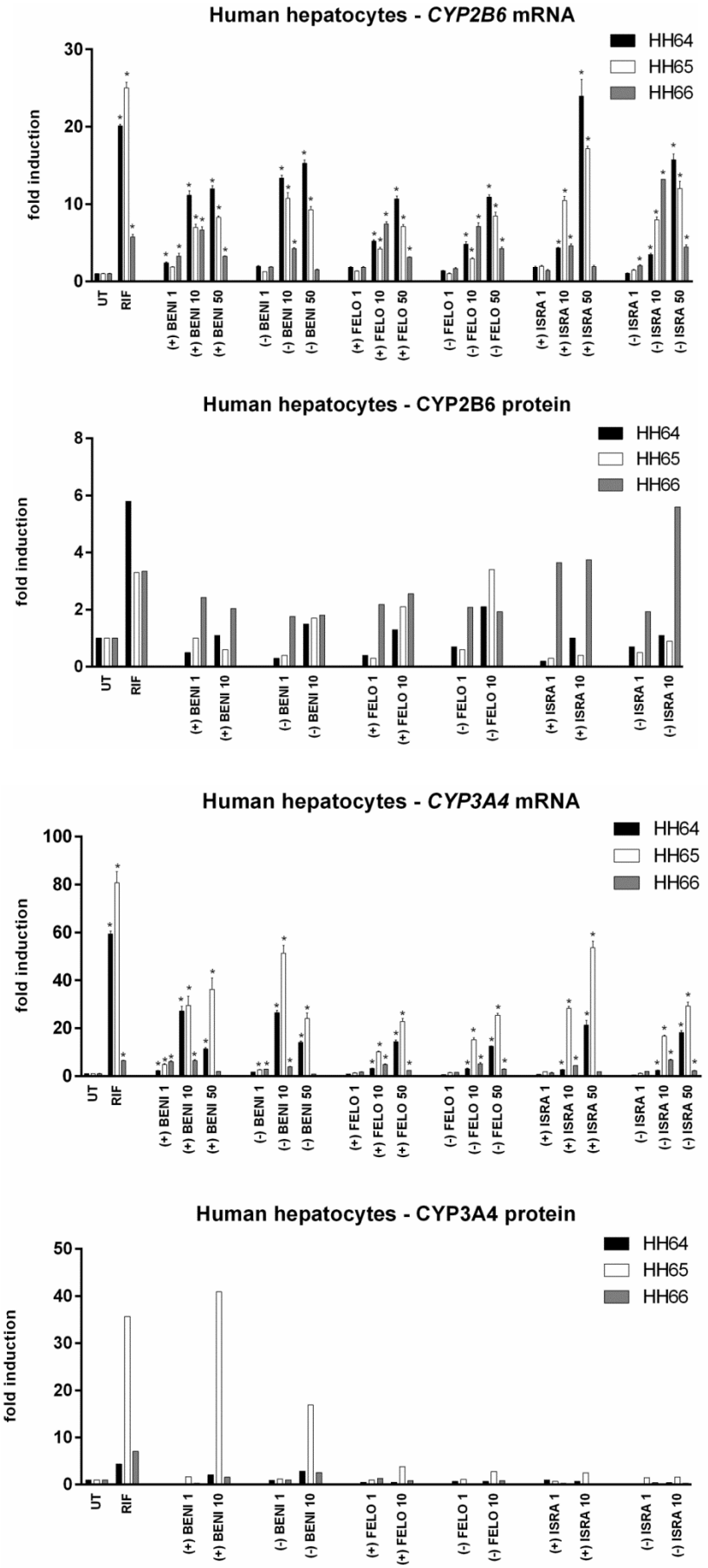

Figure 11: Effects of dihydropyridine enantiomers on the expression of CYP2B6 and CYP3A4 in primary human hepatocytes. BENI = benidipine, FELO = felodipine, ISRA = isradipine.

On the other hand, the unexpected finding was enantiospecific activation of AhR and induction of *CYP1A* genes by optical isomers of the CCBs under study. Transcriptional activity of AhR in gene reporter assays, expression of CYP1A1 in HepG2 cells, and expression of CYP1A1 and CYP1A2 in human hepatocytes were increased by CCBs as follows (descending order): (-)-benidipine > (+)-benidipine; (-)-isradipine > (+)-isradipine ; (+)-felodipine > (-)-felodipine (Figures 12-14). Because there is a common chiral centre in all three dihydropyridine CCBs, the difference between enantiospecific effects of benidipine and isradipine in contrast to felodipine is probably determined by the specific structure of each derivative. Collectively, the potential of the tested dihydropyridine CCBs to cause induction-based DDIs and other adverse effects is a result of dual activation of AhR and PXR. Activation of AhR and induction of *CYP1A* genes were found to be enantioselective, whereas activation of PXR and induction of genes *CYP2* and/or *CYP3* took place regardless of optical configuration.

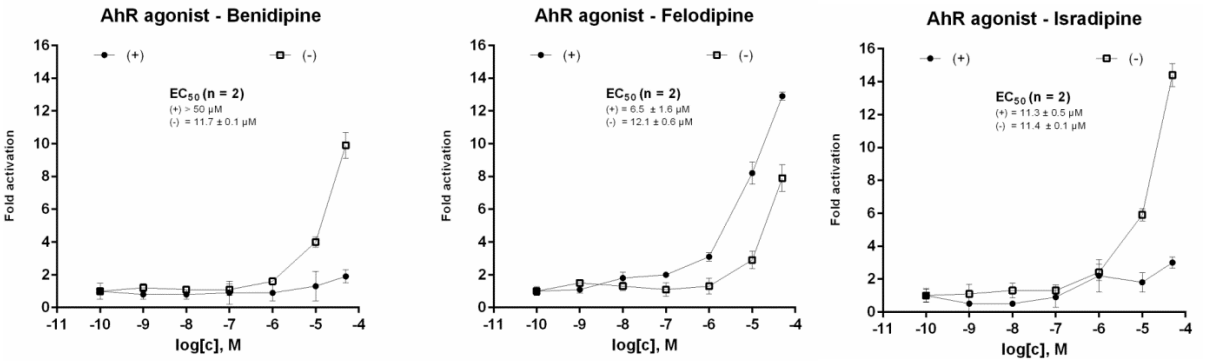

Figure 12: Effects of dihydropyridine enantiomers on transcriptional activity of human AhR (agonist mode).

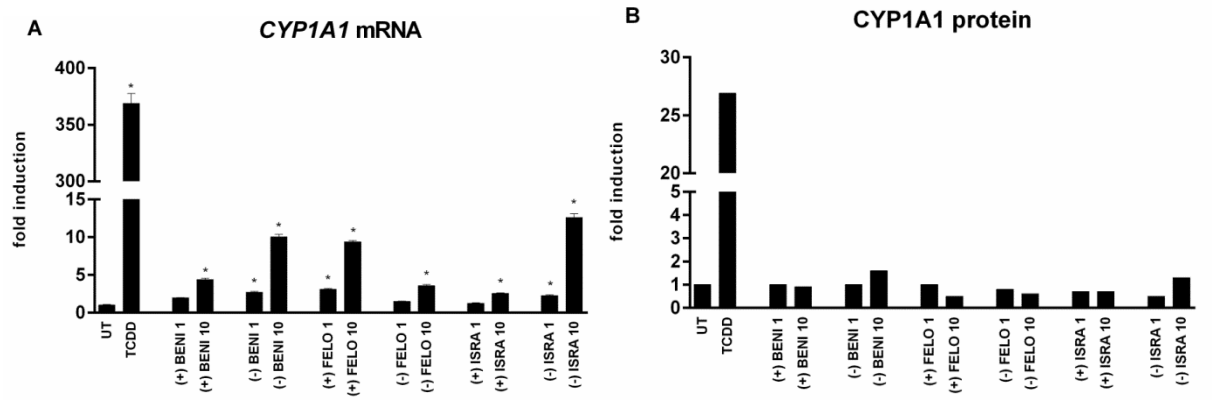

Figure 13: Effects of dihydropyridine enantiomers on CYP1A1 mRNA and protein levels in HepG2 cells. BENI = benidipine, FELO = felodipine, ISRA = isradipine.

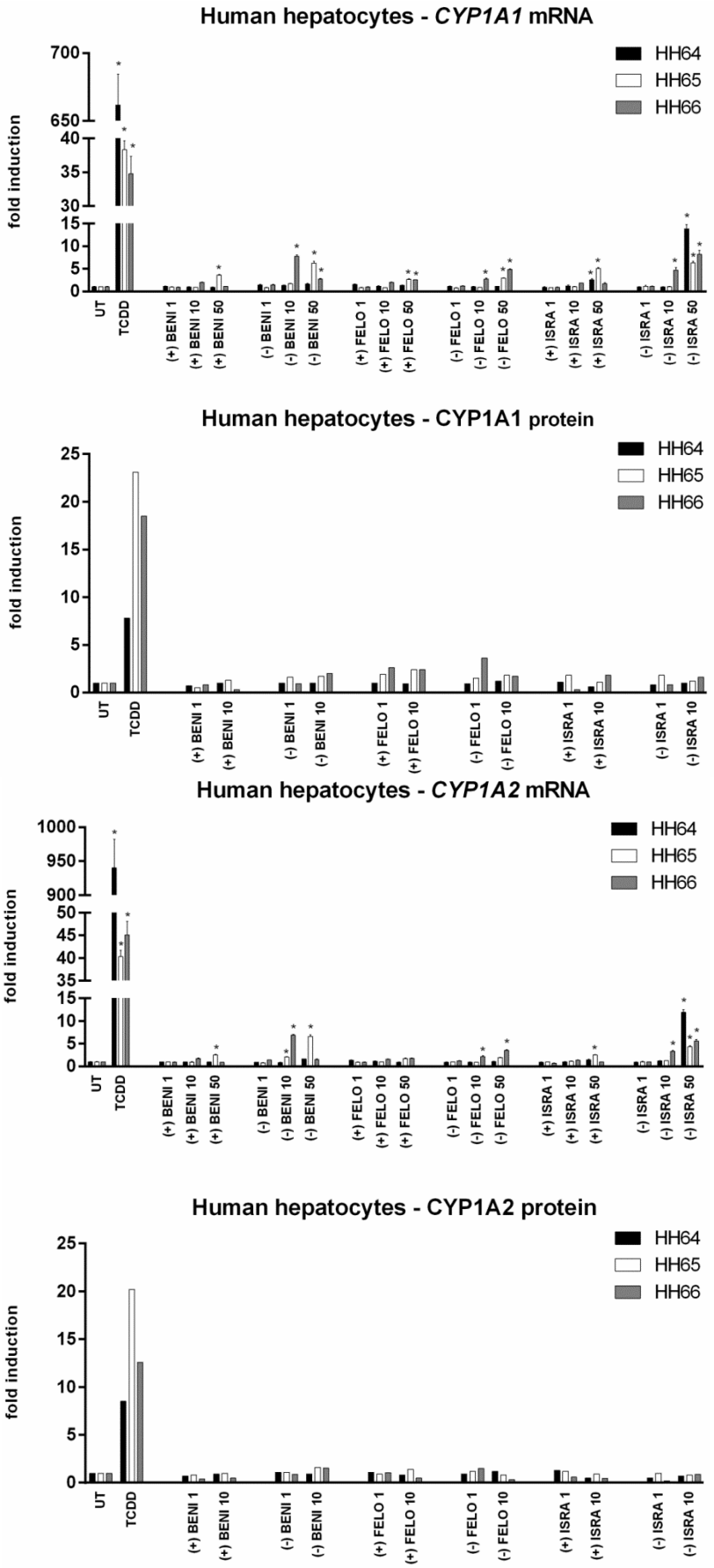

Figure 14: Effects of dihydropyridine enantiomers on the expression of CYP1A1 and CYP1A2 in primary human hepatocytes. BENI = benidipine, FELO = felodipine, ISRA = isradipine.

6 CONCLUSION

The present thesis deals with the enantiospecific effects of cholesterol-lowering drugs (statins) and antihypertensive drugs (dihydropyridine CCBs) on the transcriptional activities of human xenoreceptors and on the expression of drug-metabolizing cytochrome P450s. The major findings are

(i) atorvastatin, fluvastatin, and rosuvastatin enantiospecifically activate PXR and induce genes *CYP2A6*, *CYP2B6*, and *CYP3A4*.

(ii) benidipine, felodipine, and isradipine enantiospecifically activate AhR and induce genes *CYP1A1* and *CYP1A2*.

(iii) benidipine, felodipine, and isradipine non-enantiospecifically activate PXR and induce genes *CYP2A6*, *CYP2B6*, and *CYP3A4*.

Taking into account the broad spectrum of processes mediated by enantiospecific activation of these xenoreceptors (induction of CYPs and other XMEs, consequent DDIs, toxicity, and others), the data presented here may have clinical and toxicological implications.

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- Novotna A., Srovnalova A., Svecarova M., **Korhonova M.**, Bartonkova I., Dvorak Z. (2014): Differential effects of omeprazole and lansoprazole enantiomers on aryl hydrocarbon receptor in human hepatocytes and cell lines. *PLoS One*, 9: 1-8. [**IF_{2012} – 3.730**]. Contributions to the published work: performed data analysis, conducted experiments.

- Novotna A., **Korhonova M.**, Bartonkova I., Sashilov A.A., Denison M.S., Bogdanova K., Kolar M., Dvorak Z.: (2014) Enantiospecific effects of ketoconazole on aryl hydrocarbon receptor. *PLoS One*, 9. 101832 [**IF_{2013} – 3.534**]. Contributions to the published work: performed data analysis, conducted experiments.

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