PALACKY UNIVERSITY OLOMOUC

FACULTY OF SCIENCE

DEPARTMENT OF BIOCHEMISTRY

ROLE OF THE POST-TRANSLATIONAL MODIFICATIONS IN THE FUNCTION OF PREGNANE X RECEPTOR

Ph.D. Thesis

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Study program:	Biochemistry
Study brunch:	Biochemistry
Form of study:	Full-time study
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Submitted:	24th April 2013

OLOMOUC 2013

I declare that the Ph.D. thesis is based on my own research carried out at the Department of Cell Biology and Genetics, Faculty of Science, Palacky University, Olomouc in the period September 2009 – March 2013. Co-authors agree with the inclusion of published results. All sources quoted in this work are listed in the "Reference" section.

Olomouc, 24. 4. 2013 .....

Acknowledgement

First and foremost, I would like to thank my graduate advisor Prof. RNDr. Zdeněk Dvořák, DrSc. et Ph.D. for his advice and guidance in the completion of this dissertation. His creativity, friendship and passion for science have been an inspiration to me throughout my graduate career.

I am very grateful to Doc. Radim Vrzal, Ph.D. for his help in my research, friendship, enlightening conversations and valuable suggestions. In addition, I would like to thank all my colleagues for a pleasant working environment. I thank doc. PharmDr. Petr Pávek, Ph.D. (Laboratory of clinical and molecular pharmacotherapy, Department of Pharmacology and Toxicology, Faculty of Pharmacy in Hradec Králové, Charles University of Prague) for his co-operation in the designing of plasmids.

Finally, I would like to thank my family, specifically my husband, for their unwavering support in this pursuit.

The research presented in this dissertation was funded by grants GACR 303/07/0128, 304/10/0149, P303/12/G163, P303/12/0472 from the Grant Agency of the Czech Republic, student projects PrF 2010-002, PrF 2011-001, PrF 2012- 002 and PrF 2013-002 of the Palacky University

The data presented in this thesis is contained in the two following publications:

- **Doricakova A.**, Novotna A., Vrzal R., Pavek P., Dvorak Z. (2012) The role of residues T248, Y249 and T422 in the function of human pregnane X receptor. *Arch Toxicol* 87(2):291-301 **[IF_{2011} 4.674]**

- **Vavrova A.**, Vrzal R., Dvorak Z. (2013) A non-radioactive electrophoretic mobility shift assay for measurement of pregnane X receptor binding activity to CYP3A4 response element. *Electrophoresis (in press)* **[IF_{2011} 3,303]**

Bibliographical identification:

First name and surname	Aneta Vávrová
Title	Mgr.
Type of thesis	Ph.D.
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Supervisor	prof. RNDr. Zdeněk Dvořák, DrSc. et Ph.D.
The year of presentation	2013

Abstract

The pregnane X receptor (PXR) is a broad spectrum entero-hepatic xenobiotic “sensor” and master-regulator of drug inducible gene expression. In addition, PXR controls the expression of numerous genes involved in the metabolism of endobiotics and plays an important role in the development of specific forms of cancer. Importantly, the xenosensor PXR is often responsible for clinically important drug-drug interactions as well as for undesirable side effects underlying important physiological functions of this nuclear receptor. All PXR functions have been attributed to ligand activation. However, the activity of PXR is also modulated in a ligand-independent manner implying cross-talk between other cell signaling pathways and PXR. The transcriptional activity of PXR is modulated through post-translational modification of this receptor. This thesis is focused on PXR phosphorylation, and in particular on the study of specific target residues. The theoretical background concentrates on xenobiotic and endobiotic functions of this nuclear receptor, its target genes and its post-translational modifications. In the methodological section, methods of studying protein-protein interaction and binding to DNA were developed and optimized. Then, the putative phosphorylation sites T248, Y249 and T422 were determined by *in silico* consensus kinase site prediction analysis. The roles of these residues in the function of PXR were examined with a particular focus on PXR transcriptional activity, promoter- binding activity, and interaction with the common heterodimerization partner retinoid X receptor alpha (RXRα). Our finding would shows an important mechanistic link between the xenobiotic metabolism gene expression controlled by PXR and cellular signaling pathways.

Keywords	Drug metabolism, nuclear receptors, pregnane X receptor, cytochrome P450, phosphorylation
Number of pages	77
Number of appendices	2
Language	English

Bibliografická identifikace:

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Rok odevzdání	2013

Abstrakt

Pregnanový X receptor působí jako tzv. “senzor” širokého spektra endogenních metabolitů a exogenních chemických látek a na základě interakce s těmito látkami reguluje genovou expresi enzymů metabolizující léčiva. Kromě genů pro enzymy I. a II. fáze biotransformace a transportérů léčiv řídí PXR také transkripci důležitých genů podílejících se na metabolismu endogenních látek a na rozvoji specifických forem karcinomu. Kromě toho, aktivace tohoto nukleárního receptoru může být příčinou farmakokinetických lékových interakcí a nežádoucích vedlejších účinků. V předcházejících studiích byly veškeré vlivy PXR připisovány jeho aktivaci přímou vazbou ligandu. Kromě aktivace xenobiotiky mohou tuto transaktivační kaskádu spustit i další mechanismy nezávislé na přítomnosti ligandu. Bylo prokázáno, že transkripční aktivita PXR je modulována pomocí post-translačních modifikací tohoto receptoru. Tato práce je zaměřena na fosforylaci PXR, zejména na studium specifických aminokyselin, které mohou být modifikovány. Teoretická část této práce se zabývá xenobiotickou a endobiotickou funkcí, cílovými geny a možnými post-translačními modifikacemi tohoto receptoru. V rámci praktické části byly zavedeny a optimalizovány dvě metody umožňující studovat vzájemné interakce mezi proteiny a vazbu proteinu do specifického místa v DNA. Pomocí bioinformatické analýzy potencionálních fosforylačních míst byly navrženy mutace PXR, konkrétně v místech T248, Y249 a T422. Byly studovány změny v transkripční aktivitě, vazba do DNA a interakce s retinoidním x receptorem alfa (RXR), který vytváří s PXR heterodimer. Nové poznatky uvedené v této práci mohou přispět k objasnění souvislostí mezi regulací exprese genů metabolizujících léčiva prostřednictvím PXR a dalších buněčných signálních drah.

Klíčová slova	Metabolismus léčiv, nukleární receptory, pregnanový X receptor, cytochrome P450, fosforylace
Počet stran	77
Počet příloh	2
Jazyk	Angličtina

CONTENT

ABBREVIATIONS

αAF	Activation function alpha helix
A, ala	Alanine
ABC	ATP-binding cassette transporter family
AF-1	Activation function-1
AF-2	Activation function-2
AhR	Aryl hydrocarbon receptor
AMPK	AMP-activated protein kinase
AR	Androgen receptor
BCRP	Breast cancer resistance protein
CAR	Constitutive androstane receptor
CBP/p300	cAMP response element binding protein
CCRP	cytoplasmic CAR retention protein
CDK	Cyclin-dependent kinase
CLEM4	Constitutive liver enhancer module of CYP3A4
COS	Fibroblast-like cell line derived from monkey kidney tissue
CPT1A	Carnitine palmitoyltransferase 1A
CREB	cAMP response element-binding protein
CYP P450	Cytochrome P450
CYP3A4	Cytochrome P450, family 3, subfamily A, polypeptide 4
D, asp	Aspartic acid
DBD	DNA-binding domain
DDIs	Drug-drug interactions
DMEs	Drug-metabolising enzymes
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNR1	Distal nuclear receptor-binding element 1
DR-3	Direct repeat separated by three nucleotides
DR-4	Direct repeat separated by four nucleotides
DR-5	Direct repeat separated by five nucleotides
E, glu	Glutamic acid
E1	Ubiquitin-activating enzymes
E2	Ubiquitin-conjugating enzymes
E3	Ubiquitin ligases
EC50	Half maximal effective concentration
eNR3A4	PXR response elements DR4 in the XREM module of the CYP3A4 gene

ER-6	Everted repeat separated by six nucleotides
ER-8	Everted repeat separated by eight nucleotides
F, phe	Phenylalanine
FOXO1	Forkhead box O1
FOXA2	Forkhead box protein A2
FXR	Farnesoid X receptor
G, gly	Glycine
G6Pase	Glucose-6-phosphatase
GR	Glucocorticoid receptor
GRIP-1	Glutamate receptor interacting protein 1
GST	Glutathione S-transferase
HDAC	Histone deacetylase
HeLa	Human Negroid cervix epithelial carcinoma cell
HepG2	Human hepatoma cells
HIV	Human immunodeficiency virus
HMGCS2	3-hydroxy-3-methylglutarate-CoA synthase 2
HNF-4α	Hepatocyte nuclear factor 4 alpha
Hsp90	Heat-shock protein 90 kDa
IL-1	Interleukin 1 family
JNK	c-Jun N-terminal kinases
K, lys	Lysine
LBD	Ligand-binding domain
MDR1	Multidrug resistance 1
MRP	Multidrug resistance-associated proteins
NCoR	Nuclear receptor co-repressor
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLS	Nuclear localization signal
NR	Nuclear receptor
LXR	Liver X receptor
OATP	Organic anion-transporting polypeptide transporter
P, pro	Proline
P70 S6K	p70-ribosomal S6 kinase
PBP	Peroxisome proliferator-activated receptor (PPAR)-binding protein
PCN	Pregnenolone 16a-carbonitrile
PEPCK	phosphoenolpyruvate carboxykinase
PGC-1α	Peroxisome proliferator-activated receptor-gamma co-activator 1 alpha
PKA	Cyclic-AMP-dependent Protein Kinase

PKB	Protein Kinase B
PKC	Protein Kinase C
PPAR	Peroxisome proliferator-activated receptor
PR	Progesterone receptor
prER6	proximal ER6 response elements of the CYP3A4 gene
PTMs	Post-translational modifications
PXR	Pregnane X receptor
PXR-RE	PXR response element
Q, gln	Glutamine
RAR	Retinoic Acid Receptor
RBCK1	protein kinase C-interacting protein with a new type of RBCC (RING-B-Box-Coiled-coil) region
RIF	Rifampicin
RIP 140	Receptor-interacting protein 140
RXRα	Retinoid X receptor alpha
S, ser	Serine
SCD1	Stearoyl-CoA desaturase 1
SHP	Small heterodimer partner
SIRT1	Silent mating type information regulation 2, homolog 1
SMRT	Silencing mediator of retinoic acid and thyroid hormone receptor
SPRM	Selective PXR modulator
SR12813	Tetraethyl [2-(3,5-di-tert-butyl-4-hydroxyphenyl)ethene-1,1-diyl]bis(phosphonate)
SRC-1	Steroid receptor co-activator-1
SUG-1	Suppressor for Gal-1
SULT	Sulfotransferase
SUMO	Small Ubiquitin-like Modifier proteins
SXR	Steroid and xenobiotic receptor
T, thr	Threonine
TFIIH	Transcription factor IIH
TNF-α	Tumor necrosis factor alpha
TR	Thyroid receptor
UGTs	Uridin-5´-diphosphate-glucuronosyltransferases
V, val	Valine
VDR	Vitamin D receptor
XREM	Xenobiotic Responsive Enhancer Module
Y, tyr	Tyrosine

1 INTRODUCTION

The pregnane X receptor (PXR; NR1I2) is a ligand-activated orphan nuclear receptor that belongs to the family of steroid/thyroid/retinoid superfamily of receptors (Kliewer et al., 1998; Lehmann et al., 1998). Originally an orphan receptor was identified based on sequence homology to other nuclear receptors (NRs) and the receptor was named PXR based on its activation by pregnane (21-carbon) steroids (Kliewer et al., 1998). Subsequently it was shown to respond to a variety of eubiotics and xenobiotics including clinical drugs, with typical EC50's in the high nanomolar or low micromolar range (Bertilsson et al., 1998; Blumberg & Evans, 1998; Kliewer et al., 1998; Lehmann et al., 1998). PXR is predominantly expressed in the liver, but it has been detected in the small intestine, colon, kidney, brain, and mammary tissues (Bertilsson et al., 1998; Blumberg & Evans, 1998; Elias et al., 2013; Kliewer et al., 1998; Masuyama et al., 2003). PXR has since been firmly adopted as a xenobiotic sensor that regulates the expression of numerous genes involved drug-metabolizing enzymes, including CYP2B6, CYP2C8, CYP2C9, CYP3A4 (Pascussi et al., 2008), some phase II enzymes (Rushmore & Kong, 2002) and transporters (Klaassen & Slitt, 2005). PXR is involved in clinically important drug-drug interactions mediated through the cytochrome P450 (CYP) pathway. Therefore understanding how PXR modulates CYP expression is critical to predicting and preventing therapy-induced adverse drug interactions. In addition, PXR controls the expression of numerous genes involved in the metabolism of steroid hormones, vitamin D, lipids, carbohydrates or cholesterol (Moreau et al., 2008; Wada et al., 2009) and it plays an important role in the development of specific forms of cancer (Pondugula & Mani, 2013). In its active state, PXR forms a heterodimer with the retinoid X receptor alpha (RXRα, NR2B1) (Pascussi et al., 2008). Transcriptional activity of PXR is primarily dependent on the presence of a ligand that binds PXR in a C-terminal ligand-binding domain (LBD) flanked with a ligand-dependent activation function 2 (AF-2). However, the activity of PXR is also modulated in a ligand-independent manner implying cross-talk between other cell signaling pathways and PXR.

The transcriptional activity of PXR may be modulated through post-translational modification of this receptor, in particular by phosphorylation, acetylation, sumoylation and ubiquitination of amino acids residues. It has been known that drug-inducible CYP gene expression includes kinase signaling pathways; however, the exact mechanism by which these pathways intersect with PXR signaling is not entirely understood yet. The activation of cyclic AMP-dependent protein kinase (PKA) signaling has been shown to modulate PXR activity and kinase assays showed that PXR is subject to

phosphorylation by PKA *in vitro*. It has also been shown that PXR exists as a phospho-protein *in vivo* and that its phosphorylation status is modulated by PKA (Ding & Staudinger, 2005a; Dowless et al., 2005; Lichti-Kaiser et al., 2009b). The modulation of PXR activity by protein kinase C (PKC), cyclin-dependent kinase 2 (CDK2) and p70-ribosomal S6 kinase (p70 S6K) was demonstrated. The fact that PXR transcriptional activity is substantially regulated by various protein kinase signaling pathways was provided an impetus for the systematic investigation of the roles of potential phosphorylation sites within the PXR protein in the modulation of PXR activity by protein kinases (Lichti-Kaiser et al., 2009a; Lin et al., 2008; Pondugula et al., 2009a).

In the present thesis, putative phosphorylation sites threonine 248 (T248), tyrosine 249 (Y249) and threonine 422 (T422) were determined by *in silico* consensus kinase site prediction analysis, and the roles of these residues in the function of PXR were examined. Importantly, the hydrogen-bonding interaction of T248 with T422 in helix-12 is critical for a ligand dependent recruitment of steroid receptor co-activator-1 (SRC-1) and for PXR activation with its ligands in target gene transactivation (Ueda et al., 2005). Site-directed mutagenesis analysis was performed to generate phospho-deficient and phospho-mimetic mutants.

Basal and ligand-inducible transactivation activity of the PXR mutants in cell based gene reporter assays was examined. Mutation of the T248 residue to valine (T248V) diminished basal and rifampicin-mediated transactivation and phospho-mimetic mutation of PXR at T248 (T248D) resulted in the constitutive activation of the CYP3A4 promoter. Mutations at T422 (T422A, T422D) almost abolished the transactivation of the CYP3A4 promoter suggesting an importance of T248 and T422 residues in PXR transcriptional activity. Following this, we studied the effect of mutations on the expression of CYP3A4 mRNA and whether mutations of PXR affect the formation of PXR-RXRα heterodimer, which is a crucial step in the transcriptional function of PXR. Finally direct binding of PXR mutants to the direct repeat response element (DR3) was investigated. We found that phospho-mimetic mutation at T422 and both mutations at T248 decrease the ability of PXR to bind to its response elements DR3 within the promoter sequences of its target CYP3A4 gene.

Overall, we examined the roles of T248, Y249 and T422 mutations in PXR transcriptional activity, in its promoter- binding activity, and its interaction with the common heterodimerization partner RXRα. Our finding provides an important mechanistic link between xenobiotic metabolism gene expression controlled by PXR and cellular signaling pathways.

2 AIMS

The main goal of the present thesis was to study the roles of selected threonine and tyrosine residues in human PXR protein in the functions of this receptor. The current understanding of PXR is reviewed in the theoretical section of this thesis.

a) The design and construction of full length expression vectors for PXR, containing phospho-mimetic and phospho-deficient mutations at selected threonine and tyrosine residues

b) Studying the transcriptional activity of PXR employing transient gene reporter assays and measuring the expression of target genes by the means of qRT-PCR and western blotting

c) Studying the protein-protein interactions between PXR and transcriptional co-activators SRC-1 and RXRα by means of protein co-immunoprecipitation (the technique to be developed and optimized)

d) Studying the interactions between PXR and specific response elements in DNA by means of an electrophoretic mobility shift assay – EMSA (the technique to be developed and optimized)

3 THEORETICAL BACKGROUND

3.1 THE REGULATION OF PXR

3.1.1 PXR structure

Nuclear receptors are one of the largest groups of transcription factors comprising 48 members in the human genome that regulate diverse biological processes including metabolism, homeostasis, development and reproduction (Willson & Kliewer, 2002). The activity of many NRs is controlled by the binding of small lipophilic molecules such as hormones, fatty acids, bile acids and oxysterols, and xenobiotics. The pregnane X Receptor belongs to the group of ligand-inducible transcription factors, which share considerable amino acid sequence similarity in two highly conserved domains. These include a highly variable N-terminal domain and a central DNA binding domain (DBD) connected by a presumably flexible hinge region to a C-terminal ligand-binding domain (LBD) (Giguere, 1999; Mangelsdorf & Evans, 1995) (Figure 1). The highly conserved DBD (amino acids 41-107) that allows target gene recognition consists of two zinc-binding motifs that each coordinates a zinc ion through four precisely positioned cysteines. The pregnane X receptor binds as heterodimer with a common partner retinoid X receptor alpha (RXRα) to the response elements composed of two copies of the consensus NR binding motif AG(G/T)TCA (Bertilsson et al., 1998; Blumberg & Evans, 1998; Lehmann et al., 1998; Mangelsdorf & Evans, 1995). The LBD (amino acids 141- 434) displays the signature fold of the NR superfamily, comprising a three-layer sandwich of α-helices and a short region of β-strands which create a hydrophobic pocket that can bind a hydrophobic ligand (Watkins et al., 2001) (Figure 2). In addition to its ligand binding properties, the LBD also contains dimerization and transcriptional activation motifs, including the well characterized ligand dependent activation function-2 (AF-2) helix in the extreme C-terminal portion of the LBD. Upon the ligand binding, the LBD of PXR heterodimerizes with the LBD of RXRα employing an extensive set of polar and nonpolar interactions, similar to those seen in structures of other NR LBDs with the RXRα LBD (Gampe et al., 2000). When an activating ligand (agonist) is present, the AF-2 surface of the PXR LBD is stabilized in a conformation that allows the binding of a leucine-rich motif (e.g., NR boxes) present in transcriptional co-activators, such as the steroid receptor co-activator proteins (e.g., SRC-1, -2, and -3), leading to changes in transcription of target genes (Nolte et al., 1998; Renaud et al., 1995; Weatherman et al., 1999; Xu et al., 2002). The N-terminal domain of NRs is highly variable both in terms of length and amino acid sequence. A second

transcriptional activation function, termed the AF-1, has been characterized in the N-terminal domains of a number of the NRs, but in the case of PXR, the AF-1 domain activity is absent (Giguere, 1999; Kliewer et al., 2002; Mangelsdorf & Evans, 1995).

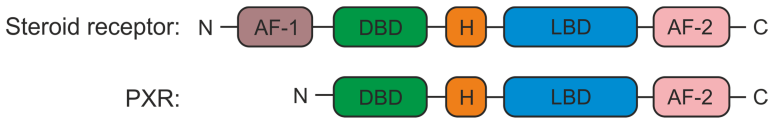

Figure 1. A schematic comparison of the domain structures of a steroid receptor and PXR.

It has so far not been possible to determine the tertiary structure of intact receptors, because of extremely difficult expression and purification of full-length NRs. Thus, conformational studies of NRs have so far involved smaller parts of the receptors. Nevertheless, these studies have obtained very valuable structural information. The structural organization of pregnane X receptor was subject of many studies where the structures of the ligand-binding domain of hPXR have been determined both for the ligand-free protein and for PXR-ligand complexes (Chrencik et al., 2005; Watkins et al., 2003a; Watkins et al., 2003b; Watkins et al., 2001; Xue et al., 2007a; Xue et al., 2007b). The PXR LBD contains the three-layer helical sandwich first described for RXRα, but modifies the LBD scaffold to include an extended five-stranded antiparallel β-sheet, instead of the two- to three-stranded β-sheet commonly observed (Figure 2) (Goodwin et al., 2002; Wada et al., 2009; Watkins et al., 2003a; Watkins et al., 2003b; Watkins et al., 2002; Watkins et al., 2001).

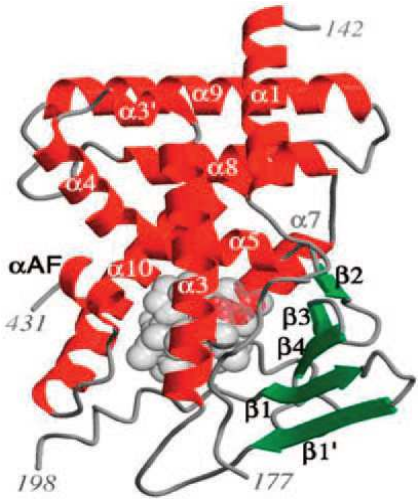

Figure 2. PXR-LBD scaffold (Mani et al., 2013).

The overall structure of the receptor could help in understanding, how PXR recognizes an array of different endogenous and exogenous compounds, how PXR modulates CYP expression and the processes by which harmful compounds are cleared from the body. New findings may help also improve our capacity to predict and avoid adverse drug-drug interactions (Watkins et al., 2002; Watkins et al., 2001).

3.1.2 PXR agonists and antagonists

Virtual screening and crystallographic studies revealed that the PXR ligand-binding domain is largely hydrophobic and extremely flexible. The binding pocket of steroid receptors are typically 369–697 Å3, in contrast, the pocket of PXR varies from 250 to 1,150 Å3 (Benoit et al., 2004; Watkins et al., 2003b; Watkins et al., 2002; Watkins et al., 2001). Unlike other nuclear receptors that interact selectively with their physiological ligands, this structural flexibility appears to be an essential aspect of the receptor's success. It has the ability to conform to a broad panel of chemically-distinct ligands with high to extremely low affinity, ranging in mass from 232 Da (phenobarbital) to >800 Da (taxol, rifampicin) (Bertilsson et al., 1998; Jones et al., 2000; Kliewer et al., 1998; Lehmann et al., 1998; Synold et al., 2001). The structures of the apo and ligand-bound forms of PXR are very similar, in contrast to more proteins that generally adapt their shape to different ligands (Watkins et al., 2001; Xiao et al.; Xue et al., 2007a; Xue et al., 2007b).

Ligand-dependent PXR activation is species specific, as evidenced by the differences in the pharmacological profiles of PXR across species (Ekins et al., 2008b; Jones et al., 2000; Reschly et al., 2007; Watkins et al., 2001). *In vitro* data between vertebrate species revealed that antibiotic rifampicin is a potent PXR activator in human and rabbits, whereas in rats it is a poor PXR activator. Conversely, synthetic antiglucocorticoid pregnenolone-16α-carbonitrile (PCN) can activate mouse and rat PXR but it has no effect on human PXR (Blumberg & Evans, 1998; Jones et al., 2000; Lehmann et al., 1998; Savas et al., 2000). The LBDs of the PXRs vary considerably in sequence between related mammalian isoforms, e.g., by as much as 76% between human and rat (Jones et al., 2000), which is uncommon in the NR superfamily. It was found that a small number of amino acid changes are responsible for this “directed promiscuity” observed between the PXRs of mammalian species (Ngan et al., 2009; Tirona et al., 2004).

PXR agonists

PXR is activated by a vast array of natural and synthetic compounds, including antibiotics such as rifampicin, rifaximine or clotrimazole (Bertilsson et al., 1998; Jones et al., 2000; Ma et al., 2007), calcium channel modulators of dihydroxypyrimidine family (Drocourt et al., 2001), steroids including some glucocorticoids (Lehmann et al., 1998), dietary supplements, vitamins K2 and E (Landes et al., 2003; Tabb et al., 2003) and bile acids and their precursors (Goodwin et al., 2003; Staudinger et al., 2001a; Xie et al., 2001). Several groups also reported that a number of environmental pollutants are PXR ligands, such as organochlorine pesticides and polybrominated diphenyl ether flame retardants (Coumoul et al., 2002; Pacyniak et al., 2007; Zhang et al., 2008). Finally, PXR can be activated by a variety of commonly used herbal medicines including the herbal antidepressant St. John's wort, gugulipid, and kava (Moore et al., 2002; Staudinger et al., 2006).

Due to the ability of PXR to bind a broad spectrum of xenobiotics, PXR is considered as a xenobiotic sensor that regulates their detoxification and elimination. On the other hand PXR increases the transcription of CYP3A4 as well as other drug-metabolising enzymes (DMEs) and transporters after the exposure to its agonists (Chen et al., 2007; Kawana et al., 2003). This may result in accelerated metabolism of endogenous compounds, such as hormones and to severe drug side effects (Kliewer et al., 2002; Staudinger et al., 2001a).

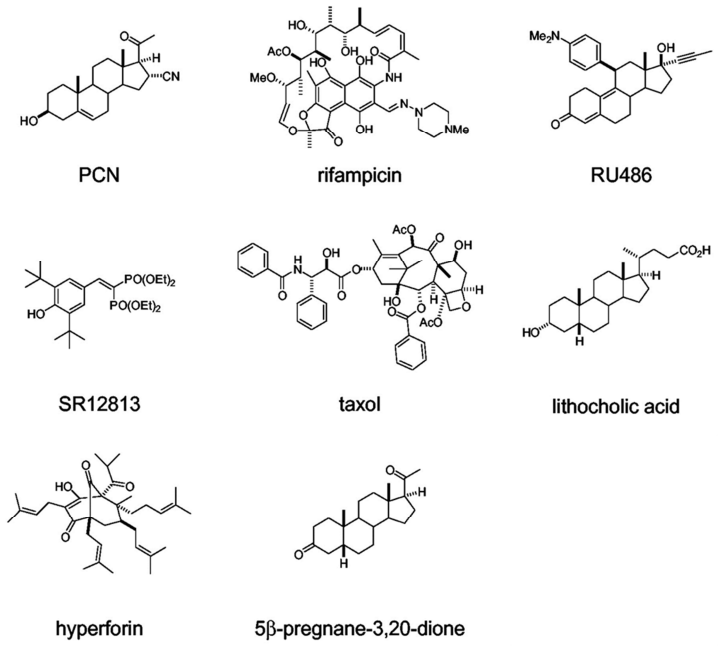

Figure 3. PXR agonists. The chemical structures of xenobiotics and endogenous chemicals that regulate PXR activity.

PXR antagonists

Although many PXR agonists have been reported, comparatively few PXR compounds antagonizing the PXR-CYP3A4 pathway have been identified. The first discussion of a PXR antagonist ET-743 (Synold et al., 2001) was subsequently followed by reports that included compounds such as polychlorinated biphenyls, camptothecin, ketoconazole, sulforaphane, fluconazole, enilconazole, A792611 (HIV protease inhibitor), metformin, sesamin, coumestrol (Duret et al., 2006; Ekins et al., 2007; Ekins et al., 2008a; Huang et al., 2007; Chen et al., 2010; Krausova et al., 2011; Lim et al., 2012; Liu et al., 2012; Mani et al., 2005; Takeshita et al., 2002; Zhou et al., 2007). The development of a suitable antagonist targeting ligand-activated PXR could well avoid adverse PXR-dependent drug interactions. Recent studies have been successful in developing nontoxic azole analogs that may be used to antagonize PXR activation *in vivo* and identifying one important site on αAF that could serve as an allosteric site that modulates PXR activation (Mani et al., 2013).

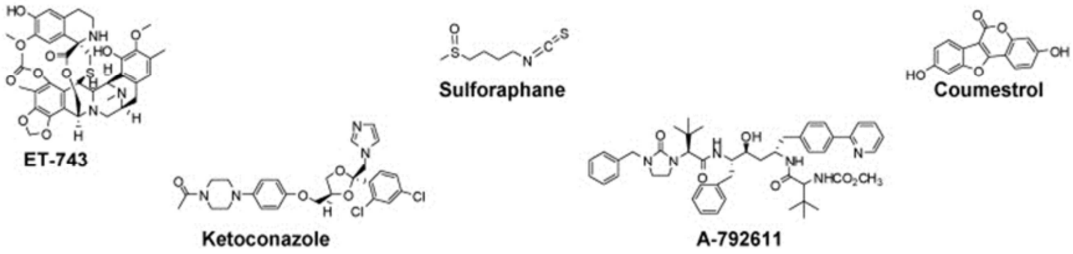

Figure 4. Chemical structures of PXR antagonists.

3.1.3 Intracellular localization

Most nuclear receptors are constitutively localized in the nucleus. However, the major proportion of steroid receptors and a few other receptors may be located in the cytoplasm in the absence of a ligand. In the cytoplasm, NRs are bound to heat shock proteins and this association prevents receptor transport through the nuclear pores and thus sequesters NRs from binding to DNA. In the nucleus, ligand-mediated activation of NRs causes redistribution of the receptor to chromatin. Several studies have suggested that nuclear localization of some NRs is a cell signaling- and phosphorylation-dependent event (Rochette-Egly, 2003; Staudinger & Lichti, 2008). For example, this was demonstrated using a human glucocorticoid receptor (GR), which is phosphorylated by c-Jun N-terminal kinases (JNK) at Ser-226 and enhances its nuclear export after dexamethasone withdrawal (Itoh et al., 2002). Moreover, phosphorylation of hepatocyte nuclear factor 4 alpha (HNF-4α) is required for DNA binding and appropriate nuclear localization (Ktistaki et al., 1995; Viollet et al., 1997). Ligand-dependent nuclear imports of aryl hydrocarbon receptor (AhR) are negatively regulated by phosphorylation for Ser-12 or Ser-36 at the two PKC sites adjacent to the bipartite NLS of AhR (Ikuta et al., 2004). On the other hand, dephosphorylation of Ser-202 is a required step that regulates the xenobiotic-dependent nuclear translocation of mouse constitutive androstane receptor (CAR) (Hosseinpour et al., 2006). Taken together, these data indicate that the phosphorylation status of NRs is intimately involved in cytoplasmic retention and nuclear translocation.

The induction of PXR target genes is mediated through the direct interactions between the receptor and response elements, located in the promoter regions of these genes. The clarification of the molecular mechanisms of PXR translocation is important for better understanding the PXR-dependent regulation of these genes. Studies on the cellular localization of PXR provided conflicting findings. PXR was originally believed to be localized in the nucleus even in the absence of ligand, but later studies have revealed that it is also present in the cytoplasm.

It is generally thought that in the absence of the ligand, the subcellular localization of PXR is affected by the cellular context. Based on immunostaining data, it was reported that human PXR is exclusively localized in the nucleus regardless of the presence or absence of rifampicin (Koyano et al., 2004). PXR ectopically expressed in cultured cells such as HepG2, COS-1, COS-7 and HeLa, was localized in the nucleus in the absence of the ligand (Echchgadda et al., 2004; Johnson et al., 2006; Kawana et al., 2003; Li et al., 2009; Pondugula et al., 2009a; Saradhi et al., 2005). However, endogenous PXR in human prostate cancer cells resides exclusively in the cytoplasm and treatment with

PXR agonist SR12813 leads to the nuclear translocation (Chen et al., 2007). PXR has been shown to reside in the nuclei of some human endometrial and prostate cancer tissues (Chen et al., 2007; Masuyama et al., 2003). Similarly, PXR was reported to be localized in the nuclei of mouse liver cells irrespective of the presence of a ligand (Saradhi et al., 2005). Kawana et al. showed that PXR was retained in the cytoplasm of hepatic cells of untreated mice and was translocated to the nucleus after administration of a ligand. They identified the bipartite nuclear localization signal (NLS) of PXR in the DBD, which guarantees the movement between the nucleus and cytoplasm (Kawana et al., 2003). Along the same lines, *in vitro* studies of PXR-mediated transduction pathways suggest that mPXR is expressed as yellow fluorescent protein fusion in the cytoplasm of untreated liver cells and is translocated in the nucleus after PCN treatment (Squires et al., 2004). PXR forms a protein complex with the cytoplasmic CAR retention protein (CCRP) and heat shock protein 90 (Hsp90), which in turn increases the cytosolic retention of PXR (Squires et al., 2004). Transient knockdown of CCRP by an RNA-silencing technique attenuated ligand-induced PXR transcriptional activation. This outlines the importance of cytosolic sequestration in regulating the PXR transcriptional activity (Squires et al., 2004).

The inconsistencies between these studies indicate that both ligand binding and also modulation of PXR by signaling pathways (i.e., by signal cross-talking) and subcellular trafficking are important regulatory processes that tune the function of this nuclear xenobiotic receptor. Because of the various observations of the subcellular localization of PXR, it is important that future studies are directed to determine the molecular mechanisms responsible for PXR localization in both physiological and pathological conditions (Pondugula et al., 2009b).

3.1.4 Transcriptional activation

The transcriptional activity of PXR is inhibited by interactions with co-repressors such as nuclear receptor co-repressors (NCoR1 and 2), receptor-interacting protein 140 (RIP 140), short heterodimer partner (SHP) or silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) (Orans et al., 2005). Upon ligand binding to PXR, the xenosensor dissociates from the multiple-protein complex that inhibits its transcriptional activity, often through the recruitment of other co-factor proteins that contain histone deacetylase (HDAC) activity. The p160 family co-activators (e.g., SRC-1 and GRIP), peroxisome proliferator-activated receptor (PPAR)-binding protein (PBP), and peroxisome proliferator-activated receptor-gamma co-activator 1 alpha (PGC1-α have intrinsic histone acetyltransferase activity. Recruitment of these leads to chromatin remodeling and subsequent transcriptional activation (Ding & Staudinger,

2005a; b; Orans et al., 2005; Synold et al., 2001; Takeshita et al., 2001; Takeshita et al., 2002; Wentworth et al., 2000). Activated PXR binds to the promoter of its target gene as a heterodimer with RXRα (Frank et al., 2005; Goodwin et al., 1999). The complex recognizes the PXR response element (PXR-RE) and confers transactivation. PXR exhibits a broad binding activity toward several DNA elements containing two copies of a half-site consensus sequence AG(G/T)TCA with various spacing. This includes direct repeats DR-3, DR-4 and DR-5 and everted repeats ER-6 and ER-8 (Goodwin et al., 2002; Kast et al., 2002; Orans et al., 2005; Sugatani et al., 2008; Toell et al., 2002). In some PXR target genes, more than one PXR-RE is present (Bertilsson et al., 2001; Goodwin et al., 1999; Pascussi et al., 1999). Studies of the CYP3A4 promoter have uncovered the complex molecular mechanisms underlying the transcriptional regulation of this gene. The CYP3A4 gene, encoding the most abundant P450 enzymes has four major functional PXR-binding response elements. PXR recognizes proximal ER6 (prER6; -169/-152) response element (Bertilsson et al., 1998; Blumberg & Evans, 1998; Lehmann et al., 1998), a xenobiotic-responsive enhancer module (XREM) with a functional DR3 nuclear receptor-binding element 1 (dNR1; -7733/ -7719) and DR4-type PXR binding element (eNR3A4; -7618/-7603) (Goodwin et al., 1999; Toriyabe et al., 2009) and finally functional PXR responsive ER6 element localized within the far constitutive liver enhancer module (CLEM4-ER6, -1368/-11351) (Liu et al., 2008). Promoter analysis further demonstrated that maximal CYP3A4 induction by rifampicin requires PXR activation of both the prER6 element and the XREM (Goodwin et al., 1999; Lehmann et al., 1998). Nuclear receptor binding sites typically have high levels of promiscuity because the binding site is not specific for one receptor. For example, PXR and CAR can bind the same xenobiotic response elements on CYP2B and CYP3A genes (Xie et al., 2000b). Moreover, in primary human hepatocytes several new PXR target genes containing PXR binding sites were identified. Interestingly, PXR binding to PXR-REs was not always significantly increased in the presence of rifampicin (Hariparsad et al., 2009).

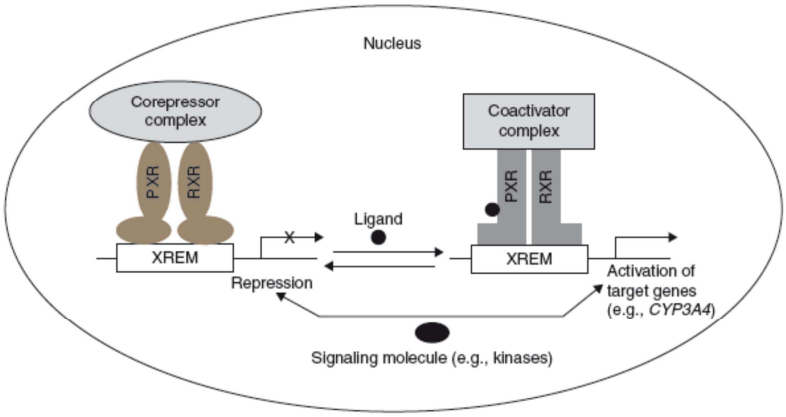

Figure 5. The mechanism of target gene induction by PXR (Pondugula et al. 2009).

3.2 PXR TARGET GENES

3.2.1 Regulation of drug-metabolizing enzymes and drug transporters

The regulation of transcriptional activity by nuclear receptors is involved in a myriad of physiological processes including reproduction, differentiation, development, metabolism, metamorphism, and homeostasis (Li et al., 2007). PXR controls key xenobiotic metabolic pathways involving phase I enzymes (oxidation) and phase II enzymes (conjugation) and transporters (Kliewer et al., 1998; Synold et al., 2001).

Specifically, PXR is a dominant regulator of CYP3A genes. In the human liver, CYP3A4 is the most highly expressed CYP enzyme and it is highly inducible by a wide variety of xenobiotics. CYP3A4 catalyzes the metabolism of more than 50% of clinically used drugs. Induction of CYP3A4 caused largely due to xenobiotic binding and activation of PXR can have serious toxicological consequences as a result of accelerated drug metabolism that contributes to drug-drug interactions, the bioactivation of xenobiotics to carcinogenic or toxic metabolites and possibly endocrine disruption (Santoliquido et al., 2005).

Moreover, PXR plays a central role in induction of CYPs such as CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP3A4 and CYP3A5 in the human liver and other organs (Capristo et al., 2005; Harmsen et al., 2007; Morere et al., 1975; Synold et al., 2001; Xie et al., 2000b). In addition, PXR regulates the expression of carboxylesterases, aldehyde and alcohol dehydrogenases, and enzymes involved in heme production and the P450 reaction cycle (Hegardt, 1999; Nakamura et al., 2007; Watkins et al., 2003a). Phase II conjugation DMEs that are up-regulated by PXR ligands include glutathione S-transferases (GST) (Staudinger et al., 2001a), sulfotransferases (SULT) (Hofmann, 1999; Louet et al., 2002; Scaglia & Igal, 2005) and UDP-glucuronosyltransferase (UGT)

essential to the clearance of bilirubin, drugs, and xenobiotics (Inoue et al., 2004; Kast et al., 2002) and carboxylesterase (Chiang, 1998) families. The expression of several drug transporters is controlled by PXR, e.g. ABC drug efflux transporters, multidrug resistance-associated proteins (MRP) and breast cancer resistance protein (BCRP) (Gu et al., 2006; Hegardt, 1999; Kast et al., 2002; Staudinger et al., 2001a; Steidl et al., 2006; Synold et al., 2001; Watkins et al., 2003b).

3.2.2 Drug-drug interactions

Xenosensor PXR is often responsible for clinically important drug-drug interactions (DDIs) as well as for undesired drug side effects (Zhou et al., 2006a; Zhou et al., 2007). Adverse drug interactions represent the fourth- to sixth-leading cause of death in the United States (Boustead et al., 2003; He et al., 1995). The induction or inhibition of drug-metabolizing cytochromes P450 represents one of the most common causes of DDIs, and many of these interactions involve CYP3A4 (Kaplan et al., 1995). The identification of the antituberculosis drug rifampicin as a potent hPXR agonist has provided an explanation as to why the use of this drug is prone to drug-drug interactions. Indeed, in situations with co-existing illnesses, PXR activation can adversely affect (i.e., inactivate) other concomitantly delivered drugs, including anti-HIV (human immunodeficiency virus) protease inhibitors, oral contraceptive, thiazolidinediones, and benzodiazepines (CYP3A substrates) (Barry et al., 1997; Biswas et al., 2009; Dowell et al., 2004; Grub et al., 2001; Chen & Zhou, 1999; Lotsch et al., 2002; Meyer, 1976; Pea et al., 2008; Poirier et al., 2007; Rosskopf et al., 2009; Schafer-Korting, 1993; Scheen, 2007; Sivertsson et al., 2013; Vethe et al., 2011). The clinical consequences of such adverse interactions are observed with cyclosporine transplant rejection (Ruschitzka et al., 2000) or loss of drug efficacy (e. g. with anti-HIV and co-administered drugs) (Curran & Ribera, 2011; Kredo et al., 2011). Drug interaction leading to liver toxicity is caused by isoniazid and 3-methylcholanthrene, which can accelerate the metabolism of acetaminophen (Burk et al., 1990; Crippin, 1993; Guo et al., 2004; Chen et al., 2012; Chen et al., 2010; Lee, 2004; Pondugula & Mani, 2013). Another example of a PXR-activation drug is paclitaxel, one of the most commonly used antineoplastic agents. Synold et al. showed that paclitaxel-activated human PXR can induce the hepatic expression of CYP3A4 and CYP2C8, as well as multidrug resistance protein 1 (MDR1) expression in intestinal tumor cells. This PXR-mediated drug clearance pathway may enhance the metabolism and efflux of paclitaxel, which may lead to increased intestinal drug excretion and drug resistance (Synold et al., 2001). It is conceivable that understanding how PXR modulates CYP expression may be useful to prevent adverse drug interactions.

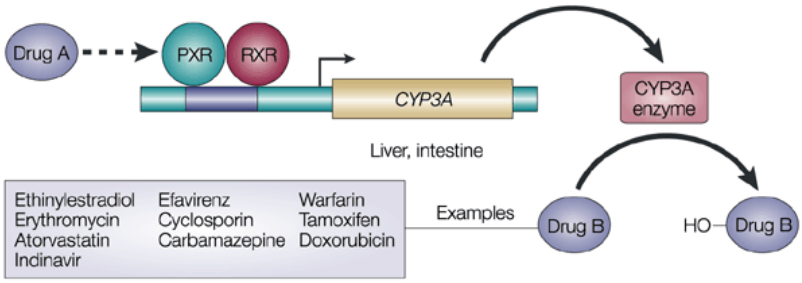

Figure 6. The molecular basis of drug-drug interaction (Willson & Kliewer, 2002).

3.2.3 The role of PXR in cancer tissue

The roles that PXR plays in the regulation of xenobiotic metabolism and transport in cancer tissue have been well-reviewed (Dai et al., 2008; Ekins et al., 2007; Elcombe et al., 2012; Igarashi et al., 2007; Kameda et al., 1996; Pondugula & Mani, 2013; Tabb et al., 2003; Xie et al., 2000a). One of the main barriers in clinical oncology is the multidrug resistance phenomenon (MDR) involving tumors which become resistant to a wide spectrum of different drugs. One tumor pathway that may allow the development of drug-resistant tumor cells to anticancer drugs is signaling via the xenobiotic nuclear receptors (PXR, CAR, FXR, LXR, and VDR) (Chawla et al., 2001). Several studies have demonstrated that PXR is activated by many different chemotherapeutic agents (e.g. paclitaxel, tamoxifen and cisplatin) (Desai et al., 2002; Huang et al., 2006; Synold et al., 2001) and it has been shown that PXR is expressed in a variety of cancer tissues (e.g., colon, ovarian, prostate, endometrial, osteosarcoma). Activation of PXR-target genes such as Mdr1/p-glycoprotein, MRPs, CYP3A, UGTs, and GSTs (Harmsen et al., 2007) accelerate the metabolism and elimination of chemotherapeutic agent, which may contribute to acquired drug resistance and multi-drug resistance. Depending on the particular cancer tissue, PXR activation or inhibition displays distinct anticancer phenotypes (i.e. sensitizing the cancer cells to anticancer drugs, preventing the induction of drug resistance, inducing apoptosis or reducing proliferation, invading and migrating of the cancer cells). This is evident in a variety of cancers, including breast (Conde et al., 2008; Dotzlaw et al., 1999; Miki et al., 2006b), prostate (Chen et al., 2007), ovarian (Gupta et al., 2008), colon (Harmsen et al., 2010; Raynal et al., 2010) and endometrial cancer (Masuyama et al., 2003).

For instance, a pattern of PXR up-regulation combined with estrogen receptor (ER) down-regulation was identified in endometrial and breast cancer cells. This suggests that PXR provides a growth advantage to neoplastic cells by processing steroid like compounds and xenobiotics (Dotzlaw et al., 1999; Masuyama et al., 2003). These observations support a possible role for PXR in breast tumor growth by enhancing the

uptake of estrogens via organic anion-transporting polypeptide (OATP) transporter, thereby increasing intracellular levels of estrogens that activate ER (Meyer zu Schwabedissen et al., 2008; Miki et al., 2006a). Furthermore, PXR regulates the expression of the proteins involved in apoptosis and anti-apoptosis in these cancers, which further contributes to altered tumor growth and drug sensitivity (Gong et al., 2006; Verma et al., 2009; Wang et al., 2011b; Zhou et al., 2008b; Zucchini et al., 2005). Even though these studies provided conflicting results, it is clear that PXR has an important role in cancer. A better understanding of receptor crosstalk which interfaces with PXR activity, the identification of compounds that limit PXR activation and the clarification of PXR PTMs may provide alternative drug therapies.

3.2.4 Endobiotic function of PXR

In addition to the regulation of the hepatic genes that encode phase I and phase II drug metabolizing enzymes and transporters, PXR plays some essential roles as an endobiotic receptor affecting glucose and lipid metabolism, bile acid and bilirubin detoxification and inflammation. This regulation of energy metabolism occurs mainly in the liver, an essential organ in metabolic homeostasis, through direct gene regulation or through crosstalk with other transcriptional regulators.

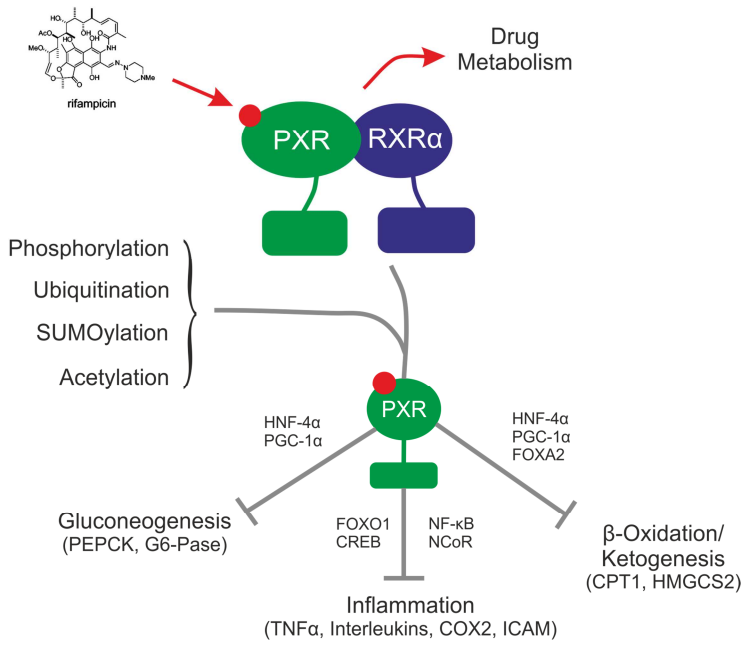

Figure 7. The endobiotic function of PXR (Staudinger et al., 2011).

PXR in glucose metabolism

Gluconeogenesis is an important metabolic pathway that results in the generation of glucose as a fuel source in central and peripheral tissues. It is tightly linked to fasting, starvation or intense exercise. In humans the main gluconeogenic precursors are pyruvate, lactate, glycerol, and the amino acids alanine and glutamine. The major gluconeogenic enzymes phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) are positively regulated by cAMP, glucocorticoids and glucagon and negatively regulated by insulin (Li & Chiang, 2005; Ourlin et al., 2003; Wang et al., 2002) Interestingly, these were dramatically suppressed in VP-PXR transgenic mice, in which the expression of an activated PXR was directed to the liver (Zhou et al., 2006c). This finding was confirmed by another study, where PXR agonist PCN down-regulated G6Pase expression in wild type but not PXR-/- mice (Krausova et al., 2011). In addition, ligand-activated PXR acts as a co-repressor of two positive regulators of gluconeogenesis, namely forkhead box protein O1 (FOXO1) and cAMP-response element-binding protein (CREB). This leads to the suppression of G6Pase and PEPCK1 gene expression and gluconeogenesis (Krausova et al., 2011; Santoliquido et al., 2005). Individuals that are healthy and well-fed have livers containing relatively low basal levels of PGC-1α. This level is readily up-regulated by fasting and diabetes mainly through an altered insulin–glucagon balance (Morere et al., 1975), which leads to activation of PEPCK and G6Pase through interactions with the transcription factor HNF-4α and FOXO1 (Boustead et al., 2003; Puigserver et al., 2003). In addition to the co-activation of PXR by insulin regulated FOXO1, it was shown that PXR expression and function is modified by fasting through overexpression of the PGC-1α pathway (Miele et al., 2005). This means, that gluconeogenesis could be negatively regulated by competition between PXR and HNF-4α, which also positively regulates gluconeogenesis by recruiting PGC-1α (Bhalla et al., 2004; Santoliquido et al., 2005).

Gluconeogenesis is a target of therapy for type II diabetes. One, such therapy involves the administration of metformin, which inhibits glucose formation and stimulates glucose uptake by cells. The mechanism of metformin action is still unclear. There is evidence that activation of the AMP-activated protein kinase (AMPK) may play a central role (Nakamura et al., 2007) and the hepatic small heterodimer partner (SHP) is implicated in the metformin-mediated activation. Importantly, SHP has been found to repress the transcriptional activity of PXR (Li & Chiang, 2005; Ourlin et al., 2003; Wang et al., 2002). This phenomena led to analysis of the effect of SHP up-regulation by metformin on PXR transcriptional regulation of its target genes (Krausova et al., 2011). The suppressive effect of metformin on CYP3A4 expression was demonstrated via

major nuclear receptors, including PXR. However, the hypothesis was refuted that this effect was caused by the role of metformin as antagonist in PXR ligand binding pocket, a putative effect of SHP expression or metformin-stimulated activation of the AMPK pathway. On the other hand, this study showed another way that this effect could be modulated. Metformin disrupted the interaction of PXR with its co-activator SRC-1 resulting in the inhibition of PXR transcriptional activity and the suppression of its target gene.

PXR in lipid metabolism and ketogenesis

When blood glucose is low, the liver increases fatty acid oxidation and ketogenesis to provide ketone bodies to extra-hepatic tissues via β-oxidation and ketogenesis. Under these conditions key enzymes carnitine palmitoyltransferase 1A (CPT1A) and mitochondrial 3-hydroxy-3-methylglutarate-CoA synthase 2 (HMGCS2) are down-regulated (Hegardt, 1999; Louet et al., 2001). Transcription of these key enzymes is positively regulated by forkhead box A2 (FOXA2) (Puigserver et al., 2003; Wolfrum et al., 2003). Stearoyl-CoA desaturase 1 (SCD1), the key enzyme in the synthesis of fatty acids is up-regulated (Dobrzyn & Ntambi, 2005). It is well known that PXR agonists affect lipid metabolism, for example rifampicin induces hepatic steatosis, characterized by the abnormal accumulation of triglycerides in liver (Morere et al., 1975). It has been suggested that ligand-activated PXR represses hepatic energy metabolism by decreasing both β-oxidation and ketogenesis. PXR agonist PCN down-regulated the expression of genes encoding CPT1A and HMGCS2 (Nakamura et al., 2007). Further, the same study shown that activated PXR and FOXA2 directly interact together. In addition, it has been shown that HNF-4α, which is a master transcriptional activator for a large number of genes in hepatocytes, directly regulates the expression of CPT1A (Louet et al., 2002) indicating that HNF4-a, PGC-1a, PXR and FOXO2 are jointly involved in the regulation of lipid metabolism (Staudinger et al., 2011).

PXR in bile acid homeostasis

Bile acids are important physiological agents that are secreted by hepatocytes as the end product of cholesterol catabolism. Biosynthesis of bile acids is essential for the elimination of excess cholesterol and hydrophobic endobiotic and xenobiotic metabolites from the body. Because extreme toxicity can lead to a variety of pathological conditions, including cholestasis (Hofmann, 1999), it is necessary that intracellular levels of bile acids be tightly regulated by multiple NRs, including LXR, FXR, HNF-4α, CAR and PXR (Inoue et al., 2004; Kalaany & Mangelsdorf, 2006; Staudinger et al., 2001a). Primarily, bile acid and cholesterol level are regulated by

LXR and FXR. The hepatoprotective function of PXR against toxic bile acids is involved when the level of endogenous compounds rise to excessive concentrations, as occurs during hypercholesterolemia and cholestasis. The first and rate-limiting step of cholesterol catabolism and bile acid formation involves hydroxylation of cholesterol at the 7α-position and is catalyzed by CYP7A1 (Chiang, 1998). In rodents, it was shown that PXR represses this enzyme and bile acid synthesis. In addition, PXR is involved in bile acid detoxification through the Phase II enzyme SULT2A (Sonoda et al., 2002). PXR also stimulates the expression of genes, such as MRP2 (Kast et al., 2002) and OATP2 (OATP1B1) (Staudinger et al., 2001b), which encode proteins that contribute to the metabolism and clearance of bile acids.

PXR in inflammation

It is known that inflammatory responses and infections decrease drug metabolism capacity and drug clearance by proinflammatory cytokines such as interleukin 1 (IL-1) and tumor necrosis factor alpha (TNF-α). It is also known that an exposure to xenobiotics has an immunosuppressive side effect. It has long been known that the PXR activator rifampicin suppresses immunological responses in liver cells (Doria & Agarossi, 1973). However, the mechanisms through which inflammatory signals down-regulate hepatic CYP genes, and through which drugs exert immunosuppressive effects remain unclear. A major regulator of inflammation is nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which plays a critical role in the expression of a diverse array of genes triggering and coordinating innate as well as adaptive immune responses (Ghosh & Karin, 2002). It has been shown that the p65 subunit of NF-κB plays an important role in the suppression of PXR-RXRα-regulated gene expression by interfering with the binding of PXR-RXRα to the regulatory DNA sequences (Gu et al., 2006). Moreover, the interaction of NF-κB with PXR has a reciprocal inhibitory effect on NF-κB activity as the expression of NF-κB target genes (Zhou et al., 2006b). These observations provide a potential molecular mechanism that links xenobiotic metabolism and inflammation.

3.3 POSTRANSLATIONAL MODIFICATIONS OF PXR

Nuclear receptors are a target for post-translational modifications (PTM), a fast way to control receptor function. Among the prominent PTMs are serine, threonine and tyrosine phosphorylation; lysine acetylation; lysine and arginine methylation, proline isomerization; lysine sumoylation and ubiquitination. Two main types of PTMs exist. The first type comprises reversible modifications that function by either the addition or removal of functional chemical groups (i.e. phosphate or acetyl) on specific amino acid residues of target proteins. The second type of modification involves the addition of other proteins or polypeptides (e.g. sumoylation or ubiquitination) (Anbalagan et al., 2012). Multiple signaling pathways modulate the activity of PXR, likely through direct alteration of the phosphorylation status of the receptor and its protein co-factors. Therefore, specific combinations of ligand binding and cell signaling pathways affect PXR-mediated gene activation and determine the overall biological response.

3.3.1 Phosphorylation

Post-translational modification of proteins by phosphorylation is a widely used mechanism to control a number of cellular processes including transcription, ubiquitination, protein degradation or protein subcellular localization. Phosphorylation usually occurs on serine, threonine, tyrosine and histidine residues by the covalent binding of a phosphate group, which changes the characteristics of the protein. In general, NRs site-specific phosphorylation by protein kinases occurs in all domains (Figure 8) and it plays a vital role in regulating all aspects of NR function, including expression, stability, sub-cellular localization, dimerization, ligand binding, DNA binding, interaction with co-regulator and transcriptional activity (Blumberg & Evans, 1998; Ismaili & Garabedian, 2004; Orti et al., 1992; Rochette-Egly, 2003; Shao & Lazar, 1999; Staudinger & Lichti, 2008; Sun et al., 2007; Weigel & Moore, 2007). The majority of phosphorylated residues lie in the N-terminal A/B region (containing AF-1) and most of the sites are serine residues surrounded by prolines. Therefore, this region is mainly target proline-dependent kinases, which include cyclin-dependent kinases (CDKs) (Morgan, 1995) and Mitogen-activated protein (MAP) kinases (Chang & Karin, 2001). The LBD domain is phosphorylated by the same proline-dependent kinases, but also other kinases are involved, such as tyrosine kinases or PKA. It is known that DBD of ERα is phosphorylated by PKA (Chen et al., 1999), the retinoic acid receptor alpha (RARα) and VDR are phosphorylated by PKC (Delmotte et al., 1999; Jurutka et al., 1993).

In general, there are different modes of phosphorylation. NRs can be phosphorylated by kinases that are associated with transcription factors (CDK7, TFIIH), activated in response to a variety of signals (MAPKs, PKA, PKB, PKC) or synergized with the concomitant phosphorylation of the associated co-regulators. But phosphorylation can also contribute to the termination of the ligand response by inducing DNA dissociation or NR degradation or through decreasing ligand affinity (Rochette-Egly, 2003).

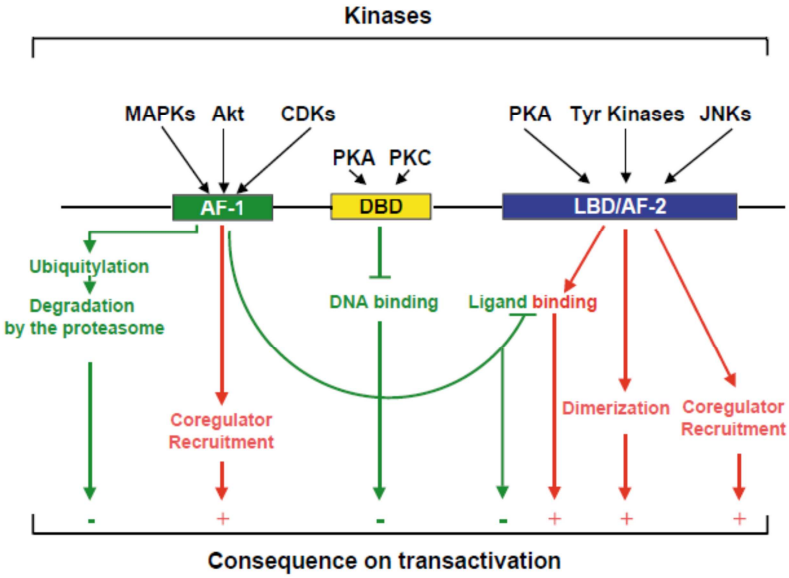

Figure 8. The positive and negative effects of NR phosphorylation on transactivation of target genes (Rochette-Egly, 2003).

Several studies revealed that PXR could be a substrate for protein kinases *in vitro*. Forskolin, an activator of PKA, induced Cyp3a in a ligand-dependent manner in mice and rat hepatocytes (Dowless et al., 2005). In addition, forskolin influenced the interaction of PXR with SRC-1 and co-repressor NCoR. PXR-GST pull-down assays revealed that PKA phosphorylates PXR in both a ligand-binding domain and a DNA-binding domain (Ding & Staudinger, 2005a; Dowless et al., 2005). In a follow-up study, the same group demonstrated that interaction between PKA and PXR occurs in a species-specific manner (Lichti-Kaiser et al., 2009b). In contrast, the activation of PKCα repressed PXR activity by increasing the strength of interaction between PXR and NCoR co-repressor and by abolishing the interaction between PXR and the SRC-1 co-activator (Ding & Staudinger, 2005b). Another study has shown that inhibition or activation of CDK2 led to the activation or attenuation of PXR-mediated CYP3A4 gene expression in HepG2 cells, respectively. A phospho-mimetic mutation (i.e., a mutation mimicking negatively charged phospho-moiety) of S350 impaired the function of PXR, whereas a phospho-deficient mutation (i.e., a mutation not allowing phosphorylation at

this site) conferred resistance to the repressive effect of CDK2 (Lin et al., 2008). Pondugula et al. showed that the phospho-mimetic T57 mutant exhibited a distinctive nuclear localization pattern, and this PXR mutant had impaired ability to bind to the CYP3A4 gene promoter. Furthermore, the role of p70 S6K kinase in regulation of PXR transactivation was demonstrated (Pondugula et al., 2009a). The same group performed systematic site-directed mutagenesis of serine and threonine residues to generate phospho-mimetic and phospho-deficient mutations at 18 *in silico* predicted consensus kinase recognition sequences in the human PXR. Mutations at T57 and T408 abolished ligand-inducible PXR activity. Mutations at S8, S208, S305, S350 and T408 decreased the formation of PXR/RXRα heterodimer, and subcellular localization of PXR was affected by mutations at T408. Mutants S208, S305, S350 and T408 displayed altered PXR protein/cofactor interactions (Lichti-Kaiser et al., 2009a).

3.3.2 Acetylation

As described above the lysine residue is a target of different PTMs, but these changes are mutually exclusive. Lysine acetylation was first reported in histones and the modifying enzymes involved were named histone acetyltransferases (HAT) and histone deacetylases (HDAC) accordingly as they respectively regulated acetylation or deacetylation (Yang & Seto, 2008). In addition to histones, a number of non-histone substrates are acetylated. Lysine acetylation has been reported for AR, ER, VDR, FXR, LXR, TR, RXRα receptors and co-regulators including SRC-1, SRC-3, PGC-1α, RIP140 and others (Fu et al., 2000; Kemper et al., 2009; Li et al., 2007; Lonard & O'Malley B, 2007; Wang et al., 2001; White et al., 2008; Whittle et al., 2007). Recently it was shown that PXR is acetylated and that the SIRT1 protein is presumably responsible for partial deacetylation of PXR. Additionally, the acetylation status of PXR regulates its selective function (i.e. lipogenesis) independently of an activation by a ligand (Biswas et al., 2009). Acetylation of NRs at conserved lysine residues raises the possibility that acetylation competes with other post-translational modifications to regulate NR action (Wang et al., 2011b). Potential crosstalk might occur between acetylation and phosphorylation. It has been shown that phosphorylation and acetylation signals can be linked. Further, they provide an important mechanism for the activation of p53-regulated genes following DNA damage through a signaling pathway linking p53 N-terminal kinase and C-terminal acetyltransferase activities (Elias et al., 2013; Lambert et al., 1998). Phosphorylation and acetylation of ERα are also interrelated events suggesting a potential cascade of posttranslational modifications of the receptor at these motifs. Potential PKA phosphorylation site at S305 blocked acetylation of K303 further enhanced the estrogen sensitivity of the mutant receptor

(Cui et al., 2004). Moreover, Sugatani et al. showed that phospho-mimetic mutation at Ser350, which was found as CDK2 phosphorylation site, alters deacetylation of PXR and the capacity to bind with RXRα in the nucleus of cells treated with roscovitine (Sugatani et al., 2012).

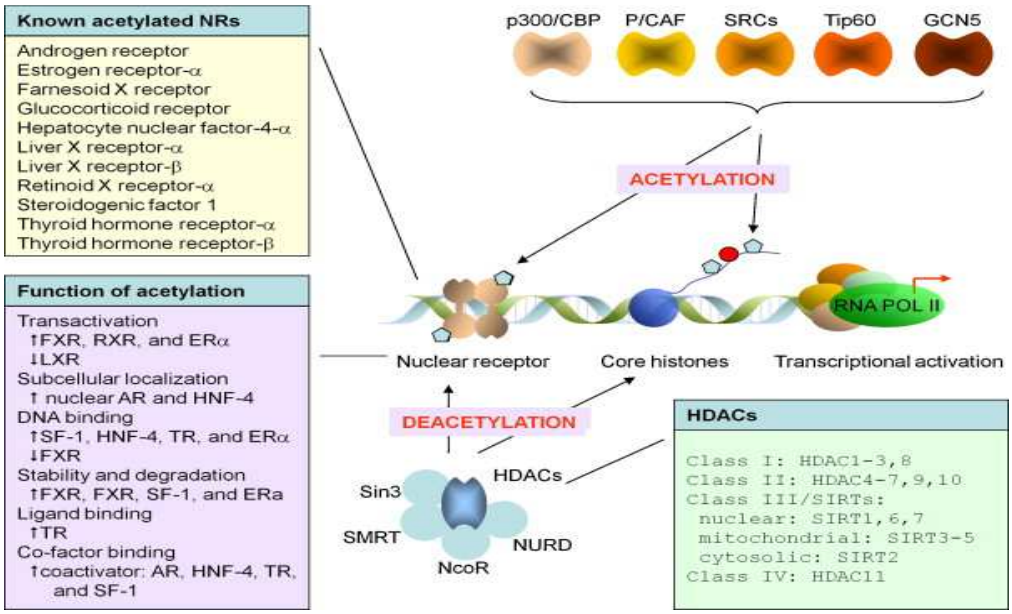

Figure 9. Post-translational modification of a nuclear receptor by acetylation (Wang et al., 2011a).

3.3.3 Ubiquitination and sumoylation

The protein modifier ubiquitin is most widely known for targeting protein substrates for proteasomal degradation, an essential and versatile housekeeping function in eukaryotic cells that maintains cellular homeostasis (Konstantinova et al., 2008). Ubiquitin is a small highly conserved protein that is reversibly conjugated to protein substrates through a complex cascade of enzymes via a three-step mechanism. This mechanism involves the ubiquitin-activating enzyme E1, followed by the ubiquitin-conjugating enzyme E2. E2 mediates transfer of ubiquitin from E1 to a member of the ubiquitin-protein ligase family E3. A number of earlier studies suggest that histone ubiquitination is involved in NR-mediated transcriptional regulation. Several steroid hormone receptors, including GR, ER, PR, AR, TR, RAR and VDR are regulated by the ubiquitin-proteasome pathway (Kinyamu et al., 2005). The first evidence, that PXR might be degraded by proteasome was published by Masuyama. They found that PXR interacts with the suppressor for gal-1, a key component of the 26S proteasome complex, in the presence of progesterone but not in the presence of endocrine disrupting chemicals. A follow up study confirmed that some steroids (including progesterone and dexamethasone) modify PXR protein levels through the proteasomal

pathway in the mice (Masuyama et al., 2000; Masuyama et al., 2002; Masuyama et al., 2005). Moreover, proteasomal inhibition by MG-132 resulted in the inhibition of PXR transactivation, suggesting an interplay between PXR and the ubiquitin pathway (Staudinger et al., 2011). Finally in recent study, a new PXR-interacting protein RBCK1 was identified (protein kinase C-interacting protein with a new type of RBCC). RBCK1 is an E3 ubiquitin ligase that regulates PXR protein expression *via* increased ubiquitination and it probably mediates PXR proteasomal degradation in human primary hepatocytes and in other cell lines (Rana et al., 2013).

Sumoylation is a process of covalent attachment of Small Ubiquitin-like Modifier proteins (SUMO) to proteins through a series of biochemical steps similar to ubiquitination. In contrast to ubiquitin, SUMO is not used to tag proteins for degradation. This kind of PTMs is involved in various cellular processes, such as nuclear–cytoplasmic transport, transcriptional regulation, apoptosis, protein stability, response to stress, and progression through the cell cycle. Several reports indicate that NRs sumoylation play an important role in the repression of the inflammatory responses in various tissue types. The most important drug-drug interaction in the treatment of HIV-related tuberculosis is between the PXR agonist rifampicin and antiretroviral drugs, where the long-term treatment of patients with RIF inhibits the inflammatory-response in the liver (Gu et al., 2006; Paunescu, 1970) and the immune response is potentially lethal. Recent publications have demonstrated a mutually repressive and negative cross-talk between the PXR and NF-κB signaling pathways (Gu et al., 2006; Zhou et al., 2006c). Moreover, it was shown that activation of the inflammatory response in hepatocytes strongly modulates SUMOylation of ligand-bound PXR and that the SUMOylated form of the PXR represses NF-κB gene expression (Hu et al., 2010). These findings suggest that coordinate regulation of genes involved in both inflammation and xenobiotic metabolism occurs.

4 METHODOLOGICAL FRAMEWORK

4.1 BIOLOGICAL MATERIAL

4.1.1 Cell lines - HepG2 and HeLa

The human hepatoma cells HepG2 (ECACC No. 85011430) and Human Negroid cervix epithelial carcinoma cells HeLa (ECACC No. 93021013) were grown in Dulbecco`s modified Eagle`s medium (DMEM) supplemented with 10% of fetal bovine serum (FBS), streptomycin (1 mg/ml), penicillin (100 U/ml), L-glutamine (2 mM), non-essential amino acids (1x) and sodium pyruvate (1mM). Cells were maintained at 37 °C and 5 % CO_2 in a humidified incubator.

4.2 REAGENTS

Dulbecco`s modified Eagle`s medium, fetal bovine serum, penicillin, streptomycin, L-glutamine, non-essential amino acids, sodium pyruvate, rifampicin, and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich (Prague, Czech Republic). TRI Reagent, M-MLV Reverse Transcriptase and oligonucleotide primers used in RT-PCR reactions in the samples from HepG2 cells were purchased from Invitrogen (Grand Island, NY). Random hexamers and ECL kit were purchased from GE Healthcare (UK). LightCycler FastStart DNA MasterPLUS SYBR Green I, FuGENE HD transfection reagent and protease and phosphatase inhibitor cocktails were purchased from Roche Diagnostic Corporation (Intes Bohemia, Czech Republic). PXR mouse monoclonal antibody (sc-48340 and sc-48340 X) and RXR-α rabbit polyclonal antibody (sc-553 and sc-553 X), secondary horseradish peroxidase conjugated antibody, and Western Blotting Luminol Reagent were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). A Light Shift Chemiluminescent EMSA Kit and Co-Immunoprecipitation Kit were purchased from Thermo Scientific (Waltham, MA, USA). TNT Quick Coupled Transcription/Translation Systems and a luciferase detection system were purchased from Promega (Southampton, UK). All other chemicals were of the highest quality commercially available.

4.3 PLASMIDS

A chimeric 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 (Promega, UK) has been described before (Goodwin et al. 1999). The expression plasmid for human PXR (pSG5-hPXR) was kindly provided by Dr. S. Kliewer (University of Texas, Dallas, TX), and the expression plasmid pSG5-hRXRα encoding human RXRα was a generous gift from Dr. C. Carlberg (University of Kuopio, Kuopio, Finland). Consensus tyrosine and threonine phosphorylation sites within the human PXR protein were identified using the *in silico* computer-based analysis on a NetPhos 2.0 server. The human PXR protein sequence has been obtained from the NCBI website (Accessions AAH17304 and AF061056). The T422 and T248 residues have been previously reported as a potential phosphorylation sites, but have not been characterized (Lichti-Kaiser et al., 2009a). The side chain of T248 is hydrogen bonded to T422 in a-helix 12 in case of PXR. This interaction is critical for SRC-1 co-activator recruitment to AF-2 domain and for proper ligand-dependent PXR function in target genes transactivation (Ueda et al. 2005). Potential tyrosine phosphorylation site Y249 (Score 0.916, NetPhos 2.0 Server) has not been characterized before. Site-directed mutagenesis of wild-type PXR (wt-PXR) was performed by GenScript (Piscataway, NJ, USA) and the following mutant plasmids were generated:

Table 1: Site –directed mutagenesis of PXR residues

Residue	**Phospho-deficient**	**Phospho-mimetic**
T248	T248V	T248D
Y249	Y249F	Y249D
T422	T422A	T422D

4.4 METHODS

4.4.1 Gene reporter assay

HepG2 cells (density 10^5/well) were transiently transfected employing lipofection with 0.3 µg/well of p3A4-luc reporter construct and 0.1 µg/well of expression plasmid encoding wild-type PXR (wt-PXR) or its mutated forms. Cells were seeded in the medium supplemented with 10% charcoal/dextran-stripped fetal bovine serum on 24-well collagen-coated dishes and stabilized for 16 hours prior to the treatments. Cells were treated for 24 hours with rifampicin (0.01–25 µM) and/or vehicle (DMSO; 0.1% v/v). After the treatments, cells were lysed using a luciferase detection system and luciferase activity was measured on an Infinite M200 luminometer (Tecan, Schoeller, Prague).

4.4.2 CYP3A4 mRNA determination in HepG2 cells

HepG2 cells (density 10^6 cells/well) were transiently transfected employing lipofection with 1.3 µg/well of expression plasmid encoding wt-PXR or its mutated forms. Cells were seeded in the medium supplemented with 10% charcoal/dextran-stripped fetal bovine serum on 6-well collagen-coated dishes and stabilized for 16 hours prior to the treatments. Cells were treated for 24 hours with rifampicin (10 µM) and/or vehicle (DMSO; 0.1% v/v). The total RNA was isolated using TRI Reagent according to the manufacturer instructions. cDNA was synthesized according to a common protocol, using M-MLV Reverse Transcriptase and random hexamers. One tenth was used for qPCR amplification using the Light Cycler apparatus (Roche Diagnostic Corporation, Meylan, France). The measurements were performed in triplicates. The following program was used: an activation step at 95°C for 10 min was followed by 40 cycles of PCR (denaturation at 95°C for 10 seconds; annealing of 7 seconds at 65°C for CYP3A4 or 68°C for GAPDH; elongation at 72°C for 17 seconds). The levels of CYP3A4 and GAPDH mRNAs were determined as described elsewhere (Pavek et al., 2007) using primers described in Table 2. Expression of CYP3A4 was normalized per glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a reference (housekeeping) gene. Data were processed by the delta–delta method.

Table 2. Oligonucleotides used for quantitative RT-PCR

Oligonucleotide name		Oligonucleotid sequence	Tm [°C]	Number of cycles
GAPDH	Forward	5′-TTCAGCAAGAAGAACAAGGACAA-3′	68	30
GAPDH	Reverse	5′-GGTTGAAGAAGTCCTCCTAAGC-3′		
CYP3A4	Forward	5′-TCCGGGACATCACAGACAGC-3′	65	40
CYP3A4	Reverse	5′-ACCCTGGGGTTCATCACCAA-3′		

4.4.3 Preparation of total and nuclear protein extracts

Preparation of total extracts

HepG2 cells (density 10^6 cells/well) were transiently transfected employing lipofection with 1.3 µg/well of the expression plasmid encoding wt-PXR or its mutated forms. Cells were seeded in the medium supplemented with 10% charcoal/dextran-stripped fetal bovine serum on 6-well collagen-coated dishes and stabilized for 16 hours prior to the treatments. After the stabilization period, the cells were treated for 48 hours with RIF (10 µM) and/or DMSO as a vehicle. The cells were washed twice with ice cold phosphate-buffered saline (PBS), scraped into PBS and centrifuged at 4,500 x g for 2 min at 4°C. The pellet was re-suspended in 170 µl of lysis buffer (10 mM Hepes pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.2% (v/v) sodium dodecylsulfate) supplemented with protease and phosphatase inhibitor cocktail. After homogenization with a pipette, the samples were vortexed for two minutes. Finally, the mixture was centrifuged at 15,000 x g for 13 min at 4°C and the supernatant was collected and stored at –80°C.

Preparation of nuclear extracts

HeLa cells (density 2.5 × 10^6/10 cm plate) were transiently transfected employing lipofection with 7 µg/well of expression plasmid encoding wild-type human PXR or RXRα. After 48 hours of incubation, adherent cells were scrapped into 1.5 ml of cold PBS. Suspension was then transferred to a microcentrifuge tube. Cells were pelleted for 5 minutes at 450 × g at 4°C and re-suspended in cold Buffer A (10 mM HEPES-KOH pH 7.9, 1.5 mM $MgCl_2$, 1 mM KCl, 1 mM DTT, protease and phosphatase inhibitor cocktail). The cells were allowed to swell on ice for 10 minutes and then vortexed for 10 seconds. Samples were centrifuged for 1 minute at 14,000 × g at 4°C. Supernatant contained the cytoplasmic fraction. The pellet containing nuclear fraction was re-suspended in 100 µl (according to the starting number of cells) of cold Buffer C (20 mM HEPES-KOH pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, protease and phosphatase inhibitor cocktail) and incubated on ice for 30

minutes with shaking. Cellular debris was removed by centrifugation for 5 minutes at 14,000 × g at 4°C. Supernatant fraction (containing DNA binding proteins) was transferred to a clean microcentrifuge tube and stored at -80°C. The yield is 50 µg to 100 µg proteins per 10^6 cells.

4.4.4 Western blotting

Total protein extracts (30 µg/well) were loaded and SDS–PAGE gels (8%) were run on a Bio-Rad apparatus according to the standard procedure. Protein transfer onto the PVDF membrane was carried out. The membrane was saturated with 5% non-fat dried milk in a TBS-T buffer for 1 hour at room temperature, followed by 2 hours of incubation with primary antibody and 1 hour of incubation with an appropriate secondary antibody at room temperature. Chemiluminescence detection was performed using horseradish peroxidase-conjugated secondary antibody and ECL kit.

4.4.5 Electrophoretic mobility shift assay

Labeling of DNA probe for EMSA

The following double-stranded 5'-biotinylated oligonucleotides containing a specific DNA-binding sequence for human PXR (DR3 motif from the XREM sequence of CYP3A4 gene promoter) were used as probes (Table 3): the wild-type unlabeled and biotinylated forms; biotin-labeled CYP3A4-DR3 with mutations (mutated bases in underlined-bold). The oligonucleotides were synthesized by Generi-Biotech (Hradec Kralove, Czech Republic). Final concentration of the labeled oligonucleotides was 10 fmol/µl and 2 pmol/µl for unlabeled probes.

Table 3. Oligonucleotides used for EMSA

	Sense	Antisense
Wild type CYP3A4-DR3	5'-GAATGAACTTGCTGACCCTCT-3'	5'-AGAGGGTCAGCAAGTTCATTC-3'
Mutated CYP3A4-DR3	5'-GAA**A**G**C**A**T**TTGC**A**GACCCTCT-3'	5'-AGAGGGTC**T**GCAA**A**T**G**C**T**TTC-3'

EMSA

The gel mobility shift assay was performed using a LightShift Chemiluminescent EMSA Kit with suitable modifications and corresponding to PXR interaction with a DR3 response element. Binding reactions were set-up according to Table 4. A nuclear protein (5 µg) from each sample was pre-incubated with 10x binding buffer (100 mM Tris, 500 mM KCl, 10 mM DTT; pH 7.5), 50% glycerol, 1% NP-40, ddH_2O and component of the kit nonspecific competitor Poly (dI.dC) to suppress strong nonspecific interaction before adding the biotin-labeled probe (10 fmol/µl). An unlabeled probe (2 pmol/µl) was added to the reaction mixture for a competitive experiment. The

complete binding reaction was incubated at room temperature 15 minutes. For supershift experiments, 2 µg of the anti-RXRα (sc-553 X) or anti-PXR (sc-48340 X) were added to the reaction mixture and incubated an additional 5 minutes at room temperature. Finally 5 µl of 5x loading buffer with bromophenol blue was added as an electrophoresis indicator to each 20 µl binding reaction (included in the kit). Native polyacrylamide gel 5% (18 x 16 cm, acrylamide:bisacrylamide 29:1) was pre-electrophoresed at 90 V for 30 minutes. Thereafter, protein-DNA complexes were loaded on pre-run gel and electrophoresed at 300 V in a 0.5 × TRIS-borate-EDTA (TBE) running buffer. When the bromophenol blue ran 2/3 of the way to the bottom of the plates, the electrophoresis was stopped and the protein DNA complexes were electro-blotted to a positively charged nylon membrane at 380 mA for 30 minutes in fresh and cold 0.5 × TBE buffer. Transferred DNA was cross-linked with the nylon membrane using a UV-light cross-linker instrument equipped with 254 nm bulbs at 120 mJ/cm^2 for 120 seconds exposure. Biotin-labeled DNA was detected using a streptavidin-horseradish peroxidase conjugate and chemiluminescent substrate contained in LightShift Chemiluminescent EMSA Kit.

Table 4. Binding Reaction set-up for the study of hPXR/RXRα interaction with DR3 motif

	Final Amount	**1. [µl]**	**2. [µl]**	**3. [µl]**	**4. [µl]**	**5. [µl]**
Ultrapure water	-	13	5	3	4	5
10 x binding buffer	1x	2	2	2	2	2
50 % glycerol	2.5 %	1	1	1	1	1
1 µg/µL Poly (dI.dC)	50 ng/µl	1	1	1	1	1
1% NP-40	0.05 %	1	1	1	1	1
unlabeled CYP3A4-DR3 (2 pmol/µl)	4 pmol	-	-	2	-	-
PXR/RXRα nuclear extract (1:1)	system-dependent	-	8	8	8	8
Biotin-CYP3A4-DR3 (10 fmol/µl)	20 fmol	2	2	2	2	-
Mutated Biotin-CYP3A4-DR3 (10 fmol/µl)	20 fmol	-	-	-	-	2
Antibody	0.1 µg/µl	-	-	-	1	-
Total reaction volume	-	20	20	20	20	20

Negative control containing only biotin labeled CYP3A4-DR3 probe; 2. Positive control containing sufficient target protein to effect binding and shift of biotin labeled CYP3A4-DR3 probe; 3. Competitive reaction control to prevent shift by competition from excess non-labeled CYP3A4-DR3 probe (200x molar excess); 4. Supershift reaction containing sufficient antibody which recognizes the protein; 5. Nuclear protein extract is incubated with mutant biotin-DR3 probe

4.4.6 Protein Co-Immunoprecipitation

Human PXR, its mutants and RXRα were translated *in vitro* using TNT Quick Coupled Transcription/Translation Systems. The following co-immunoprecipitation (Co-IP) experiment was optimized.

Co-Immunoprecipitation using Protein A/G Plus-Agarose

The antibody against PXR (2 µg; sc-48340) was incubated with 100 µl of diluted *in vitro* translated protein mixture of PXR and RXR-α (200 µg, PBS) for 2 hours at 4°C. As a negative control *in vitro* translated RXR-α (100 µg, PBS) was used. Then 20 µl of resuspended volume of Protein A/G PLUS-Agarose was added and incubated at 4°C overnight. The immunoprecipitation mixture was centrifuged at 500 x g for 5 minute at 4°C. The Supernatant was carefully discarded. The pellet was washed 3 times with 1 ml of PBS (100 mM sodium phosphate, 150 mM NaCl, pH 7.2) or more stringent Triton (1% Triton X-100 in TBS pH 7.4) or an RIPA buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% Na-deoxycholate, 0.1% SDS, 2 mM EDTA, 1% NP-40). Finally, the pellet was resuspended in 20 µl of 2x Laemmli sample buffer, boiled for 3 minutes at 95°C and 20 µl aliquots were analyzed by SDS-PAGE followed by Western blot and immunodetection with appropriate antibody.

Co-Immunoprecipitation using Pierce Co-IP Kit

A Co-IP Kit was used according to the manufacturer's protocol. Fifteen micrograms of the polyclonal RXRα antibody (sc-553 X) were incubated with the delivered resin and covalently coupled. The antibody-coupled resin was incubated with 200 µL of diluted PXR-RXRα protein mixture overnight at 4 °C. The resin was washed and the protein complexes bound to the antibody were eluted. Western blot analyses were performed as described above. Proteins were boiled in 2x Laemmli sample buffer and resolved on 8% SDS-PAGE gels followed by Western blot analysis and immunodetection with anti-PXR (sc-48340).

4.4.7 Statistical analysis

Results were expressed as mean ± SD. A one-way ANOVA test followed by the Dunnett's multiple comparison post hoc test or the paired Student's t-test were performed using GraphPad Prism software (GraphPad Software Inc., San Diego, CA).

5 RESULTS

5.1 Optimization of non-radioactive EMSA

5.1.1 Binding of human PXR to DR3 motif of human CYP3A4 gene promoter using *in vitro* translated PXR and RXRα

In vitro translated human PXR and RXRα proteins were incubated with biotin-labeled probe containing a human PXR binding site corresponding to the DR3 response element in the CYP3A4 promoter and resolved on non-denaturing gel. In order to demonstrate the effectiveness of EMSA and to ensure that the system was properly set, a control EBNA system including a complete set of three reactions (60 bp biotin-EBNA Control DNA, Epstein-Barr Nuclear Antigen EBNA Extract) was used as recommended by manufacturer (Figure 1a). Even though the control system worked perfectly, EMSA performed with *in vitro* translated nuclear receptors failed. High background was observed in each binding reaction except to negative control, where no *in vitro* translated PXR was present in the reaction mixture (Figure 1b). The results were completely unreadable because of shifted biotinylated proteins that occur in reticulocyte lysate (Figure 1c). This indicates that *in vitro* translated protein is unsuitable for detection and a preferable alternative must be found.

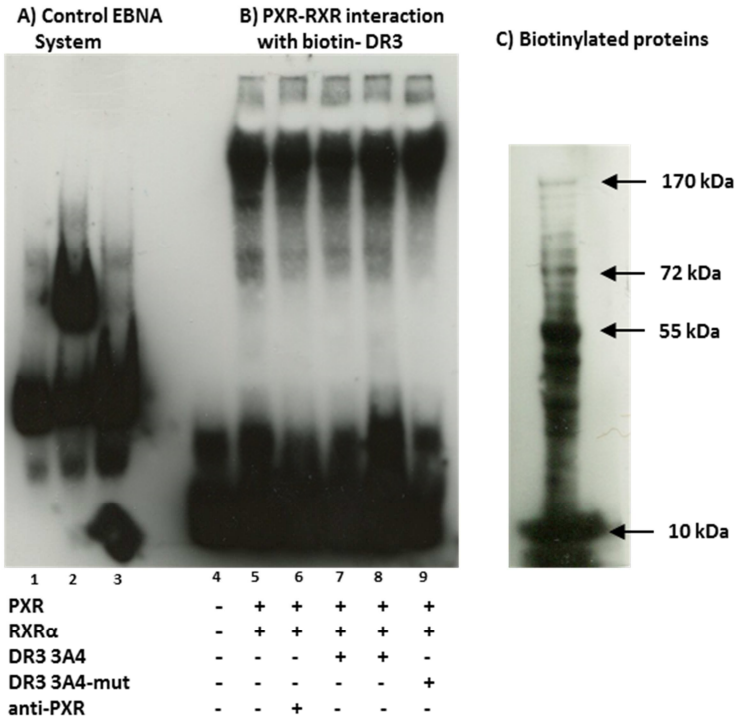

Figure 1. EMSA employing *in vitro* translated PXR and RXRα; a) The Control EBNA System. 1- Biotin-EBNA Control DNA, 2 - Biotin-EBNA Control DNA + EBNA Extract, 3 - Biotin-EBNA Control DNA + EBNA Extract + 200-fold molar excess of unlabeled EBNA DNA; **b)** Binding of *in vitro* translated hPXR to DR3 motif of human CYP3A4 promoter. *In vitro* translated hPXR and RXR-α were incubated with a biotin-labeled CYP3A4-DR3 probe and electrophoresed on 5 % polyacrylamide gel as described under the "Materials and methods" section. There are no shifted bands corresponding to PXR-DR3 interaction because of the shifts of a biotinylated proteins present in reticulocyte lysate; **c)** Identification of biotinylated proteins. The presence of biotinylated proteins in reticulocyte lysate were showed by SDS-PAGE and western blot using streptavidin-horseradish peroxidase conjugate.

5.1.2 Binding of human PXR to DR3 motif of human CYP3A4 gene promoter using nuclear extracts from HeLa cells

Because of the high background in experiments using *in vitro* translated proteins, HeLa cells were transiently transfected with human PXR and RXRα vectors. Nuclear extracts were isolated and used as a source of proteins for a binding reaction. Nuclear extracts were incubated with biotin-labeled double-stranded oligonucleotides corresponding to the DR3 response element from the CYP3A4 promoter (Figure 2). It is evident that PXR-RXRα heterodimer bound to the DR3 motif very strongly (Figure 2a). The specificity of binding to DNA was verified by a competing experiment, where an excess of unlabeled DR3 probe was mixed together with the DR3 labeled probe. Specific DNA binding was diminished by an excess (1-, 20-, 200-fold excess) of

unlabeled probe (unlabeled specific competitor). In addition, the “supershift” experiment was performed. A specific antibody against RXRα or PXR was added to the gel shift reaction, resulting in the formation of an antibody-PXR-RXRα-DNA complex with further decreased mobility (Figure 2a). PXR alone binds the DR3 probe very weakly as shown in Figure 2b. It is evident that the formation of PXR-RXRα heterodimer is essential for the binding of PXR to the DR3 response element. The specificity of protein-DNA interaction was confirmed also by incubation with a biotin-labeled probe containing a mutation in the DR3 sequence (Figure 2b).

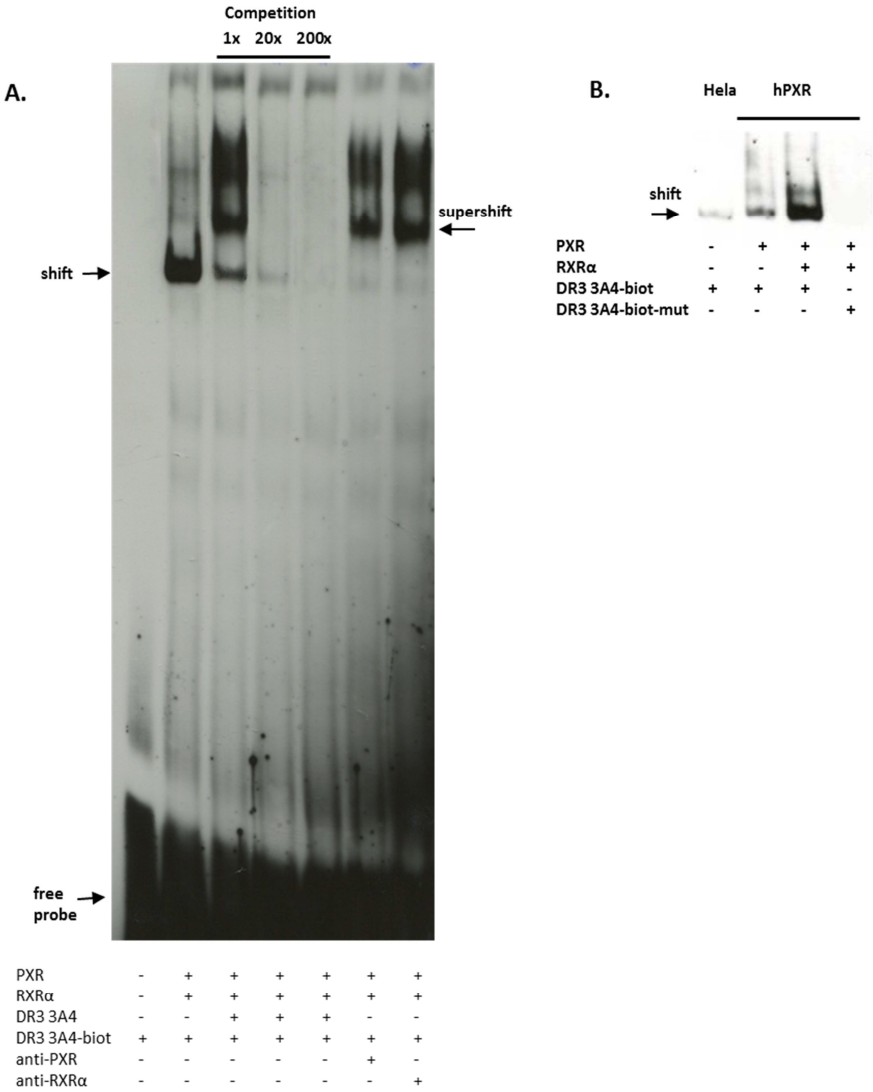

Figure 2. Binding of hPXR isolated from HeLa cells to DR3 motif of human CYP3A4 promoter. Nuclear extracts from HeLa cells transfected with PXR or RXR-α were incubated with a biotin-labeled CYP3A4-DR3 probe and electrophoresed on 5 % polyacrylamide gel as described in the “Materials and methods” section. The complex formation of the DR3 response element with hPXR alone or hPXR-RXR-α heterodimer was examined; a**)** In a competitive reaction, a biotin-labeled probe was incubated with a nuclear extract containing hPXR and

RXR-α proteins and 1-, 20- and 200-fold excess of unlabeled oligonucleotides in the binding reaction. The last two lines represent a supershift experiment with an antibody against PXR or RXR-α, respectively; **b)** A biotin-labeled CYP3A4-DR3 probe was incubated with nuclear extract from non-transfected HeLa cells, hPXR alone or together with RXR-α; The last line represents the control experiment with a mutated biotin-labeled CYP3A4-DR3 probe that was incubated with a nuclear extract containing hPXR and RXR-α proteins;

5.2 Optimization of Co-Immunoprecipitation

Co-Immunoprecipitation (Co-IP) is a common approach to the study protein-protein interactions that uses an antibody to immunoprecipitate the antigen (bait protein) and co-immunoprecipitate any interacting proteins (prey proteins).

Protein A/G Plus-Agarose

First we used traditional Co-IP employing Protein A/G Plus-Agarose to identify the interaction between PXR and RXR-α protein. The antibody against PXR formed an immune complex with *in vitro* translated PXR (bait) in the sample containing both interacting partners (Figure 3, line 2). The correct size of the detected protein was verified using an *in vitro* translated RXRα (line 3). RXR-α (prey) alone was added as a negative control, because the specific interaction between a bound PXR antibody and an RXRα protein cannot occur. Surprisingly, immunodetection resulted in a strong signal in the negative control (line 1) as well as in the sample containing bait and prey proteins (Figure 3a). First of all, we tried to manage a stringency of the buffer during binding and washing steps to break all nonspecific interactions while to preserving the specific interactions between antibody and antigen. A PBS buffer (low-stringency), a 1% Triton buffer (medium-stringency; line 4 and 5) or a RIPA buffer (high stringency; line 6 and 7) were used for this purpose. It is clear that with an increasing amount of detergent, the intensity of signal of the negative control as well as PXR-RXRα complex were decreased (Figure 3a). This indicates the presence of non-specific bindings of RXRα protein to the A/G Plus agarose and weak interaction between PXR and RXRα. Another reason of the masking of these results could be co-elution of the antibody heavy and light chains that may co-migrate with relevant bands and interfere with the interpretation of the results from the Western blot analysis. Further, we also tested only clear agarose beads (line 8) and beads incubated with PXR antibody alone (line 9) to eliminate the possibility of cross-reactivity between antibodies. The heavy chains (50 kDa) of PXR antibody were detected (Figure 3B), but cross-reactivity between anti-PXR and anti-RXRα was not confirmed (Figure 3A). Even further tests did not show a cross-reactivity of all used antibodies (data not shown).

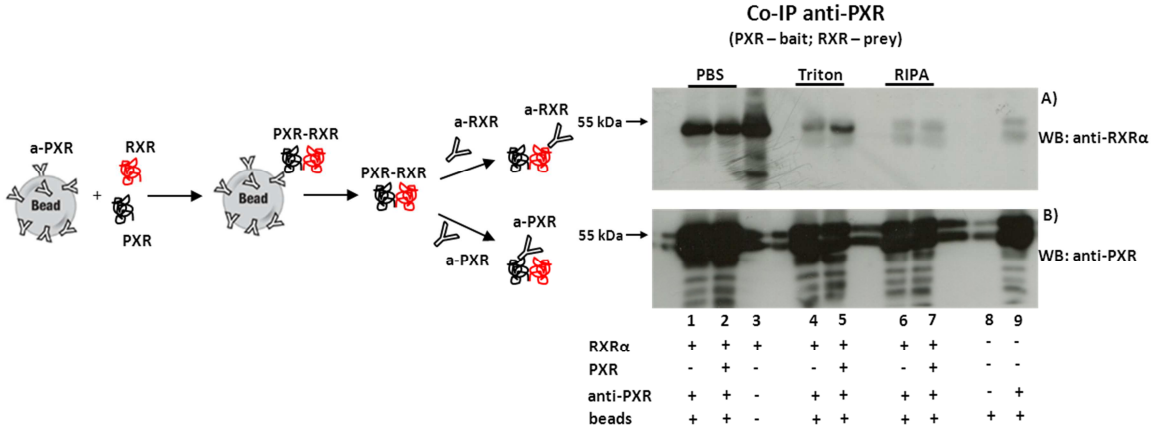

Figure 3. Detection of interaction between PXR and RXRα using Protein A/G Plus-Agarose. The antibody against human PXR was added to the sample containing *in vitro* translated protein mixture of PXR and RXR-α, and then agarose beads were added. Co-IP was performed with Protein A/G Plus-Agarose as described in the ‘‘Materials and methods” section. Co-Immunoprecipitate containing RXRα alone or complex of RXRα-PXR were resolved using 4–8 % SDS-PAGE and immunoblotted with **a)** antibody detecting RXR (prey protein) **b)** antibody detecting PXR (bait protein). A different stringency of buffers was used in washing steps (PBS, 1% Triton, RIPA).

AminoLink Plus Coupling Resin

The fastest way to avoid this problem was to use covalently coupling antibodies onto an amine-reactive resin by means of a Pierce Co-IP Kit. Immobilization to AminoLink Plus Coupling Resin was performed for both PXR (Figure 4) as well as RXRα antibodies (Figure 5) to be sure that Co-IP worked correctly. The co-immunoprecipitated complexes were eluted in two fractions, and the presence of prey proteins was detected using anti-PXR or anti-RXRα depending on the order of the antibodies (diagrams in Figure 4 and 5). We observed that the wild-type PXR protein was co-immunoprecipitated with the RXRα protein, as expected. We further verified the specificity of the co-immunoprecipitation in detecting PXR-RXRα heterodimerization by co-immunoprecipitation of RXRα alone, PXR alone and free reticulocyte lysate using the corresponding antibody. The correct size of the detected protein was verified using *in vitro* translated PXR and RXRα proteins.

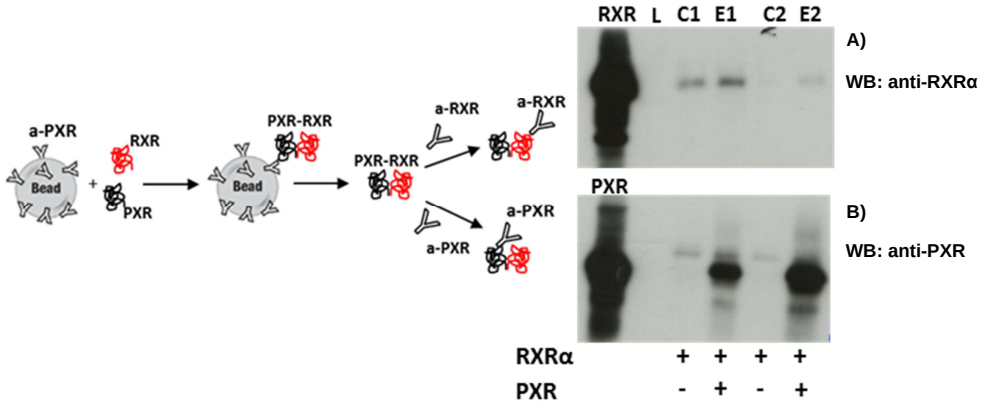

Figure 4. Detection of interactions between PXR and RXRα. The antibody against human PXR was coupled to AminoLink Plus Coupling Resin. *In vitro* translated RXRα and PXRs were incubated at laboratory temperature for 30 minutes. Co-immunoprecipitation was performed as described in the ‘‘Materials and methods” section. Eluted proteins containing RXRα alone as a control (C1, C2) and a complex of PXR-RXRα protein fractions (E1, E2) were resolved using 4-8 % SDS-PAGE and immunoblotted with **a)** antibody detecting RXR (prey protein), **b)** antibody detecting PXR (bait protein). **L** – Free reticulocyte lysate.

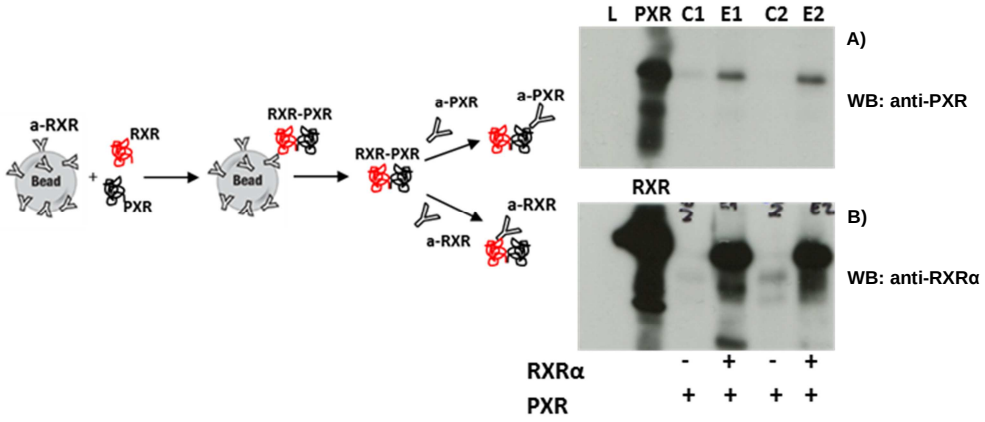

Figure 5. Detection of interactions between PXR and RXRα. The antibody against human RXRα was coupled to AminoLink Plus Coupling Resin. *In vitro* translated RXRα and PXRs were incubated at laboratory temperature for 30 minutes. Co-immunoprecipitation was performed as described in the ‘‘Materials and methods” section. Eluted proteins containing PXR alone as a control (C1, C2) and complex of PXR-RXRα protein fractions (E1, E2) were resolved using 4-8 % SDS-PAGE and immunoblotted with **a)** antibody detecting PXR (prey protein), **b)** antibody detecting RXR (bait protein). **L** – Free reticulocyte lysate.

5.3 Transcriptional activity of PXR mutants

Threonine and tyrosine residues within the human PXR protein were mutated to either a negatively charged phospho-mimetic residue aspartic acid (T422D, T248D, Y249D) or a phospho-deficient residue containing a hydrophobic side chain (T422A, T248V, Y249F) (Figure 6).

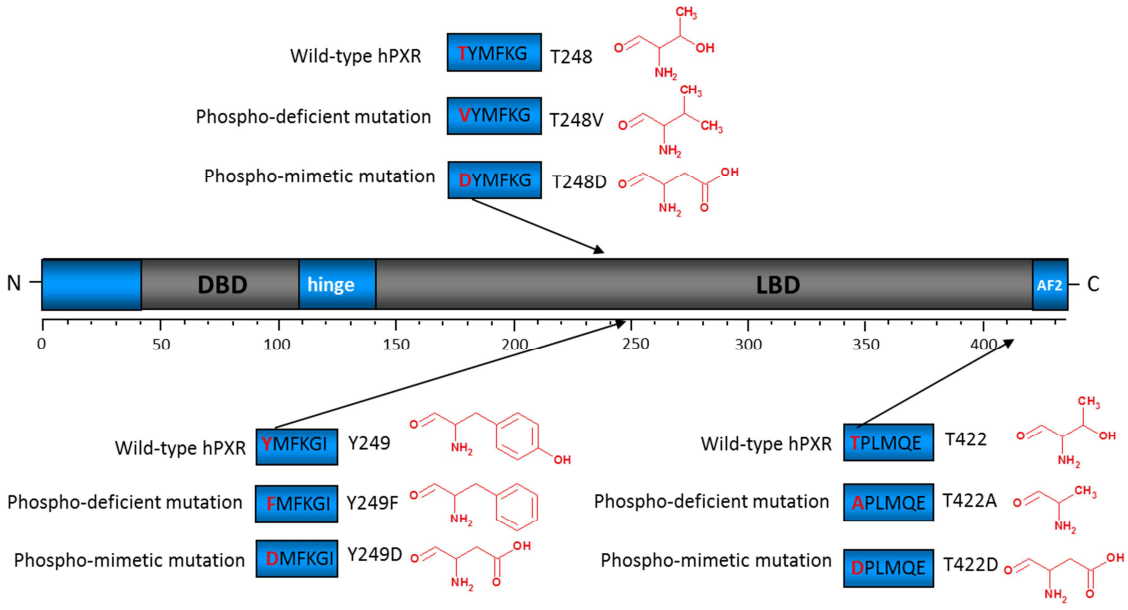

Figure 6. Schematic representation of mutations in human PXR. Site-directed mutagenesis was performed to generate phospho-deficient (T248V, Y249F, T422A) and phospho-mimetic (T248D, Y249D, T422D) PXR mutants, as described in detail in the ‘‘Materials and methods’’ section.

First, we investigated the effects of these mutations on basal and ligand-inducible transcriptional activity of PXR in cell-based gene reporter assays. For this purpose, we transiently transfected HepG2 cells with expression plasmids encoding either wild-type or mutated PXR and a p3A4-luc reporter plasmid, containing the basal promoter (-362/+53) with a proximal response element and the distal xenobiotic-responsive enhancer module (-7,836/-7,208) of the CYP3A4 gene 5´-flanking region. Cells were treated for 24 hours with increasing concentrations of the PXR agonist rifampicin (RIF). Transfection with the luciferase gene reporter construct p3A4-luc alone showed weak, concentration-dependent activity after the treatment with RIF. This was due to the activation of the CYP3A4 promoter construct by endogenous PXR present in HepG2 cells (Figure 7a). In subsequent experiments, we co-transfected wt-PXR cDNA together with the p3A4-luc reporter construct. Activation of wt-PXR by RIF was dose dependent, and maximal activity (about 15-fold) was attained at the concentration of 10 µM (Figure 7b). A hydrophobic mutation of the T248 residue to valine (T248V)

diminished basal and rifampicin-mediated transactivation of CYP3A4 promoter, suggesting an importance of T248 residue in PXR transcriptional activity (Figure 7c). In contrast, phospho-mimetic mutation of PXR at T248 (T248D) resulted in constitutive activation of the CYP3A4 promoter independently of the presence of PXR ligand (Figure 7d). Phospho-deficient mutation of Y249 (Y249F) did not reveal significant change as compared to wt-PXR (Figure 7e). Interestingly, phospho-mimetic mutation of Y249 (Y249D) decreased a transactivation potency of rifampicin to PXR receptor in transactivation of the CYP3A4 gene reporter construct (Figure 7f). With the construct, RIF at a concentration of 25 µM had the most significant effect on transactivation of the p3A4-luc reporter construct. However, at low concentrations (0.1 µM), RIF was not able to significantly activate the reporter construct in cells expressing Y249D mutated PXR, implying a decreased potency of RIF against this mutant (Figure 7f). Mutations at T422 (T422A, T422D) almost abolished the transactivation of the CYP3A4 promoter gene reporter construct (Figure 7g, h). We further studied the quantitative effects of different ratios of wt-PXR/T422A and wt-PXR/T422D proteins co-expressed in HepG2 cells on CYP3A4 promoter transactivation. In the experiments, we examined whether T422 PXR mutants function as dominant negative mutants. For both mutants (T422A, T422D), transcriptional activity of the plasmids mix decreased in a dose-dependent manner with a decreasing portion of wt-PXR in the mix (Figure 8 a, b).

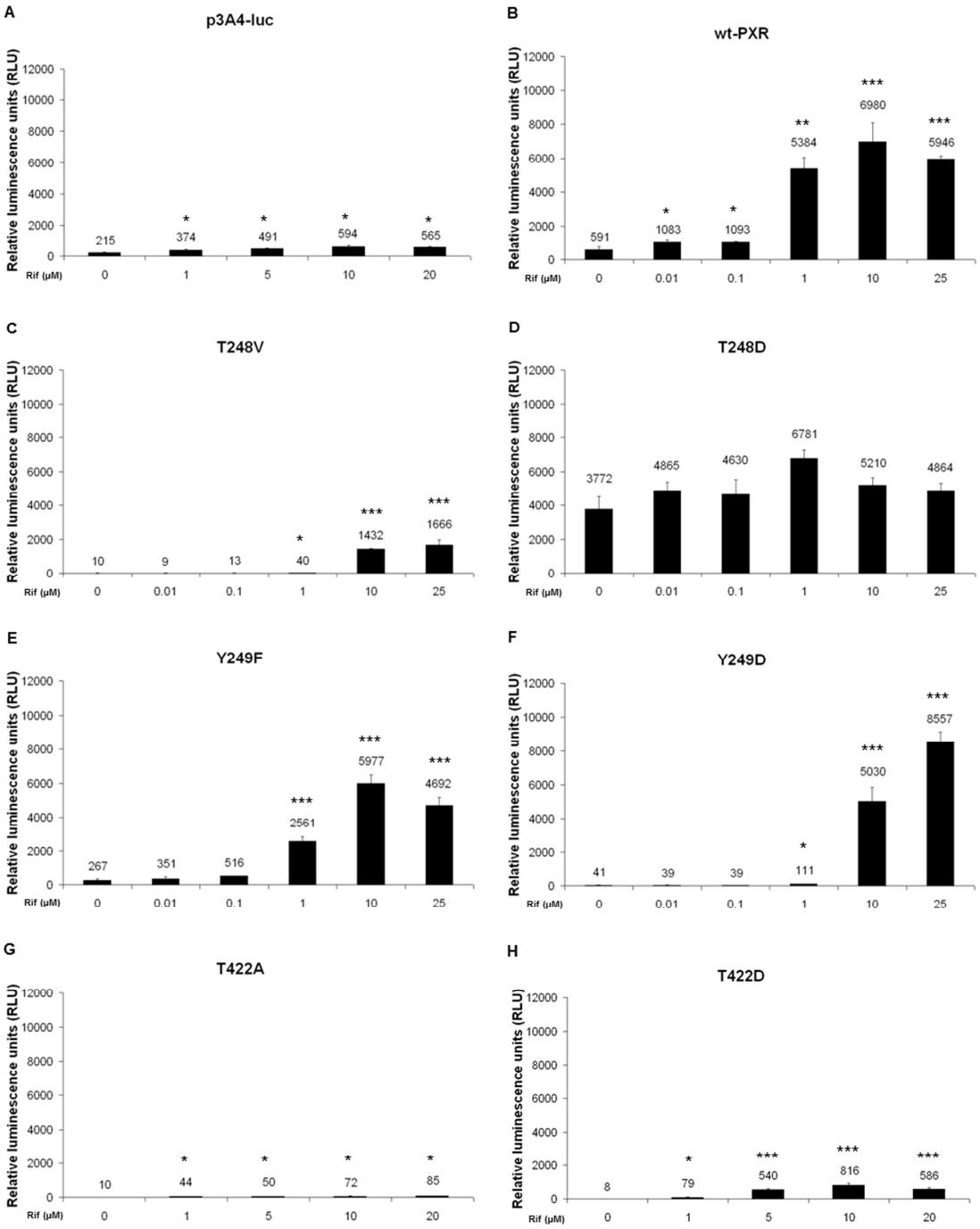

Figure 7. Transactivation of the CYP3A4 gene reporter construct with PXR mutants in HepG2 cells. HepG2 cells were transiently transfected by lipofection either with 0.3 µg of reporter plasmid p3A4-luc alone **(a)**, combined with 0,1 µg of expression vector encoding wild-type PXR **(b)** or PXR mutants **(c–h)**. Cells were treated for 24 hours with a vehicle (DMSO; 0.1 % v/v) or rifampicin in a range of concentrations from 0.01 to 25 µM. The data are expressed as relative luminescence units (RLU). Differences were considered statistically significant for *p<0.05, **p<0.01, ***p<0.001.

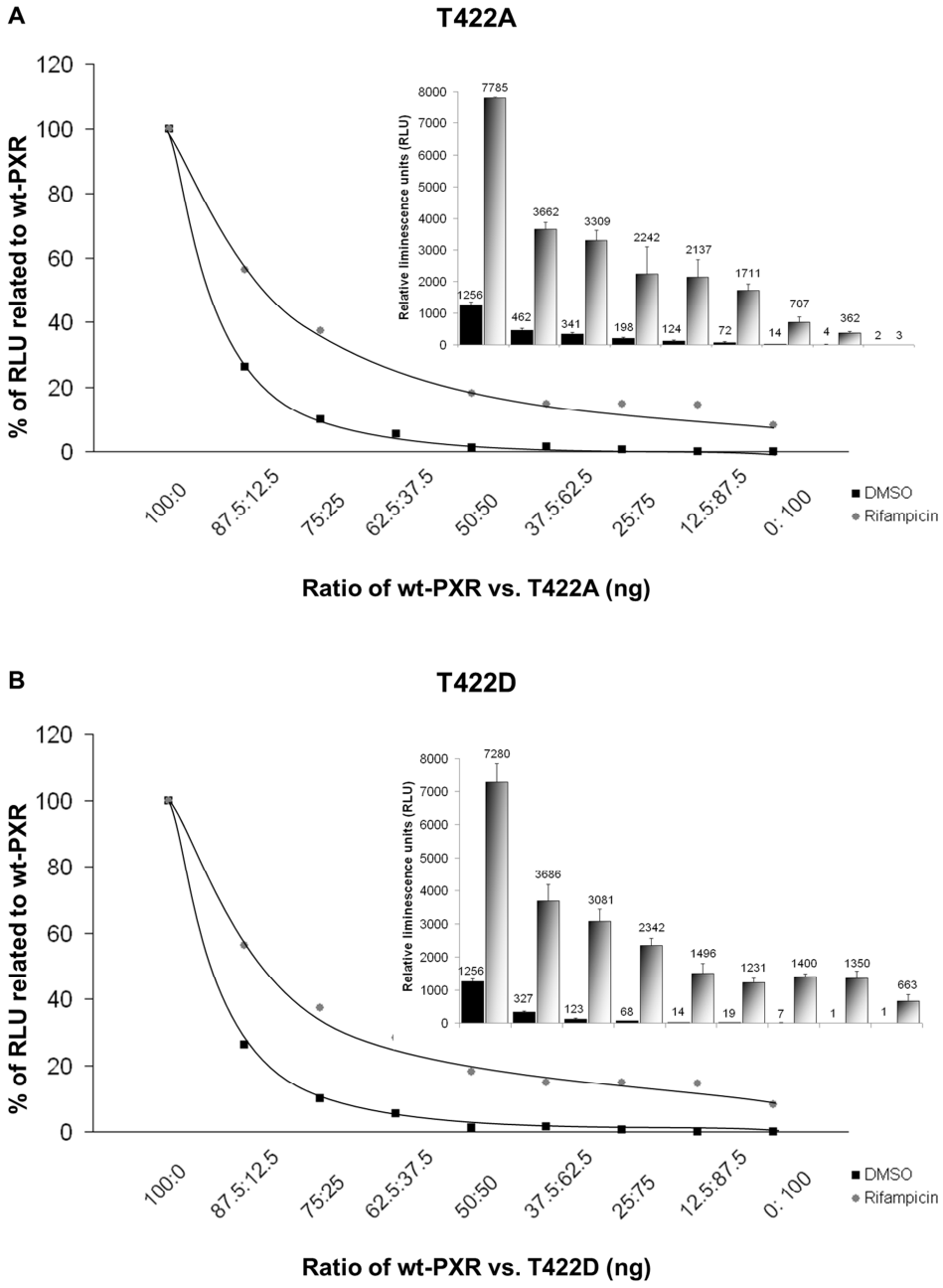

Figure 8. Dominant-negative effects of T422 mutations on transcriptional activity of PXR in HepG2 cells. HepG2 cells were transiently transfected by lipofection with 0.3 µg of p3A4-luc plasmid and with different combinations of PXR expression vectors in a total amount of 0,1 µg. The mixture contained different ratios of wt-PXR and mutant T422A **(a)** or T422D **(b)** expression constructs. Cells were treated for 24 hours with a vehicle (DMSO; 0.1 % v/v) or rifampicin (10 µM). After the treatments, cells were lysed and luciferase activity was measured. The data are presented as mean ± SD from triplicate measurements and they are expressed as a percentage of RLU relative to wt-PXR transfected cells with or without RIF (set to 100 %, plot graphs) or as absolute RLU (inserted bar graphs).

5.4 Effects of mutations in PXR on the expression of CYP3A4 mRNA and protein

Next, we examined the impact of phospho-mimetic and phospho-deficient mutations of PXR on basal and ligand-inducible expression of CYP3A4 mRNA and protein. HepG2 cells were transiently transfected with an empty pcDNA3 plasmid as a control or a wt-PXR plasmid or individual PXR mutants. Following 16 hours of incubation, the cells were treated for 24 and 48 hours with vehicle (DMSO) or 10 µM rifampicin. Expression of CYP3A4 mRNA in cells overexpressing the wild-type PXR was significantly induced by RIF as compared with the empty plasmid pcDNA3. This effect of RIF in pcDNA3 mock-transfected cells suggests transcriptional activity of endogenous PXR. Basal and rifampicin-inducible CYP3A4 mRNA expression was significantly ($p<0.05$) decreased for T422D; for T422A mutant, the effect was not statistically significant. This finding correlates well with the proposed dominant negative effects of T422 mutants (Figure 8). Cells overexpressing phospho-deficient mutants T248V or Y249F had similar level of CYP3A4 mRNA as those transfected with wild-type PXR. Transfection of HepG2 cells with T248D significantly ($p<0.05$) increased the basal level of CYP3A4 mRNA, but decreased the rifampicin-mediated induction of CYP3A4 mRNA. This could have occurred due to the constitutive activation of the CYP3A4 promoter without the presence of a ligand, as observed in Figure 7. Interestingly, phospho-mimetic mutation at Y249 abolished the rifampicin-mediated induction of CYP3A4 mRNA (Figure. 4a). Basal levels of CYP3A4 protein were significantly increased in HepG2 cells transfected with wt-PXR, T422A, T422D or T248D plasmids as compared to non-transfected HepG2 cells. Nevertheless, basal expression of CYP3A4 protein was lower in cells expressing T422D and T248D mutants in comparison with wt-PXR. Rifampicin strongly induced CYP3A4 protein expression in HepG2 cells transfected with wt-PXR, T422A, T422D, T248V or Y249F plasmids (Figure 9b). The basal and rifampicin inducible expression of CYP3A4 protein was decreased in cells transfected with the Y249D plasmid, which is in agreement with the CYP3A4 mRNA expression data. Induction of CYP3A4 protein was weak in cells transfected with T248D plasmid, which corresponds to the effects observed at mRNA level (Figure 9a).

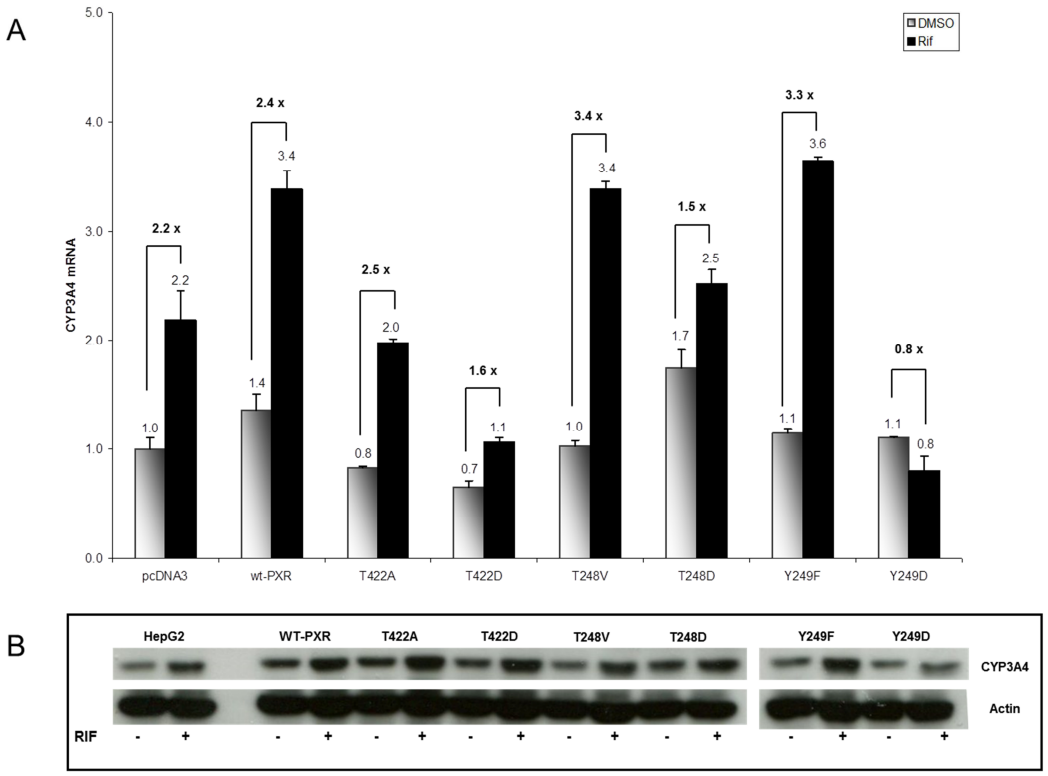

Figure 9. The effects of PXR mutants on CYP3A4 mRNA and protein expression in HepG2 cells. HepG2 cells were transiently transfected with 1.3 µg expression vector encoding either wild type or PXR mutants. Cells were treated with a vehicle (DMSO; 0.1 % v/v) or rifampicin (10 µM) for 24 or 48 hours, respectively. qRT-PCR analyses of CYP3A4 mRNA (bar graphs) **(a)** and Western blots of CYP3A4 (autoradiograms) **(b)** are shown. The qRT-PCR data are presented as mean ± SD from triplicate measurements and are expressed as fold induction over DMSO-treated cells transfected with an empty pcDNA3 plasmid. qRT-PCR data were normalized per GAPDH mRNA levels of a reference housekeeping gene. Differences were considered statistically significant for $p<0.05$ to wt-PXR transfected cells treated either with DMSO (0.1 %) or RIF (10 µM).

5.5 Protein–protein interaction between PXR mutants and RXRα

The nuclear receptor PXR is transcriptionally active in the form of a protein complex with several transcriptional co-activators and a heterodimerization partner RXRα (Frank et al., 2005; Goodwin et al., 1999). Lichti-Kaiser et al. showed that phospho-mimetic mutations of S305, S350 and T408 within the PXR ligand-binding domain interfere with PXR-RXRα dimerization (Lichti-Kaiser et al., 2009a). Therefore, we examined whether the mutations of residues forming a connection between PXR LBD and AF2 domain influence the interaction of PXR with RXRα. Expression constructs encoding RXRα, wild-type PXR and mutant PXR proteins were transcribed and translated *in vitro*. The interaction between PXR proteins and RXRα was studied by co-immunoprecipitation, using an anti-RXRα. The co-immunoprecipitated complexes

were eluted in two fractions, and the presence of PXR proteins was detected using an anti-PXR employing Western blot analysis. *In vitro* translated wild type PXR was used as control. We observed that the wild-type PXR protein was co-immunoprecipitated with the RXRα protein, as expected. We further verified the specificity of the co-immunoprecipitation in detecting PXR-RXRα heterodimerization by co-immunoprecipitation of RXRα alone, PXR alone and free lysate using the anti-PXR. We found that there are not substantial differences in the ability of individual PXR mutants to form heterodimers with RXRα, as compared to wt-PXR (Figure 10). These data suggest that tested mutations do not significantly affect the interactions of PXR with its heterodimer partner RXRα and that the effects of the mutations on CYP3A4 gene transactivation (Figure 10) are not due to impaired PXR-RXRα interaction.

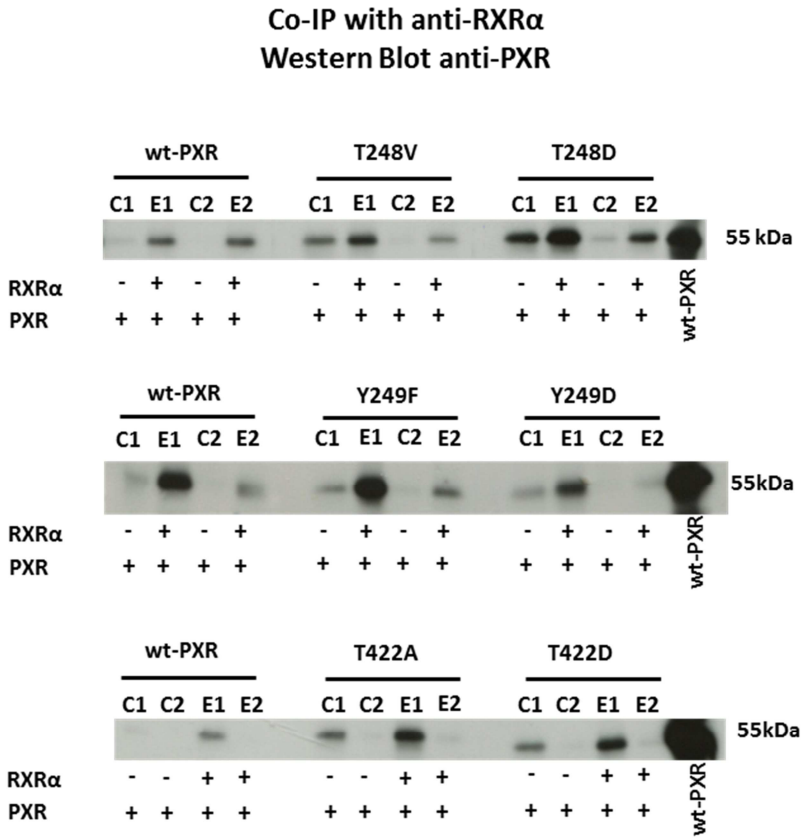

Figure 10. Protein–protein interactions between tested mutants of PXR and RXRα. RXRα antibody was coupled to AminoLink Plus Coupling Resin. *In vitro* translated RXRα, wild-type and mutated PXRs were combined and incubated at laboratory temperature for 30 minutes. Co-immunoprecipitation was performed as described under ‘‘Materials and methods” section. Eluted proteins containing PXR alone as a control (C1, C2) and complex of RXRα-PXR protein fractions (E1, E2) were resolved using 4–8 % SDS-PAGE and immunoblotted with antibody detecting PXR.

5.6 Binding of PXR mutants to DR3 motif of human CYP3A4 gene promoter

PXR-mediated gene activation requires direct binding of the PXR-RXRα heterodimeric complex to the response elements in the gene promoter. A xenobiotic-responsive enhancer module (XREM) located between -7.8 and -7.2 kb upstream of the CYP3A4 transcription start site containing a functional DR3 nuclear receptor-binding element 1 (dNR1; -7733/-7719) (Goodwin et al. 1999). The response element is crucial for both ligand-dependent PXR-inducible and basal transactivation of CYP3A4 (Goodwin et al. 1999). Nuclear extracts from HeLa cells transfected with wild-type PXR and/or mutated PXR proteins together with RXRα protein 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 demonstrated by competition with non-labeled double-stranded DR3 oligonucleotide or the mutant DR3 oligonucleotide (Figure 11a). Furthermore, we confirmed that incubation with an antibody against RXRα or PXR supershifted the PXR-RXRα oligonucleotide complex (Figure 11b). The binding of T422A, Y249D and Y249F mutant proteins to the DR3 sequence was comparable with that of wt-PXR. On the other hand, T422D, T248V and T248D mutations caused a strong decrease in DNA-binding activity of mutated PXR proteins (Figure 11c). Immunoblot analysis verified that equal amounts of PXR proteins were loaded in the gel shift assay (Figure 11d). Taken together, the phospho-mimetic mutation at T422 and both mutations at T248 decrease the ability of PXR to bind to its response elements DR3 within the promoter sequences of its target CYP3A4 gene.

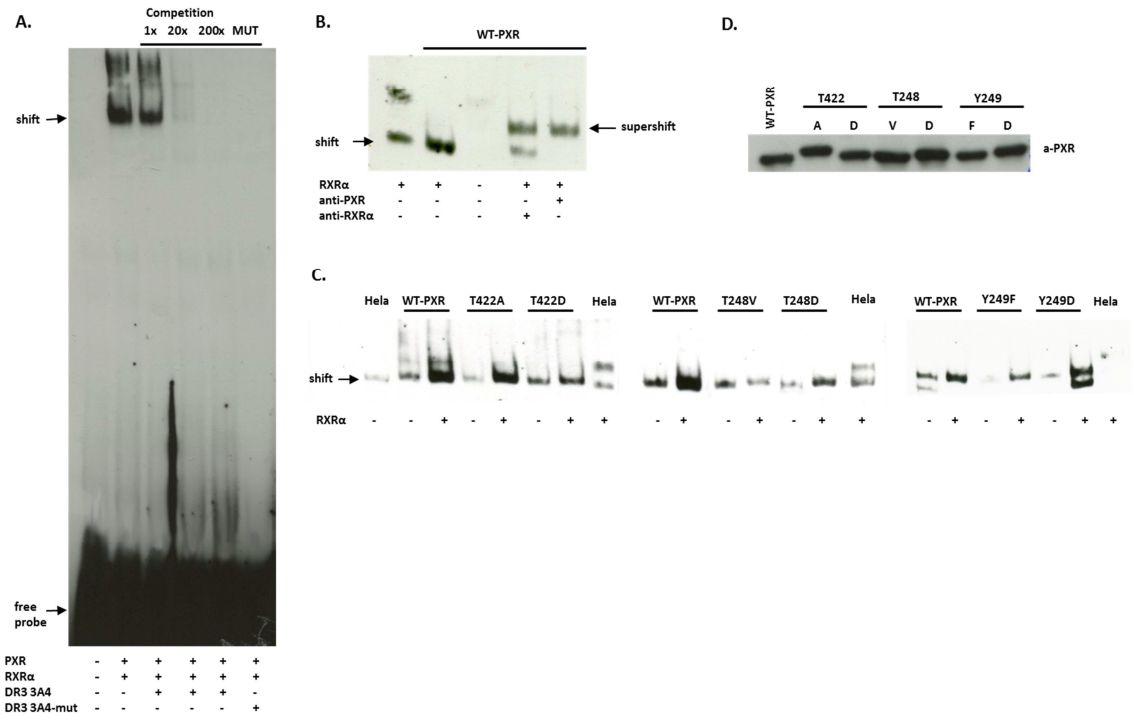

Figure 11. Binding of PXR mutants to DR3 motif of human CYP3A4 promoter as determined using an electrophoretic mobility shift assay. Nuclear extracts from HeLa cells transfected with PXR, PXR mutants or RXRα cDNAs, respectively, were incubated with a biotin-labeled CYP3A4-DR3 probe and electrophoresed on 5 % polyacrylamide gel as described in the ‘‘Materials and methods” section. The complex formation of the CYP3A4 DR3 response element with PXR alone or PXR-RXRα heterodimer was examined. **a)** In competition experiments, a biotin-labeled probe was incubated with nuclear extract containing PXR and RXRα proteins and 1-, 20- and 200-fold excess of unlabeled oligonucleotides in the binding reaction. The last line (MUT) represents the control experiment with a mutated biotin-labeled probe that was incubated with a nuclear extract containing PXR and RXRα proteins; **b)** supershift experiment with an antibody against PXR or RXRα; **c)** biotin-labeled CYP3A4-DR3 probe was incubated with nuclear extract from non-transfected HeLa cells, PXR or PXR mutants alone or together with RXRα; **d)** PXR protein level in used nuclear extracts of studied PXR mutants was analyzed by Western blot.

6 DISCUSSION

In the present thesis, I have described an optimized non-radioactive electrophoretic mobility shift assay, allowing studying interactions between pregnane X receptor and promoter DNA sequences. Traditionally, radiolabeled DNA-probes were used in studies of PXR-mediated mechanism transactivation of its target genes. Because of health, safety and environmental issues, non-radioactive methods are increasingly being developed. We used a chemiluminescent gel shift assay technique, where the biotin labeled probe is bound to a streptavidin-horseradish peroxidase, eliminating the need for labeling with radioactive compounds and allowing very sensitive chemiluminescence detection (~ 5-50 pg/band). The main advantages of this method are safety, low cost and shorter time of detection. It is important to note that reticulocyte lysate used to prepare proteins *in vitro* is unsuitable for a non-radioactive EMSA, because of the presence of biotinylated proteins that interfere with the assay. A further benefit of biotinylated DNA is that the same DNA used for EMSA can be coupled to streptavidin-coated agarose beads and used in affinity purification of DNA-binding proteins (Hagenbuchle & Wellauer, 1992).

It was also necessary to optimize a method for the study of protein-protein interactions between wild-type or mutated PXR with RXRα. For this purpose, we choose co-immunoprecipitation, one of the most widely used methods, where the target protein complex is captured by a specific antibody. First, an A/G Plus-Agarose beaded support was used. Unfortunately that did not work as expected. The main problem was a high positive signal in the negative control experiment probably caused by the non-specific binding of prey protein with Protein A/G Plus agarose. The co-elution of antibody light and heavy chains (25 and 50 kDa bands in reducing SDS-PAGE gels, respectively) from support together with the protein complex was also detected. While the co-IP methodology is straightforward, performing a co-IP reaction and identifying physiological protein-protein interactions can be difficult. This is because of the nature of the interaction, nonspecific binding to IP components and antibody contamination, all of which may mask detection. Amine-reactive resin allowing covalent antibody immobilization was used to perform a properly controlled co-IP experiment without false positive results during gel analysis.

Pregnane X receptor is a metabolic (Xie et al., 2001) and toxicological ‘‘sensor’’ (Harmsen et al., 2007; Lehmann et al., 1998) that regulates the expression of drug-metabolizing enzymes, but also genes involved in lipid and carbohydrate metabolism

(Dai et al., 2008; Lee et al., 2008; Roth et al., 2008; Zhou et al., 2008a; Zhou et al., 2006c). PXR is transcriptionally activated by a variety of structurally unrelated ligands, including drugs (Bertilsson et al., 1998; Drocourt et al., 2001; Jones et al., 2000; Lehmann et al., 1998), environmental pollutants (Coumoul et al., 2002; Pacyniak et al., 2007; Zhang et al., 2008), and synthetic and natural compounds (Goodwin et al., 2003; Moore et al., 2002; Staudinger et al., 2001a; Staudinger et al., 2006). Transcriptional activation of nuclear receptors, including PXR, may also occur in the absence of a ligand, and the role of covalent protein modification by phosphorylation was anticipated in both ligand-dependent and ligand-independent activation of PXR (Graves & Krebs, 1999). While ligand binding is the primary mechanism of PXR activation in hepatocytes, it is likely that posttranslational modification of PXR and PXR-associated proteins is involved in fine-tuning its activity in response to environmental stimuli and alterations in disease status (Lichti-Kaiser et al., 2009a). Indeed, phosphorylation of hepatic nuclear receptors regulates their functions including transactivation capacity, DNA binding, subcellular location, capacity to interact with protein cofactors and protein stability (Hunter & Karin, 1992; Karin, 1994). Therefore, in the present thesis, potential phosphorylation sites within the human PXR protein that have not been characterized before were examined. Recently, Lichti-Kaiser with co-workers performed a systematic analysis of predicted consensus serine and threonine phosphorylation sites in PXR (Lichti-Kaiser et al., 2009a). However, some residues with low score and potential phosphorylation sites at tyrosine have not been analyzed so far. A sequence alignment revealed that T248 is conserved in the other NR1I subfamily members. In the crystal structure of human PXR (Watkins et al., 2001), the side chain of T248 is hydrogen bonded to T422 in a-helix 12. Published data also indicate that the hydrogen-bonding interaction of T248 with T422 is critical for PXR, if not essential, to retain the activation by rifampicin (Ueda et al., 2005). Therefore, the role of residues T422, T248 and tyrosine residue Y249 were studied in the present thesis. Phospho-deficient mutant T248V exhibited very low basal and ligand-inducible transactivation capacity in reporter gene assay analysis. Contrarily, phospho-mimetic mutation at position T248 resulted in constitutive activation of the CYP3A4 promoter independently of the presence of a ligand (Figure 7), indicating that the position of T248 is critical for transcriptional activity of PXR. The ligand independent constitutive activation also occurred with the phospho-mimetic T248D mutant in the expression of CYP3A4 mRNA and protein (Figure 9). In contrast, both the phospho-deficient and the phospho-mimetic mutant of T422 drastically diminished the transactivation of the CYP3A4 promoter. Ueda et al. showed that the interaction of T176 with T350 in mCAR is responsible for constitutive activity of mCAR, and it is analogous to the interaction between T248 and T422 in α-helix 12 of

human PXR (Wang et al., 2007; Watkins et al., 2001). Since wild-type PXR is not constitutively active, it is surprising that its constitutive activity was achieved by phospho-mimetic mutation of T248 of PXR. These facts indicate that residues T248 and T422 could be the structural determinants of PXR activity. We also investigated whether mutations of PXR affect the formation of PXR-RXRα heterodimer, which is a crucial step in the transcriptional function of PXR. We show that all studied PXR mutants formed a heterodimer with RXRα at a similar level to that observed with wt-PXR. However, it was demonstrated elsewhere that mutations at S8, S305, S350 and T408 decrease the ability of PXR to form a heterodimer with RXRα (Lichti-Kaiser et al., 2009a). Replacement of T248 with Ala or Val abrogated the interaction of PXR with SRC-1 (Ueda et al., 2005), which is consistent with our findings that T248V mutation diminished the receptor transcriptional activity and binding to DNA, as revealed by gene reporter assay analysis and EMSA, respectively. We used the technique of EMSA to examine the ability of PXR mutants to bind to DR3 motif of CYP3A4 promoter. The mutant protein T422A bound to DR3 with similar strength as wt-PXR, whereas the binding of T422D was substantially decreased. It was shown elsewhere that mutation T57D, a potential phosphorylation site for p70 S6K, completely abolished the binding of PXR to the promoter sequences of its target genes (Lichti-Kaiser et al., 2009a). The primary reason for a loss of PXR function by mutation of T422 is probably not the phosphorylation, because both phospho-deficient and phospho-mimetic mutation aborted the PXR activity. Decrease in transcription activity could be caused by another mechanism, for instance, changes or loss in co-activator binding (Wang et al., 2007). We found that both T248V and T248D mutants retained their ability to bind to DNA *in vitro*, but to a lesser extent as compared to wt-PXR. While the mutation of residue Y249 had an impact on PXR transcriptional activity, we did not find the changes in DNA binding and therefore this is probably not a critical residue playing a role in phosphorylation. We can conclude that additional experiments are needed to confirm whether any serine/threonine or tyrosine kinases are involved in a cellular signaling pathway phosphorylate PXR at T248, Y249 or T422 residues. This finding would bring an important mechanistic link between xenobiotic metabolism gene expression controlled by PXR and cellular signaling pathways.

7 CONCLUSION

In my thesis I characterized the function of three putative phosphorylation sites within human PXR and optimized two methods, namely Co-IP and non-radioactive EMSA, which were necessary for this study. In particular, I observed the following:

1. The best arrangement to study the binding of PXR to DNA is non-radioactive EMSA using a biotin-labeled probe and nuclear extracts from HeLa cells, which were transfected by an appropriate plasmid (PXR, RXRα). In contrast I found that *in vitro* translated proteins are not suitable for non-radioactive detection by a biotin-labeled probe, because of the presence of biotinylated proteins in reticulocytes lysate
2. The co-IP employing Protein A/G Plus Agarose exhibited high positive signal in the negative control experiment. This was probably caused by the non-specific binding of prey protein with support and co-elution of antibody light and heavy chains. This problem was solved by using resin activated with aldehyde groups to allow the covalent immobilization of antibodies
3. Examination of potential phosphorylation sites within the human PXR protein at residue T248, Y249 and T422 showed:
    a. Position T248 is critical for transcriptional activity of PXR. Phospho-deficient mutant T248V exhibited very low basal and ligand inducible transactivation capacity. Contrarily, phosphor-mimetic mutant T248D resulted in the constitutive activation of the CYP3A4 promoter independent of the presence of a ligand. This indicate the importance of PXR activation in a ligand-independent manner
    b. T248V and T248D mutants retained their ability to bind to DNA in vitro, but to a lesser extent compared to wt-PXR
    c. Loss of PXR function by mutation of T422 is probably not due to the phosphorylation, because both phospho-deficient and phospho-mimetic mutation aborted the PXR activity
    d. Constitutive activity achieved by the phospho-mimetic mutant indicates that residues T248 and T422 could be the structural determinants of PXR activity
    e. All studied PXR mutants formed a heterodimer with RXRα at a similar level to that observed with wt-PXR
    f. Y249 is probably not a critical residue playing a role in phosphorylation

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9 Curriculum Vitae

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Education

Present study 2009-2013:

Postgraduate studies in biochemistry in the Department of Cell Biology and Genetics, Faculty of Science, Palacky University Olomouc

Thesis topic: Role of post-translational modifications in a function of pregnane X receptor (academic advisor Prof. Zdeněk Dvořák, moulin@email.cz)

Completed study 2007-2009:

Master's program in Biochemistry, Palacky University in Olomouc

Thesis topic: *Substrate specificity of isopentenyltransferases for de novo cytokinin synthesis*

Completed study 2004-2007:

Bachelor's program in Biochemistry, Palacky University in Olomouc

Thesis topic: *Effect of individual isomers of cytokinin isopentanyladenin on expression of ARR5 gene in receptor mutants of Arabidopsis thaliana*

Educational activity

Practical courses from cell biology I.

Practical courses from molecular biology II.

Practical courses from selected methods of studying cellular processes

Overview of research internship

August – November 2012

Short-term research stays at Albert Einstein College of Medicine, NYC, USA (3 months)

August 2011

FEBS Advanced Lecture Course co-sponsored by CRESCENDO

June 2010

Short-term research stays at Chemical Research Institute of Hungarian Academy of Sciences, Budapest (1 month)

Participation in projects (principal investigator)

FRVŠ 10/2012/G3 – Study of protein expression in models of human cancer cell lines *in vitro* (2012)

Participation in projects (researcher)

1. 13-07711S - The role of Hippo signaling pathways and deregulation of organic toxicants in liver cell function and metabolism of xenobiotic. Czech Scientific Foundation (2013 – 2016)
2. P303/12/G163 – Center of Excellence: Centre of Drug-Dietary Supplements Interactions and Nutrigenetics. Czech Scientific Foundation (2012 – 2018)
3. P303/12/0472 - Novel mechanisms of CYP3A4 enzyme regulation based on posttranslational modification of nuclear receptors - implications for pharmacotherapy. Czech Scientific Foundation (2012 – 2016)
4. GA303/07/0128 - Study on tissue-specific aspects of transcriptional regulation of selected P-450 enzymes and drug transporters (2007 – 2011)
5. GA304/10/0149 - Generation of hepatocyte-derived cellular models for preclinical testing of drugs (2010 – 2012)
6. OPVK CZ.1.07/2.2.00/28.0088 - Implementation of laboratory medicine in the education system at Palacky University in Olomouc (2012 – 2014)
7. OPVK CZ.1.07/2.3.00/20.0062 – Antabuse (disulfiram) as a pilot case of nonprofit drug, (2011 – 2014)
8. OPVK CZ 1.07/2.2.00/07.0354 - Innovation studies of molecular and cellular biology (2009 – 2012)

Publications

1. **Vavrova A.**, Vrzal R., Dvorak Z. (2013) A non-radioactive electrophoretic mobility shift assay for measurement of pregnane X receptor binding activity to CYP3A4 response element. *Electrophoresis (in press)* **[IF_{2011} 3.303]**

2. **Doricakova A.**, Novotna A., Vrzal R., Pavek P., Dvorak Z. (2012) The role of residues T248, Y249 and T422 in the function of human pregnane X receptor. *Arch. Toxicol*. **87**:291-301 **[IF_{2011} 4.674]**

3. Novotna A., **Doricakova A.**, Petr P., Dvorak Z. (2012) Construction and characterization of peroxisome proliferator-activated receptor-gamma co-activator 1 alpha (PGC-1α over-expressing cell line derived from human hepatocyte carcinoma HepG2 cells) *Biomed Papers (in press).* **[IF_{2011} 0.702]**

4. Novotna A, **Doricakova A.,** Vrzal R, Pavek P, Dvorak Z. (2011) Construction and characterization of hepatocyte nuclear factor HNF4alpha1 over-expressing cell line derived from human hepatoma HepG2 cells. Eur J Pharmacol. **669**:45-50 **[IF_{2011} 2.516]**

5. Vrzal R., **Doricakova A.**, Novotna A., Bachleda P., Bitman M., Pavek P., Dvorak Z. (2010) Valproic acid augments vitamin D receptor-mediated induction of CYP24 by vitamin D3: a possible cause of valproic acid-induced osteomalacia? *Toxicol Lett.* **200**:146-53 **[IF_{2011} = 3.230]**

6. Novotna A., **Doricakova A.**, Vrzal R., Maurel P., Pavek P., Dvorak Z. (2010) Investigation of Orlistat effects on PXR activation and CYP3A4 expression in primary human hepatocytes and human intestinal LS174T cells. *Eur J Pharm Sci* **41**:276-280 **[IF_{2011} = 3.291]**

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1. Vrzal R., Novotna A., **Doricakova A.,** Bitmann M., Pavek P., Dvorak Z.: Valproic acid potentiates vitamin D receptor-mediated induction of CYP24 gene – A consequence for drug-induced osteomalacia. *FEBS Journal* **277(Suppl 1)**, p153 (2010. 35th FEBS Congress, Molecules of Life, June 26th – July 1st, 2010, Gothenburg, Sweden.
2. Vrzal R., **Doricakova A.,** Novotna A., Pavek P., Dvorak Z.: Examination of Orlistat effects on PXR-CYP3A4 signaling in hepatic and intestinal cells. 15th Interdisciplinary Toxicological Conference – TOXCON 2010, Stara Lesna, Slovensko, 8.9.-11.9.2010. *Interdisciplinary Toxicology* **3(3)**:p. A91-A92, 2010.
3. Novotna A., **Doricakova A.,** Vrzal R., Pavek P., Dvorak Z.: Construction of stably-transfected reporter cell lines. 15th Interdisciplinary Toxicological Conference – TOXCON 2010, Stará Lesná, Slovensko, 8.9.-11.9.2010. *Interdisciplinary Toxicology* **3(3)**:p. A71, 2010.
4. **Doricakova A.,** Novotna A., Vrzal R., Pavek P., Dvorak Z.: Role of Thr422 residue in PXR transcrriptional activity. 15th Interdisciplinary Toxicological Conference – TOXCON 2010, Stara Lesna, Slovensko, 8.9.-11.9.2010. *Interdisciplinary Toxicology* **3(3)**:p. A40-A41, 2010.
5. **Doricakova A.,** Novotna A., Vrzal R., Pavek P., Dvorak Z.: The role of residues Thr248, Tyr249 and Thr422 in the functions of human pregnane X receptor. 16th Interdisciplinary Toxicological Conference – TOXCON 2011, Praha, 17.5.-20.5.2011. *Interdisciplinary Toxicology* **4(2)**:p. A28, 2011.
6. Novotna A., **Doricakova A.,** Vrzal R., Pavek P., Dvorak Z.: Construction of stable cell line over-expressing hepatocyte nuclear factor HNF4alpha. 16th Interdisciplinary Toxicological Conference – TOXCON 2011, Praha, 17.5.-20.5.2011. *Interdisciplinary Toxicology* **4(2)**:p. A52, 2011.
7. **Doricakova A.,** Novotna A., Vrzal R., Pavek P., Dvorak Z.: The role of residues T248, Y249 and T422 in the function of human pregnane X receptor. 19th International Symposium on Microsomes and Drug Oxidations; 12th European Regional ISSX Meeting; Noordwijk aan Zee, The Netherlands; 17-21 June 2012; p221-222.
8. **Vavrova A**., Vrzal R., Pavek P., Dvorak Z.: Binding of pregnane X receptor mutants to DR3 motif of human CYP3A4 gene promoter with the use of non-radioactive electrophoretic mobility shift assay (EMSA). Programme and Abstracts from international conference Chemical Biology: Methods and Progress, Vienna, Austria, 11.-12.2.2013; p.41

APPENDIX I.

Doricakova A., Novotna A., Vrzal R., Pavek P., Dvorak Z. (2012) The role of residues T248, Y249 and T422 in the function of human pregnane X receptor. *Arch Toxicol* 87(2):291-301

Arch Toxicol
DOI 10.1007/s00204-012-0937-9

MOLECULAR TOXICOLOGY

The role of residues T248, Y249 and T422 in the function of human pregnane X receptor

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Received: 14 June 2012 / Accepted: 28 August 2012

Abstract The pregnane X receptor (PXR) is a key xenobiotic receptor that regulates the expression of numerous drug-metabolizing enzymes. Some posttranslational mechanisms modulate its transcriptional activity. Although several kinases have been shown to directly phosphorylate this receptor, little is known about phosphorylation sites of PXR. In the present work, we examined T248, Y249 and T422 putative phosphorylation sites determined based on in silico consensus kinase site prediction analysis. T248 and T422 residues are critical for the interaction of the PXR ligand-binding domain and the activation function-2 (AF2) domain. Site-directed mutagenesis analysis was performed to generate phospho-deficient and phospho-mimetic mutants. We examined transactivation activity of the PXR mutants in gene reporter assays, formation of PXRmutant/RXRα heterodimer, binding of PXR mutants to the CYP3A4 gene response element DR3 and CYP3A4 expression in HepG2 cells after expression of the mutants. We found that T248D mutant activated CYP3A4 transactivation constitutively regardless of the presence or absence of a ligand. Contrary, T248V mutant exhibited low basal and ligand-inducible transactivation capacity as compared to wild-type PXR. Dose–response analysis revealed reduced ligand-dependent transactivation potency of PXR Y249D mutant. Transactivation of the CYP3A4 promoter was abolished with T422A/D mutants. All PXR mutants formed heterodimer with RXRα at a similar level to that observed with wild-type PXR. The ability to bind to DNA in vitro was substantially decreased in case of T248D, T422D and T248V mutants. Our data thus indicate that phosphorylation of T248, Y249 and T422 residues may be critical for the both basal and ligand-activated function of PXR.

Keywords Drug metabolism · Pregnane X receptor · Cytochrome P450 · Phosphorylation

Abbreviations

Cdk2	Cyclin-dependent kinase 2
DBD	DNA-binding domain
EMSA	Electrophoretic mobility shift assay
LBD	Ligand-binding domain
PKA	Protein kinase A
PKCα	Protein kinase C alpha
PXR	Pregnane X receptor
RXRα	Retinoid X receptor alpha
SRC-1	Steroid receptors coactivator 1

Introduction

Pregnane X receptor (PXR; NR1I2) is a ligand-activated orphan nuclear receptor that belongs to the family of steroid/thyroid/retinoid superfamily of receptors (Kliewer et al. 1998; Lehmann et al. 1998). In its active state, PXR forms a heterodimer with retinoid X receptor alpha (RXRα, NR2B1) (Pascussi et al. 2008). PXR is a master regulator of drug-metabolizing enzymes, including CYP 2B6, CYP2C8, CYP2C9, CYP3A4 (Pascussi et al. 2008), some phase II enzymes (Rushmore and Kong 2002) and

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Published online: 14 September 2012

transporters (Klaassen and Slitt 2005). In addition, PXR controls the expression of numerous genes involved in metabolism of steroid hormones, vitamin D, lipids, carbohydrates or cholesterol (Moreau et al. 2008; Wada et al. 2009). PXR is activated by a variety of structurally unrelated xenobiotics (drugs, food constituents, chemicals) (Ngan et al. 2009; Waxman 1999). In addition, PXR is activated also by eubiotics, for example, vitamin E, vitamin K, bile acids and beta-carotene (Igarashi et al. 2007; Ruhl 2005; Traber 2004). Transcriptional activity of PXR is primarily dependent on the presence of a ligand that binds PXR in a C-terminal ligand-binding domain (LBD) flanked with a ligand-dependent activation function 2 (AF2).

However, ligand-dependent activation is not the sole determinant of PXR transcriptional activity. There are many indications on the role of phosphorylation in the modulation of PXR transcriptional activity. Several studies revealed that PXR could be a substrate for protein kinases in vitro. Forskolin, an activator of protein kinase A (PKA), induced Cyp3a in a ligand-dependent manner in mice and rat hepatocytes (Dowless et al. 2005). In addition, forskolin influenced the interaction of PXR with steroid receptors coactivator 1 (SRC-1) and corepressor NCoR. PXR-GST pull-down assay revealed that PKA phosphorylates PXR in both ligand-binding domain and DNA-binding domain (Ding and Staudinger 2005a; Dowless et al. 2005). In follow-up study, the same group demonstrated that interaction between PKA and PXR occurs in a species-specific manner (Lichti-Kaiser et al. 2009b). In contrast, activation of protein kinase C alpha (PKCα) repressed PXR activity by increasing the strength of interaction between PXR and NCoR corepressor and by abolishing the interaction between PXR and SRC-1 coactivator (Ding and Staudinger 2005b). Another study has shown that inhibition or activation of cyclin-dependent kinase 2 (Cdk2) led to the activation or attenuation of PXR-mediated CYP3A4 gene expression in HepG2 cells, respectively. A phospho-mimetic mutation (i.e., a mutation mimicking negatively charged phospho-moiety) of S350 impaired the function of PXR, whereas a phospho-deficient mutation (i.e., a mutation not allowing phosphorylation at this site) conferred resistance to the repressive effect of Cdk2 (Lin et al. 2008). Pondugula et al. showed that the phospho-mimetic T57 mutant exhibited a distinctive nuclear localization pattern, and this PXR mutant had impaired ability to bind to the CYP3A4 gene promoter. Furthermore, the role of p70 S6K kinase in regulation of PXR transactivation was demonstrated (Pondugula et al. 2009). The same group performed systematic site-directed mutagenesis of serine and threonine residues to generate phospho-mimetic and phospho-deficient mutations at 18 in silico predicted consensus kinase recognition sequences in the human PXR. Mutations at T57 and T408 abolished ligand-inducible PXR activity. Mutations at S8, S208, S305, S350 and T408 decreased the formation of PXR/RXRα heterodimer, and subcellular localization of PXR was affected by mutations at T408. S208, S305, S350 and T408 mutants displayed altered PXR protein/cofactor interactions (Lichti-Kaiser et al. 2009a).

In the current study, we have generated phospho-mimetic and phospho-deficient mutants of human PXR at residues T248, Y249 and T422. The putative phosphorylation sites were chosen based on in silico consensus kinase site prediction using NetPhos 2.0 server. Effect of phosphorylation at these sites has not been evaluated before. Importantly, the hydrogen-bonding interaction of T248 with T422 in helix-12 is critical for a ligand-dependent recruitment of SRC1 and for PXR activation with its ligands in target gene transactivation (Ueda et al. 2005). We examined the roles of T248, Y249 and T422 mutations in PXR transcriptional activity, in its promoter-binding activity, and interaction with the common heterodimerization partner retinoid X receptor alpha (RXRα).

Materials and methods

Materials

Oligonucleotide primers for qRT-PCR were purchased from Invitrogen (Grand Island, NY). LightCycler FastStart DNA Master^{PLUS} SYBR Green I and FuGENE HD transfection reagent were from Roche Diagnostic Corporation (Intes Bohemia, Czech Republic). Rifampicin and DMSO were from Sigma-Aldrich (Prague, Czech Republic). All other chemicals were of the highest quality commercially available.

Cell lines

Human Caucasian hepatocellular carcinoma cells HepG2 (ECACC No. 85011430) and Human Negroid cervix epitheloid carcinoma cells *HeLa* (ECACC No. 93021013) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % of fetal calf 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.

Plasmids

A chimeric p3A4-luc reporter construct containing the basal promoter (−362/+53) with proximal PXR response element (ER6) and the distal xenobiotic-responsive enhancer module (−7,836/−7,208) of the CYP3A4 gene 5′-flanking region inserted to pGL3-Basic reporter vector (Promega, Madison, WI) was described before (Goodwin et al. 1999). The expression plasmid for human PXR (pSG5-hPXR) was kindly provided by Dr. S. Kliewer (University of Texas, Dallas, TX), and expression plasmid pSG5-hRXRα encoding human RXRα was a generous gift from Dr. C. Carlberg (University of Kuopio, Kuopio, Finland).

Consensus tyrosine and threonine phosphorylation sites within the human PXR protein were identified using the in silico computer-based analysis with NetPhos 2.0 server. The human PXR protein sequence has been obtained from the NCBI website (Accessions AAH17304 and AF061056). The T422 and T248 residues have been previously reported as a potential phosphorylation sites, but have not been characterized (Lichti-Kaiser et al. 2009a). The side chain of Thr248 is hydrogen bonded to Thr422 in α-helix 12 in case of PXR. This interaction is critical for SRC1 coactivator recruitment to AF2 domain and for proper ligand-dependent PXR function in target genes transactivation (Ueda et al. 2005). Potential tyrosine phosphorylation site Y249 (Score 0.916, NetPhos 2.0 Server) has not been characterized before.

Site-directed mutagenesis of wild-type PXR (**wt-PXR**) was performed by GenScript (Piscataway, NJ, USA) and the following mutant plasmids were generated:

Position 248: Thr → Val (**T248V**), Thr → Asp (**T248D**)
Position 249: Tyr → Phe (**Y249F**), Tyr → Asp (**Y249D**)
Position 422: Thr → Ala (**T422A**), Thr → Asp (**T422D**)

Gene reporter assays

HepG2 cells (2×10^5/well) were transiently transfected employing lipofection (FuGENE HD, Roche) with 0.3 μg/well of p3A4-luc reporter construct and 0.1 μg/well of expression plasmid encoding wild-type PXR (wt-PXR) or its mutated forms. Cells were seeded in the medium supplemented with 10 % charcoal/dextran-stripped fetal bovine serum on 24-well plates and stabilized for 16 h prior to the treatments. Cells were treated for 24 h with rifampicin (RIF; 0.01–25 μM) and/or vehicle (DMSO; 0.1 % v/v). After the treatments, cells were lysed using luciferase detection system (Promega, Southampton, UK) and luciferase activity was measured on Infinite M200 luminometer (Tecan, Schoeller, Prague).

mRNA determination and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

Total RNA was isolated using TRI Reagent® (Invitrogen). cDNA was synthesized according to a common protocol, using M-MLV Reverse Transcriptase (Invitrogen) and random hexamers (Amersham Biosciences). qRT-PCR was carried out on Light Cycler apparatus 480 II (Roche Diagnostic Corporation, Prague, Czech Republic). The levels of CYP3A4 and GAPDH mRNAs were determined as described elsewhere (Pavek et al. 2007). The measurements were performed in triplicates. Expression of CYP3A4 was normalized *per* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a reference (housekeeping) gene. Data were processed by delta–delta method.

Western blotting

Preparation of total protein extracts was described elsewhere (Henklova et al. 2008). SDS–PAGE gels (8 %) were run on a Bio-Rad apparatus according to the general procedure. Protein transfer onto PVDF membrane was carried out. The membrane was saturated with 5 % non-fat dried milk in TBS-T buffer for 1 h at room temperature, followed by 2 h incubation with primary antibody and 1 h incubation with appropriate secondary antibody at room temperature. Chemiluminescence detection was performed using horseradish peroxidase-conjugated secondary antibody and Amersham (GE Healthcare) ECL kit.

Electrophoretic mobility shift assay (EMSA)

HeLa cells transfected with wild-type PXR cDNA, mutants of PXR (T248V, T248D, Y249F, Y249D, T422A, T422D) and/or RXRα cDNA, and the nuclear fraction was isolated as described elsewhere (Andrews and Faller 1991). The following double-stranded 5′-biotinylated oligonucleotides containing specific DNA-binding sequence for PXR (DR3 motif from the XREM sequence of *CYP3A4* gene promoter) were used as probes: CYP3A4-DR3 wild-type sense 5′-GAATGAACTTGCTGACCCTCT-3′; CYP3A4-DR3 antisense 5′-AGAGGGTCAGCAAGTTCATTC-3′ and their biotinylated forms; biotin-labeled CYP3A4-DR3 with mutations sense 5′-GAA**A**G**C**A**T**TTGC**A**GACCC TCT-3′ and antisense 5′-AGAGGGTC**T**GCAA**A**T**G**C**T** TTC-3′ (mutated bases in underlined-bold). The oligonucleotides were synthesized by Generi-Biotech (Hradec Kralove, Czech Republic). Gel mobility shift assay was performed using LightShift® Chemiluminescent EMSA Kit (Thermo Scientific, Waltham, MA, USA). For supershift experiments, 1 μg of the anti-RXRα rabbit polyclonal IgG antibody (sc-553 X, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or mouse monoclonal anti-PXRα

(sc-48340 X, Santa Cruz Biotechnology, Inc., USA) was added to the reaction mixture.

Protein co-immunoprecipitation (Co-IP)

Human PXR, its mutants and RXRα were translated in vitro using TNT Quick Coupled Transcription/Translation Systems (Promega, Southampton, UK). Following co-immunoprecipitation (Co-IP) experiment was performed with the Pierce Co-IP Kit (Thermo Scientific) according to the manufacturer's protocol. Fifteen micrograms of the polyclonal RXRα antibody (sc-553 X, Santa Cruz Biotechnology Inc.) were incubated with the delivered resin and covalently coupled. The antibody-coupled resin was incubated with 200 µL of diluted PXR:RXRα protein mixture overnight at 4 °C. The resin was washed and the protein complexes bound to the antibody were eluted. Western blot analyses were performed as described above. Proteins were boiled off the beads in 2× Laemmli sample buffer and resolved on 8 % SDS-PAGE gels followed by Western blot analysis and immunodetection with appropriate antibodies: anti-PXR (sc-48340 X, Santa Cruz Biotechnology, Inc., USA).

Statistical analyses

Results were expressed as mean ± SD. The one-way ANOVA followed by the Dunnett's multiple comparison post hoc test or the paired Student's *t*-test were used for statistical analyses using GraphPad Prism software (GraphPad Software Inc., San Diego, CA).

Results

Transcriptional activity of PXR mutants

Threonine and tyrosine residues within the human PXR protein were mutated to either a negatively charged phospho-mimetic residue aspartic acid (T422D, T248D, Y249D) or a phospho-deficient residue containing a hydrophobic side chain (T422A, T248V, Y249F) (Fig. 1).

First, we investigated the effects of these mutations on basal and ligand-inducible transcriptional activity of PXR in cell-based gene reporter assays. For this purpose, we transiently transfected HepG2 cells with expression plasmids encoding either wild-type or mutated PXR and p3A4-luc reporter plasmid, containing the basal promoter (−362/+53) with proximal response element (ER6) and the distal xenobiotic-responsive enhancer module (−7,836/−7,208) of the CYP3A4 gene 5′-flanking region. Cells were treated for 24 h with increasing concentrations of PXR agonist rifampicin (RIF).

Transfection with the luciferase gene reporter construct p3A4-luc alone shows weak, concentration-dependent activity after the treatment with RIF due to the activation of the CYP3A4 promoter construct by endogenous PXR present in HepG2 cells (Fig. 2a). In next experiments, we

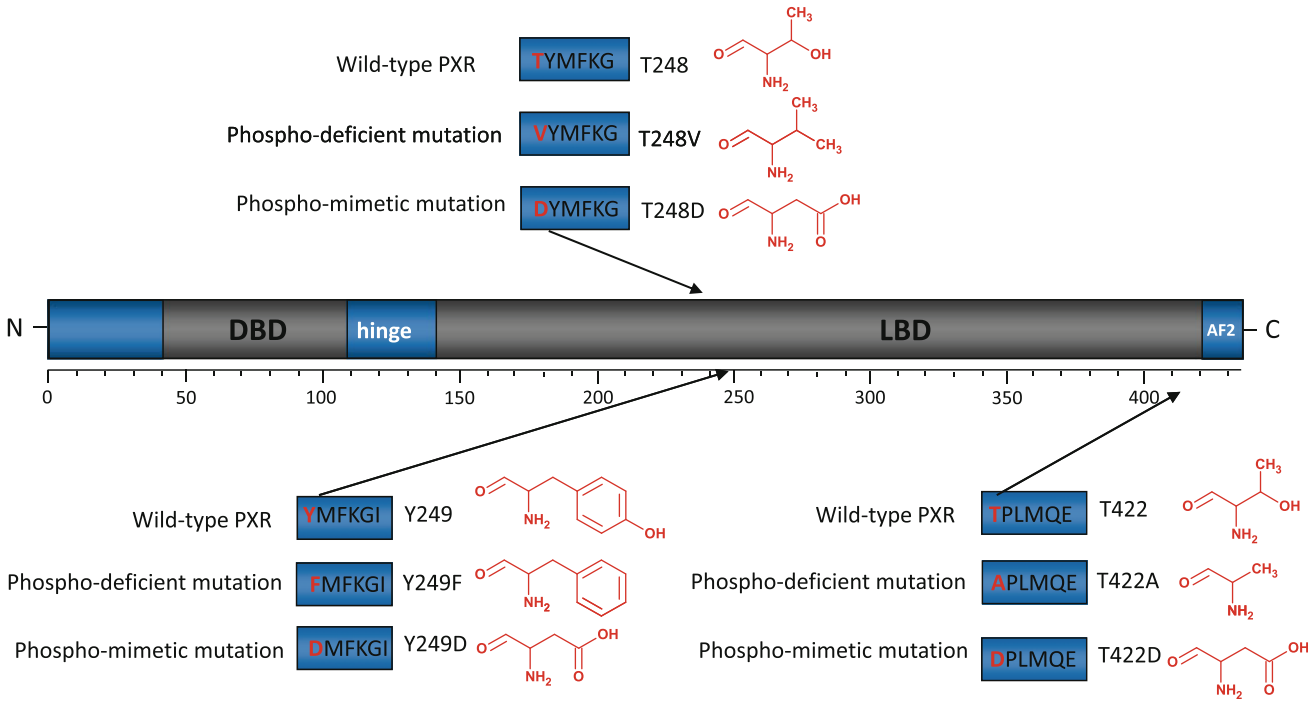

Fig. 1 Schematic representation of mutations in human PXR. Site-directed mutagenesis was performed to generate phospho-deficient (T248V, Y249F, T422A) and phospho-mimetic (T248D, Y249D, T422D) PXR mutants, as described in detail in "Materials and methods" section

cotransfected wt-PXR cDNA together with p3A4-luc reporter construct. Activation of wt-PXR by RIF was dose dependent, and maximal activity (about 15-fold) was attained at the concentration of 10 μM (Fig. 2b). A hydrophobic mutation of the T248 residue to Val (T248V) diminished basal and rifampicin-mediated transactivation of CYP3A4 promoter, suggesting an importance of T248 residue in PXR transcriptional activity (Fig. 2c). In contrast, phospho-mimetic mutation of PXR at T248 (T248D) resulted in constitutive activation of CYP3A4 promoter independently of the presence of PXR ligand (Fig. 2d). Phospho-deficient mutation of Y249 (Y249F) did not reveal significant change as compared to wt-PXR (Fig. 2e). Interestingly, phospho-mimetic mutation of Y249 (Y249D) decreased a transactivation potency of rifampicin to PXR receptor in transactivation of CYP3A4 gene reporter construct (Fig. 2f). With the construct, RIF at a concentration of 25 μM has the most significant effect on transactivation of p3A4-luc reporter construct. However, at low concentrations (0.1 μM), RIF was not able to significantly activate the reporter construct in cells expressing Y249D mutated PXR (Fig. 2f). Mutations at T422 (T422A, T422D) almost abolished the transactivation of the CYP3A4 promoter gene reporter construct (Fig. 2g, h).

We further studied quantitative effects of different ratios of wt-PXR/T422A and wt-PXR/T422D proteins co-expressed in HepG2 cells on CYP3A4 promoter transactivation. In the experiments, we examined whether T422 PXR mutants function as dominant negative mutants. For both mutants (T422A, T422D), transcriptional activity of the plasmids mix decreased in dose-dependent manner with decreasing portion of wt-PXR in the mix (Fig. 3a, b).

Effects of mutations in PXR on the expression of CYP3A4 mRNA and protein

Next, we examined the impact of phospho-mimetic and phospho-deficient mutations of PXR on basal and ligand-inducible expression of CYP3A4 mRNA and protein. HepG2 cells were transiently transfected with empty pcDNA3 plasmid as a control or wt-PXR plasmid or individual PXR mutants. Following 16 h of incubation, the cells were treated for 24 and 48 h with vehicle (DMSO) or 10 μM rifampicin.

Expression of CYP3A4 mRNA in cells overexpressing the wild-type PXR was significantly induced by RIF as compared with empty plasmid pcDNA3. This effect of RIF in pcDNA3 mock-transfected cells suggests transcriptional activity of endogenous PXR. Basal and rifampicin-inducible CYP3A4 mRNA expression was significantly ($p < 0.05$) decreased for T422D; for T422A mutant, the effect was not statistically significant. This finding well correlates with proposed dominant negative effects of T422 mutants (Fig. 3). Cells overexpressing phospho-deficient mutants T248V or Y249F had similar level of CYP3A4 mRNA as those transfected with wild-type PXR. Transfection of HepG2 cells with T248D increased significantly ($p < 0.05$) the basal level of CYP3A4 mRNA, but decreased rifampicin-mediated induction of CYP3A4 mRNA. This could refer to constitutive activation of the CYP3A4 promoter without the presence of a ligand, as observed in Fig. 2. Interestingly, phospho-mimetic mutation at Y249 abolished rifampicin-mediated induction of CYP3A4 mRNA (Fig. 4a).

Basal levels of CYP3A4 protein were significantly increased in HepG2 cells transfected with wt-PXR, T422A, T422D or T248D plasmids as compared to non-transfected HepG2 cells. Nevertheless, basal expression of CYP3A4 protein was lower in cells expressing T422D and T248D mutants in comparison with wt-PXR. Rifampicin strongly induced CYP3A4 protein expression in HepG2 cells transfected with wt-PXR, T422A, T422D, T248V or Y249F plasmids (Fig. 4b). The basal and rifampicin-inducible expression of CYP3A4 protein was decreased in cells transfected with Y249D plasmid, which is in agreement with CYP3A4 mRNA expression data. Induction of CYP3A4 protein was weak in cells transfected with T248D plasmid, which corresponds to the effects observed at mRNA level (Fig. 4a).

Protein–protein interaction between PXR mutants and RXRα

Nuclear receptor PXR is transcriptionally active in the form of protein complex with several transcriptional coactivators and heterodimerization partner retinoid X receptor-α (RXRα) (Frank et al. 2005; Goodwin et al. 1999). Lichti-Kaiser et al. (2009a) showed that phospho-mimetic mutations of S305, S350 and T408 within the PXR ligand-binding domain interfere with PXR-RXRα dimerization. Therefore, we examined whether the mutations of residues forming connection between PXR LBD and AF2 domain influence the interaction of PXR with RXRα. Expression constructs encoding RXRα, wild-type PXR and mutant PXR proteins were transcribed and translated in vitro. The interaction between PXR proteins and RXRα was studied by co-immunoprecipitation, using an anti-RXRα antibody. The co-immunoprecipitated complexes were eluted in two fractions, and the presence of PXR proteins was detected using an anti-PXR antibody employing Western blot analysis. In vitro translated wild-type PXR was used as control. We observed that wild-type PXR protein was co-immunoprecipitated with the RXRα protein, as expected. We further verified the specificity of the co-immunoprecipitation in detecting PXR-RXRα

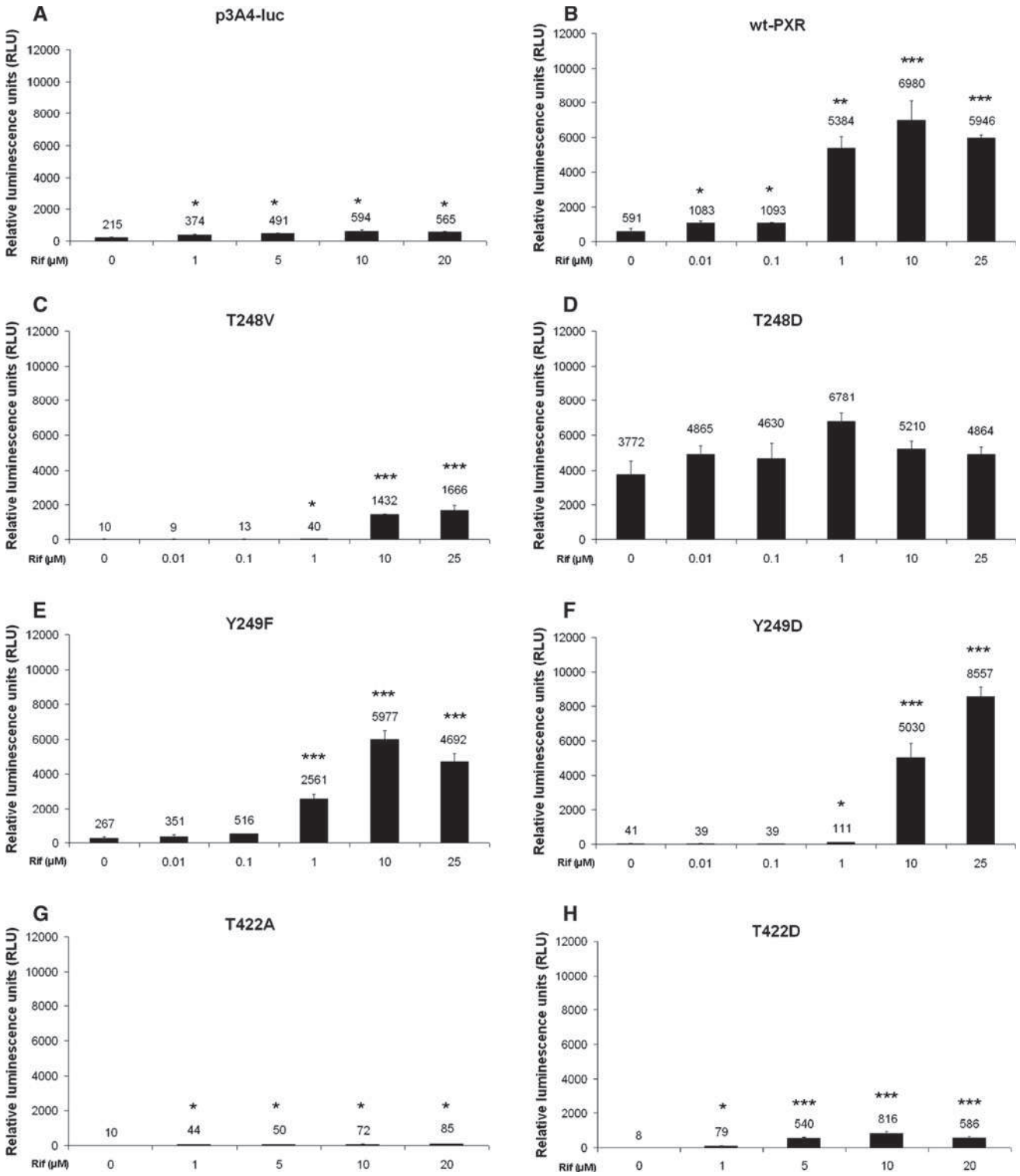

Fig. 2 Transactivation of CYP3A4 gene reporter construct with PXR mutants in HepG2 cells. HepG2 cells were transiently transfected by lipofection (FuGENE HD) either with 300 ng of reporter plasmid p3A4-luc alone (**a**), together with 100 ng of expression vector encoding wild-type PXR (**b**) or PXR mutants (**c**–**h**). Cells were treated for 24 h with vehicle (DMSO; 0.1 % v/v) or rifampicin (RIF) in a range of concentrations from 0.01 to 25 μM. The data are expressed as relative luminescence units (RLU). Differences were considered statistically significant for $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

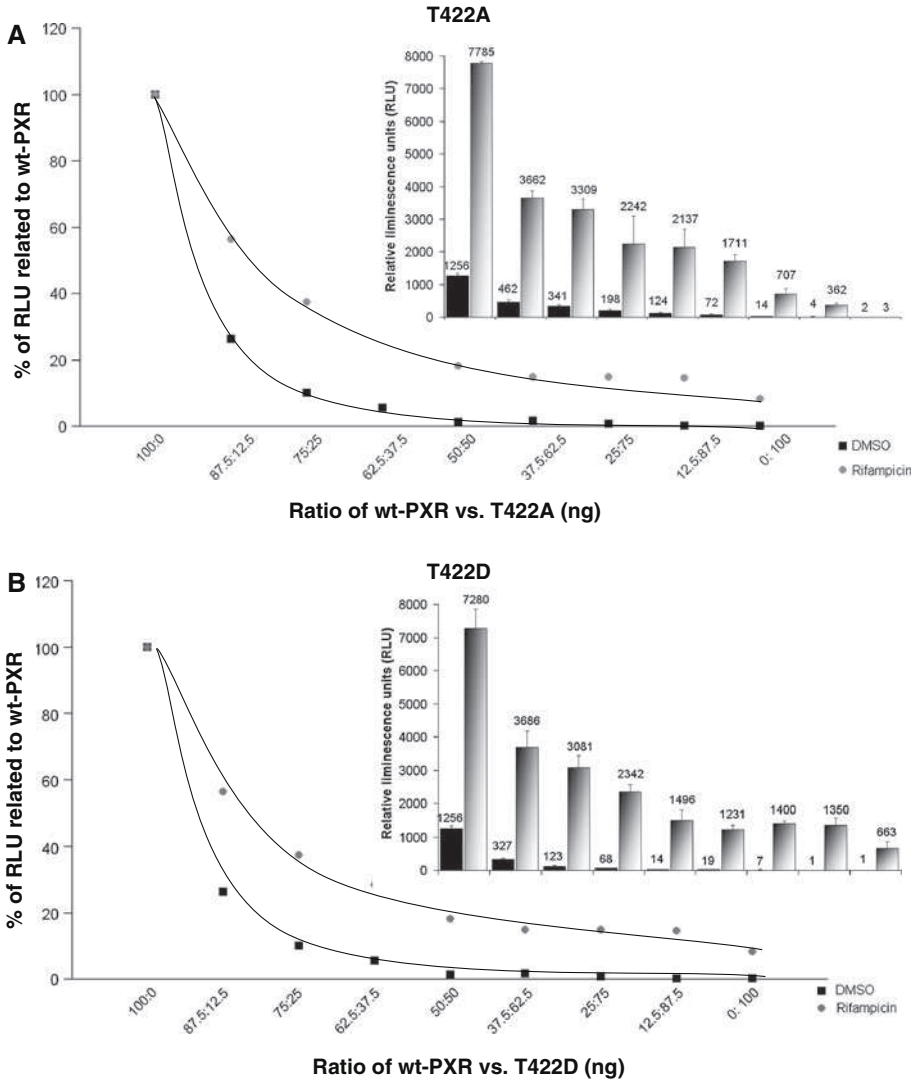

Fig. 3 Dominant-negative effects of T422 mutations on transcriptional activity of PXR in HepG2 cells. HepG2 cells were transiently transfected by lipofection with 300 ng of p3A4-luc plasmid and with different combinations of PXR expression vectors in a total amount of 100 ng. The mixture contained different ratios of wt-PXR and mutant T422A (**a**) or T422D (**b**) expression constructs. Cells were treated for 24 h with vehicle (DMSO; 0.1 % v/v) or rifampicin (RIF; 10 μM). After the treatments, cells were lysed and luciferase activity was measured. The data are presented as mean ± SD from triplicate measurements and they are expressed as percentage of RLU relative to wt-PXR transfected cells with or without RIF (set to 100 %, *plot graphs*) or as absolute RLU (*inserted bar graphs*)

heterodimerization by co-immunoprecipitation of RXRα alone, PXR alone and free lysate using anti-PXR antibody.

We found that there are not substantial differences in the ability of individual PXR mutants to form heterodimers with RXRα, as compared to wt-PXR (Fig. 5). These data suggest that tested mutations do not significantly affect the interactions of PXR with its heterodimer partner RXRα and that the effects of the mutations on CYP3A4 gene transactivation (Fig. 2) are not due to impaired PXR-RXRα interaction.

Binding of PXR mutants to DR3 motif of human CYP3A4 gene promoter

PXR-mediated gene activation requires direct binding of the PXR-RXRα heterodimeric complex to the response elements in the gene promoter. Xenobiotic-responsive enhancer module (XREM) located between −7.8 and −7.2 kb upstream of the CYP3A4 transcription start site containing a functional DR3 nuclear receptor-binding element 1 (dNR1;−7,733/−7,719) (Goodwin et al. 1999). The response element is crucial for both ligand-dependent PXR-inducible and basal transactivation of CYP3A4 (Goodwin et al. 1999). Nuclear extracts from HeLa cells transfected with wild-type PXR and/or mutated PXR proteins together with RXRα protein 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 demonstrated by competition with non-labeled double-stranded DR3 oligonucleotide or mutant DR3 oligonucleotide (Fig. 6a). Furthermore, we confirmed that incubation with antibody against RXRα or PXR supershifted PXR-RXRα oligonucleotide complex (Fig. 6b). Binding of T422A, Y249D and Y249F mutant proteins to DR3 sequence was comparable with that of wt-PXR. On the other hand, T422D, T248V and T248D mutations caused a strong decrease in

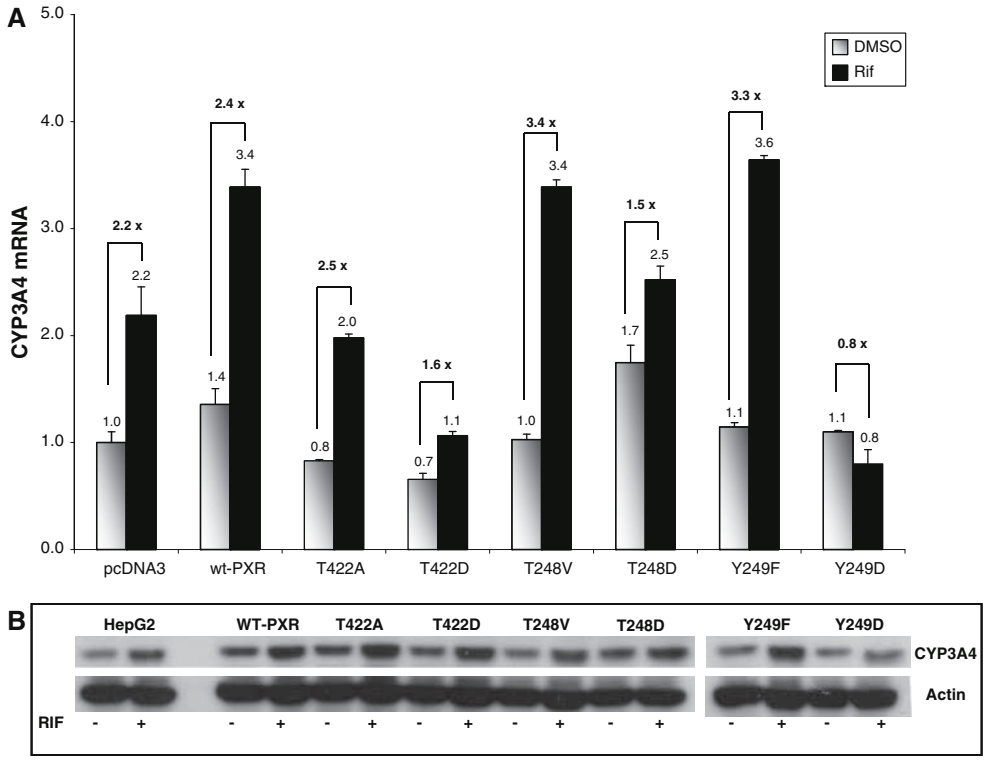

Fig. 4 Effects of PXR mutants on CYP3A4 mRNA and protein expression in HepG2 cells. HepG2 cells were transiently transfected by FuGENE HD with 3 μg expression vector encoding either wild-type or PXR mutants. Cells were treated with vehicle (DMSO; 0.1 % v/v) or rifampicin (RIF; 10 μM) for 24 or 48 h, respectively. qRT-PCR analyses of CYP3A4 mRNA (*bar graphs*) (**a**) and Western blots of CYP3A4 (*autoradiograms*) (**b**) are shown. The qRT-PCR data are presented as mean ± SD from triplicate measurements and are expressed as fold induction over DMSO-treated cells transfected with empty pcDNA3 plasmid. qRT_PCR data were normalized per GAPDH mRNA levels of a reference housekeeping gene. Differences were considered statistically significant for $p < 0.05$ to wt-PXR transfected cells treated either with DMSO (0.1 %) or RIF (10 μM)

DNA-binding activity of mutated PXR proteins (Fig. 6c). Immunoblot analysis verified that equal amounts of PXR proteins were used in the gel shift assay (Fig. 6d). Taken together, phospho-mimetic mutation at T422 and both mutations at T248 decrease the ability of PXR to bind to its response elements DR3 within the promoter sequences of its target CYP3A4 gene.

Discussion

Pregnane X receptor is a metabolic (Xie et al. 2001) and toxicological "sensor" (Harmsen et al. 2007; Lehmann et al. 1998) that regulates the expression of drug-metabolizingenzymes, but also genes involved in lipid and carbohydrate metabolism (Dai et al. 2008; Lee et al. 2008; Roth et al. 2008; Zhou et al. 2008; Zhou et al. 2006). PXR is transcriptionally activated by a variety of structurally unrelated ligands, including drugs (Bertilsson et al. 1998; Drocourt et al. 2001; Jones et al. 2000; Lehmann et al. 1998), environmental pollutants (Coumoul et al. 2002; Pacyniak et al. 2007; Zhang et al. 2008), synthetic and natural compounds (Goodwin et al. 2003; Moore et al. 2002; Staudinger et al. 2001; Staudinger et al. 2006). Transcriptional activation of nuclear receptors, including PXR, may also occur in the absence of a ligand, and the role of covalent protein modification by phosphorylation was anticipated in both ligand-dependent and ligand-independent activation of PXR (Graves and Krebs 1999). While ligand binding is the primary mechanism of PXR activation in hepatocytes, it is likely that posttranslational modification of PXR and PXR-associated proteins is involved in fine-tuning its activity in response to environmental stimuli and alterations in disease status (Lichti-Kaiser et al. 2009a). Indeed, phosphorylation of hepatic nuclear receptors regulates their functions including transactivation capacity, DNA binding, subcellular location, capacity to interact with protein cofactors and protein stability (Hunter and Karin 1992; Karin 1994).

Therefore, in the current study, we examined potential phosphorylation sites within the human PXR protein that have not been characterized before. Recently, Lichti-Kaiser with coworkers performed a systematic analysis of predicted consensus serine and threonine phosphorylation sites

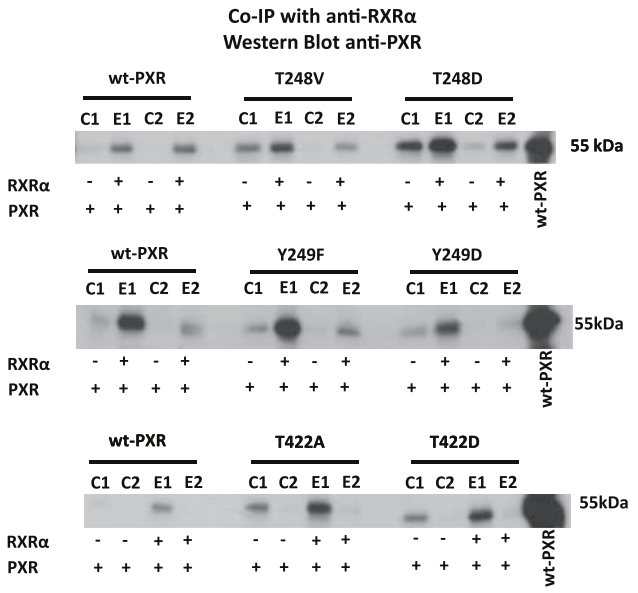

Fig. 5 Protein–protein interaction between tested mutants of PXR and RXRα. Rabbit antibody against human RXRα was coupled to AminoLink Plus Coupling Resin. In vitro translated RXRα, wild-type and mutated PXRs were combined and incubated at laboratory temperature (cca 25 °C) for 30 min. Co-immunoprecipitation was performed as described under "Materials and methods section." Eluted proteins containing PXR alone as a control (C1, C2) and complex of RXRα-PXR protein fractions (E1, E2) were resolved using 4–8 % SDS-PAGE and immunoblotted with antibody detecting PXR

in PXR (Lichti-Kaiser et al. 2009a). However, some residues with low score and potential phosphorylation sites at tyrosines have not been analyzed so far.

A sequence alignment revealed that T248 is conserved in the other NR1I subfamily members. In the crystal structure of human PXR (Watkins et al. 2001), the side chain of T248 is hydrogen bonded to T422 in α-helix 12. Published data also indicate that the hydrogen-bonding interaction of T248 with T422 is critical for PXR, if not essential, to retain the activation by rifampicin (Ueda et al. 2005). Therefore, the role of residues T422, T248 and tyrosine residue Y249 were studied in the current paper.

Phospho-deficient mutant T248V exhibited very low basal and ligand-inducible transactivation capacity in reporter gene assay analysis. Contrary, phospho-mimetic mutation at position T248 resulted in constitutive activation of CYP3A4 promoter independently of the presence of a ligand (Fig. 2), indicating that the position of T248 is critical for transcriptional activity of PXR. The ligand-independent constitutive activation also occurred with phospho-mimetic T248D mutant in the expression of CYP3A4 mRNA and protein (Fig. 4). In contrast, both phospho-deficient and phospho-mimetic mutant of T422 drastically diminished the transactivation of the CYP3A4 promoter. Ueda et al. showed that the interaction of T176 with T350 in mCAR is responsible for constitutive activity of mCAR, and it is analogical to the interaction between T248 and T422 in α-helix 12 of human PXR (Wang et al. 2007; Watkins et al. 2001). Since wild-type PXR is not constitutively active, it is surprising that we achieved its constitutive activity by phospho-mimetic mutation of T248 of PXR. These facts indicate that residues T248 and T422 could be the structural determinants of PXR activity.

We also investigated whether mutations of PXR affect the formation of PXR-RXRα heterodimer, which is a crucial step in the transcriptional function of PXR. We show that all studied PXR mutants formed heterodimer with RXRα at a similar level to that observed with wt-PXR. However, it was demonstrated elsewhere that mutations at S8, S305, S350 and T408 decrease the ability of PXR to form heterodimer with RXRα (Lichti-Kaiser et al. 2009a). Replacement of T248 with Ala or Val abrogated the interaction of PXR with SRC1 (Ueda et al. 2005), which is consistent with our findings that T248V mutation diminished the receptor transcriptional activity and binding to DNA, as revealed by gene reporter assay and EMSA, respectively. We used the technique of EMSA to examine the ability of PXR mutants to bind to DR3 motif of CYP3A4 promoter. The mutant protein T422A bound to DR3 with similar strength as wt-PXR, whereas the binding of T422D was substantially decreased. It was shown elsewhere that mutation T57D, a potential phosphorylation site for p70 S6K, completely abolished the binding of PXR to the promoter sequences of its target genes (Lichti-Kaiser et al. 2009a). The primary reason for a loss of PXR function by mutation of T422 is not probably the phosphorylation, because both phospho-deficient and phospho-mimetic mutation aborted the PXR activity. Decrease in transcription activity could be caused by other mechanism, for instance, changes or loss in coactivator binding (Wang et al. 2007). We found that both T248V and T248D mutants retained their ability to bind to DNA in vitro, but to a lesser extent when compared to wt-PXR.

While the mutation of residue Y249 had an impact on PXR transcriptional activity, we did not find the changes in DNA binding and this is probably not a critical residue playing a role in phosphorylation.

We can conclude that additional experiments are needed to confirm whether any serine/threonine or tyrosine kinases involved in a cellular signaling pathway phosphorylate PXR at T248, Y249 or T422 residues. This finding would bring an important mechanistic link between xenobiotic metabolism genes expression controlled by PXR and cellular signaling pathways.

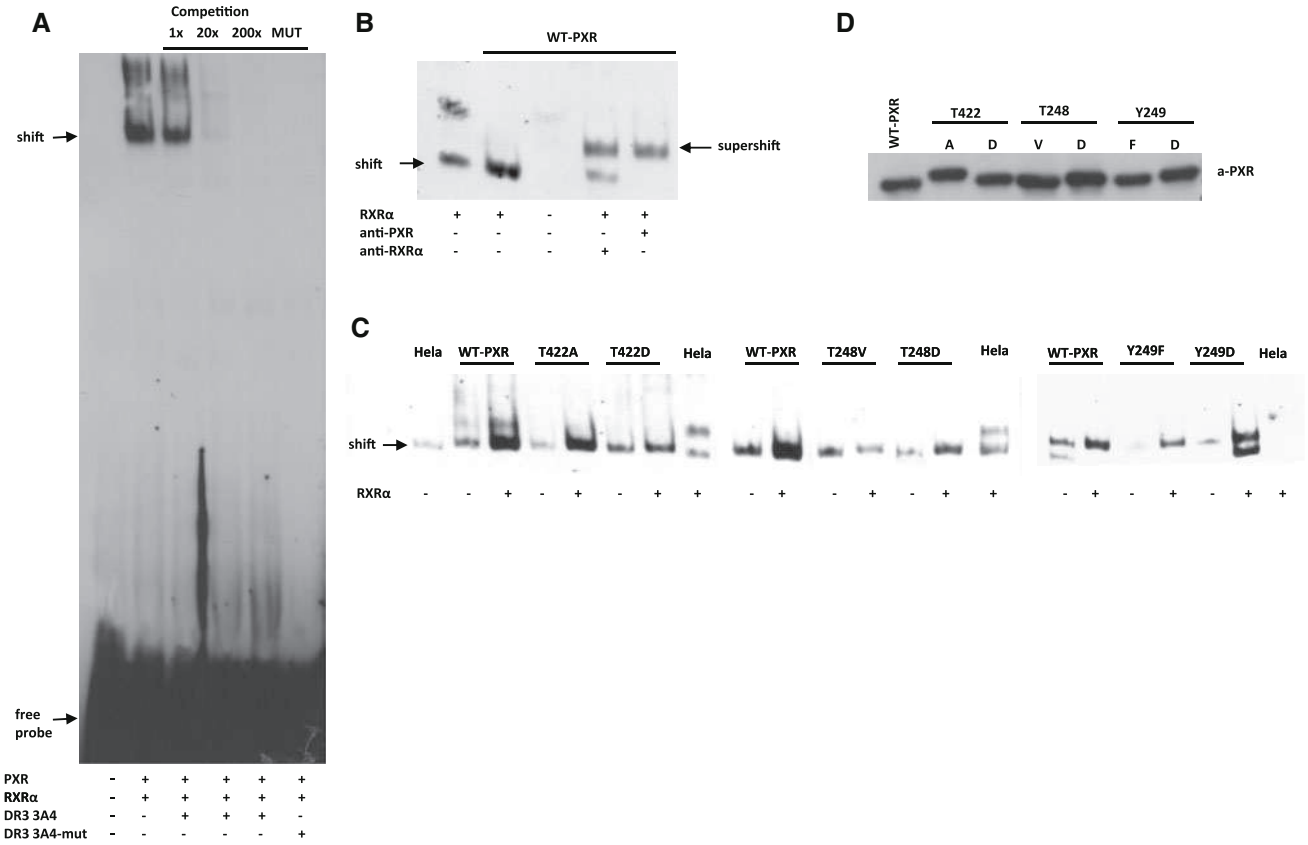

Fig. 6 Binding of PXR mutants to DR3 motif of human CYP3A4 promoter as determined using electrophoretic mobility shift assay (EMSA). Nuclear extracts from HeLa cells transfected with PXR, PXR mutants or RXRα cDNAs, respectively, were incubated with a biotin-labeled CYP3A4-DR3 probe and electrophoresed on 5 % polyacrylamide gel as described under "Materials and methods section." The complex formation of CYP3A4 DR3 response element with PXR alone or PXR-RXRα heterodimer was examined. **a** In competition experiments, a biotin-labeled probe was incubated with nuclear extract containing PXR and RXRα proteins and 1-, 20- and 200-fold excess of unlabeled oligonucleotides in the binding reaction. The *last line* (MUT) represents the control experiment with mutated biotin-labeled probe that was incubated with nuclear extract containing PXR and RXRα proteins; **b** supershift experiment with antibody against PXR or RXRα; **c** biotin-labeled CYP3A4-DR3 probe was incubated with nuclear extract from non-transfected HeLa cells, PXR or PXR mutants alone or together with RXRα; **d** PXR protein level in used nuclear extracts of studied PXR mutants was analyzed by Western blot

Acknowledgments Our laboratories are supported by the grant GACR 303/12/G163 from the Czech Scientific Agency.

Conflict of interest The authors declare that they have no conflict of interest.

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APPENDIX II.

Vavrova A., Vrzal R., Dvorak Z. (2013) A non-radioactive electrophoretic mobility shift assay for measurement of pregnane X receptor binding activity to CYP3A4 response element. *Electrophoresis (in press)*

A non-radioactive electrophoretic mobility shift assay for measurement of pregnane X receptor binding activity to CYP3A4 response element

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Key words: electrophoretic mobility shift assay (EMSA); cytochrome P450; drug metabolism; pregnane X receptor; response elements

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/elps.201300079

Received: February 7, 2013; Revised: March 13, 2013; Accepted: March 20, 2013

ABBREVIATIONS

CYP, cytochrome P450, **DMEM**, Dulbecco's modified Eagle's medium; **DR3**, direct repeats separated by 3 nt; **DR4**, direct repeats separated by 4 nt; **DTT**, dithiothreitol, **EMSA**, electrophoretic mobility shift assay; **EBNA**, Epstein-Barr Nuclear Antigen EBNA Extract; **ER6**, everted repeats separated by 6 nt; **HeLa**, Human Negroid cervix epitheloid carcinoma cells; **PBS**, phosphate-buffered saline; **PXR**, Pregnane X Receptor; **PXRE**, PXR specific response elements; **RXR-α**, Retinoic X Receptor alpha; **XREM**, Xenobiotic-Responsive Enhancer Module

Total number of words including figure and table legends: 3 291

ABSTRACT

The electrophoretic mobility shift assay (EMSA) is a method for the study of specific DNA–protein interactions *in vitro*. The pregnane X receptor is a key xenobiotic sensor that regulates the expression of drug-metabolizing enzymes and many other genes. Radiolabeled ^{32}P-DNA-probes had been used in studies of PXR-DNA interactions. There is an increasing need for non-radioactive assays, due to the health, safety and environmental issues. In the current study, we present a protocol for the non-radioactive electrophoretic mobility shift assay, allowing studying interactions between human PXR with promoter DNA sequences.

1. INTRODUCTION

Pregnane X receptor (PXR, NR1I2) is considered as a pivotal factor in the body's response to foreign chemicals and for complex regulation of cellular metabolic profile. Transcriptional activity of PXR and PXR-mediated gene expression is influenced by a number of chemicals. The majority of these chemicals are the ligands for PXR and often the substrates for PXR target enzymes [1]. As a xenobiotic sensor, PXR regulates the expression of numerous genes involved in a drug metabolism, including CYP2B6, CYP2C8, CYP2C9, CYP3A4 [2], some phase II enzymes [3] and transporters [4]. In addition, PXR controls the expression of genes

involved in the metabolism of steroid hormones, vitamin D, lipids, carbohydrates or cholesterol [5, 6] and plays an important role in the development of specific forms of cancer [7]. Activation of PXR modulates several key biochemical pathways including gluconeogenesis, β-oxidation of fatty acids, fatty acids uptake, cholesterol homeostasis, and lipogenesis. PXR-mediated gene expression requires direct binding of the PXR-RXRα heterodimeric complex to the response elements in the gene promoter. Importance of PXR-PXR homodimer in the regulation of drug metabolism genes was established [8]. For instance the CYP3A4 gene, encoding the most abundant P450 enzyme has several PXR specific response elements (PXRE) in the 5' upstream region of the gene: (i) PXRE located in the proximal promoter region (prER6) that was later identified as a PXR binding response element [9-11]; (ii) a xenobiotic-responsive enhancer module (XREM) with a functional DR3 nuclear receptor-binding element 1 (dNR1; 7733/ 7719) [12]; (iii) functional PXR responsive ER6 element localized within the module (CLEM4- ER6 at -11368/-11351) [13]; (iv) DR4-type PXR binding *cis*-element (eNR3A4) in the CYP3A4 gene 50-flanking region (-7618/-7603) [13, 14]. Changes in ability of human PXR phospho-mutants to bind to its response elements within the promoter sequences of its target genes were observed and this finding together with other results suggested that PXR transcriptional activity can potentially be regulated by phosphorylation at specific amino acid residues [15, 16].

Defining the precise promoter DNA sequence motifs, where nuclear receptors and other transcription factors bind, is an essential prerequisite for understanding how these proteins modulate the expression of their specific target genes. The electrophoretic mobility shift assay (EMSA) is a suitable, convenient and effective method for the study of specific DNA–protein interactions *in vitro* [17], which is often performed with very little modifications to the original technique. Main advantages of EMSA are: (i) a detection of low abundance DNA binding proteins from lysates; (ii) investigating the binding site mutations using many probe

configurations with the same lysate; (iii) a study of binding affinity by DNA probe mutational analysis. The technique is based on the fact that protein-DNA complexes migrate more slowly than free DNA molecule when subjected to non-denaturing polyacrylamide or agarose gel electrophoresis. The specificity of an observed DNA binding reaction can be verified using competition assays in which an excess of unlabeled probe is added together with the labeled probe. So called "supershift" experiment can confirm protein identity by adding a protein-specific antibody to the binding components that forms even larger complex (antibody-protein-DNA) which migrates even more slowly than protein-DNA complex, during electrophoresis.

Traditionally, gel shift assay is performed by incubating a protein or complex mixture of proteins with highly radioactive ^{32}P labeled DNA fragment containing putative protein binding site. Following the electrophoretic separation, resulting complexes are visualized by autoradiography. For an overview of the "classical" radioisotope EMSA technique, several reviews were published [18, 19]. Radiolabeled DNA-probes were used in several studies focused on characterization of PXR-mediated mechanism transactivation of its target genes. In most cases, PXR protein was transcribed and translated in vitro using cell-free expression system, which is considered to be quick and elegant tool for study of several proteins in many methods and suitable in shift assay using radiolabeled probes [1, 15, 20-23].

Radioactive techniques for the labeling of nucleic acids are largely replaced by non-radioactive ones, mainly due to health and safety concerns, short half-life of [^{32}P]-radioisotope and the costs of waste disposal. In the current study, we present a protocol for the non-radioactive electrophoretic mobility shift assay, allowing studying interactions between human PXR (and also other nuclear receptors) and promoter DNA sequences. In order to analyze the interaction between human PXR and DR3 motif in CYP3A4 gene, we decided to use biotinylated probe and chemiluminescent detection system where the biotin labeled probe

is bound to a streptavidine-horseradish peroxidase, eliminating the need for labeling with radioactive compounds.

2. MATERIALS AND METHODS

2.1. Cell line

Human Negroid cervix epitheloid carcinoma cells HeLa (ECACC No. 93021013) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % of fetal calf serum, 100 U/mL streptomycin, 100 ug/mL penicillin, 4 mM L-glutamine, 1 % non-essential amino acids and 1 mM sodium pyruvate. Cells were maintained at 37 °C and 5 % CO2 in a humidified incubator.

2.2. Plasmids

The expression plasmid for human PXR (pSG5-hPXR) was kindly provided by Dr. S. Kliewer (University of Texas, Dallas, TX), and expression plasmid pSG5-hRXRα encoding human RXRα was a generous gift from Dr. C. Carlberg (University of Kuopio, Kuopio, Finland).

2.3. In vitro translation

Human PXR (pSG5-hPXR) and RXRα (pSG5-RXRα) were generated by *in vitro* transcription coupled to translation using TNT Quick Coupled Transcription/Translation Systems according to standard protocol (Promega, Southampton, UK).

2.4. Transfection and nuclear extraction

Hela cells (2.5×10^6/10 cm plate) were transiently transfected employing lipofection (Fugene HD, Roche) with 7 µg/well of expression plasmid encoding wild-type human PXR or RXRα. After 48 hours of incubation, adherent cells were scrapped into 1,5 mL of cold phosphate-

buffered saline (PBS). Suspension was then transferred to microcentrifuge tube. Cells were pelleted for 5 minutes at 450 × g at 4°C and re-suspended in cold Buffer A (10 mM HEPES-KOH pH 7.9, 1.5 mM $MgCl_2$, 1 mM KCl, 1 mM DTT, complete protease and phosphatase inhibitor cocktail (Roche, France). The cells were allowed to swell on ice for 10 minutes and then vortexed for 10 seconds. Samples were centrifuged for 1 minute at 14000 × g at 4°C. Supernatant contained the cytoplasmic fraction. The pellet containing nuclear fraction was re-suspended in 100 µL (according to starting number of cells) of cold Buffer C (20 mM HEPES-KOH pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, complete protease and phosphatase inhibitor cocktail (Roche, France) and incubated on ice for 30 minutes with shaking. Cellular debris was removed by centrifugation for 5 minutes at 14,000 × g at 4°C. Supernatant fraction (containing DNA binding proteins) was transferred to a clean microcentrifuge tube and stored at -70°C. The yield is 50 µg to 100 µg proteins per 10^6 cells.

2.5. Labeling of DNA probe for EMSA

The following double-stranded 5'-biotinylated oligonucleotides containing specific DNA-binding sequence for human PXR (DR3 motif from the XREM sequence of CYP3A4 gene promoter) were used as probes: CYP3A4-DR3 wild-type sense 5'-GAATGAACTTGCTGACCCTCT-3'; CYP3A4- DR3 antisense 5'-AGAGGGTCAGCAAGTTCATTC-3' and their biotinylated forms; biotin-labeled CYP3A4-DR3 with mutations sense 5'-GAA**A**G**C**A**T**TTGC**A**GACCC TCT-3' and antisense 5'-AGAGGGTC**T**GCAA**A**T**G**C**T**TTC-3' (mutated bases in underlined-bold). The oligonucleotides were synthesized by Generi-Biotech (Hradec Kralove, Czech Republic). Final concentration of the labeled oligonucleotides was 10 fmol/µL and 2 pmol/µL for unlabeled probes.

2.6. Electrophoretic Mobility Shift Assay (EMSA)

Gel mobility shift assay was performed using LightShift Chemiluminescent EMSA Kit with modifications suitable and corresponding to PXR interaction with DR3 response element (Thermo Scientific, Waltham, MA, USA). Binding reactions were set-up according to Table 1. Nuclear protein (5 μg) from each sample was pre-incubated with 10x binding buffer (100 mM Tris, 500 mM KCl, 10 mM DTT; pH 7.5), 50% glycerol, 1% NP-40, ddH_2O and component of the kit nonspecific competitor Poly (dl.dC) (1 μg/μL in 10 mM Tris, 1 mM EDTA; pH 7.5) to suppress strong nonspecific interaction 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 15 minutes. For supershift experiments, 2 μg of the anti- RXRα rabbit polyclonal IgG antibody (sc-553 X, Santa Cruz, CA, USA) or mouse monoclonal anti-PXR (sc-48340 X, Santa Cruz, USA) was added to the reaction mixture and incubated additional 5 minutes at room temperature. Finally 5 μL of 5x loading buffer with bromophenol blue was added as electrophoresis indicator to each 20 μL binding reaction (included in the kit).

Native polyacrylamide gel 5% (18 x 16 cm, acrylamide:bisacrylamide 29:1) was pre-electrophoresed at 90 V for 30 minutes. Thereafter, protein-DNA complexes were loaded on pre-run gel and electrophoresed at 300 V in 0.5 × TRIS-borate-EDTA (TBE) running buffer. When the bromophenol blue run 2/3 of the way to the bottom of the plates, the electrophoresis was stopped and the protein DNA complexes were electro-blotted to positively charged nylon membrane at 380 mA for 30 minutes in fresh and cold 0.5 × TBE buffer. Transferred DNA was cross-linked with nylon membrane using a UV-light cross-linker instrument equipped with 254 nm bulbs at 120 mJ/cm^2 for 120 second exposure. Biotin-labeled DNA was detected

using streptavidin-horseradish peroxidase conjugate and chemiluminescent substrate contained in LightShift Chemiluminescent EMSA Kit (Pierce Biotechnology, USA).

3. RESULTS AND DISCUSSION

3.1. Binding of human PXR to DR3 motif of human CYP3A4 gene promoter using *in vitro* translated PXR and RXRα

In vitro translated human PXR and RXRα proteins were incubated with biotin-labeled probe containing human PXR binding site corresponding to the DR3 response element in the CYP3A4 promoter and resolved on non-denaturing gel. In order to demonstrate the effectiveness of EMSA and to ensure that the system was properly set, a control EBNA system including a complete set of three reactions (60 bp biotin-EBNA Control DNA, Epstein-Barr Nuclear Antigen EBNA Extract) was used as recommended by manufacturer (Figure 1A). Even though a control system worked perfectly, EMSA performed with *in vitro* translated nuclear receptors failed. High background was observed in each binding reaction except to negative control, where no *in vitro* translated PXR was present in the reaction mixture (Figure 1B). The results were completely unreadable because of shifted biotinylated proteins that occur in reticulocyte lysate (Figure 2). This indicates that *in vitro* translated protein is unsuitable for detection and preferable system have to be found.

3.2. Binding of human PXR to DR3 motif of human CYP3A4 gene promoter using nuclear extracts from HeLa cells

Because of high background in experiments using *in vitro* translated proteins, HeLa cells were transiently transfected with human PXR and RXRα vectors. Nuclear extracts were isolated and used as a source of proteins required for binding reaction. Nuclear extracts were incubated with biotin-labeled double-stranded oligonucleotides corresponding to the DR3

response element from the CYP3A4 promoter (Figure 3). It is evident that PXR-RXRα heterodimer bound to DR3 motif very strongly (Figure 3A). The specificity of binding to DNA was verified by competition experiment, where an excess of unlabeled DR3 probe was mixed together with the DR3 labeled probe. Specific DNA binding was diminished by an excess (1-, 20-, 200-fold excess) of unlabeled probe (unlabeled specific competitor). In addition, the "*supershift*" experiment was performed. Specific antibody against RXRα or PXR was added to the gel shift reaction, resulting in formation of antibody-PXR-RXRα-DNA complex with further decreased mobility (Figure 3A). PXR alone binds the DR3 probe very weakly as shown in Figure 3B. It is evident that formation of PXR-RXRα heterodimer is essential for binding of PXR to DR3 response element. Specificity of protein-DNA interaction was confirmed also by incubation with biotin-labeled probe containing mutation in DR3 sequence (Figure 3B).

4. CONCLUDING REMARKS

In the current study we describe optimized non-radioactive electrophoretic mobility shift assay, allowing studying interactions between pregnane X receptor and promoter DNA sequences. Traditionally, radiolabeled DNA-probes were used in studies focused on characterization of PXR-mediated mechanism transactivation of its target genes. Because of health, safety and environmental issues, non-radioactive methods are increasingly discovered and invented. We used chemiluminescent gel shift assay technique, where the biotin labeled probe is bound to a streptavidine-horseradish peroxidase, eliminating the need for labeling with radioactive compounds and allowing very sensitive chemiluminescence detection (~ 5-50 pg/band). Main advantages of this method are safety, low cost and shorter time of detection. It is important to note that reticulocyte lysate used to prepare proteins *in vitro* is unsuitable for a non-radioactive EMSA, because of presence of biotinylated proteins that

interfere with the assay. Further benefit of biotinylated DNA is that the same DNA used for EMSA can be coupled to streptavidin-coated agarose beads and used in affinity purification of DNA-binding proteins [24].

ACKNOWLEDGEMENTS

Our laboratory is supported by the grants from Czech Science Agency GACR 13-01809S (to Z.D.) and GACR 303/12/0472 (to R.V.), and by a student grant from Palacky University Olomouc PrF-2013-002 (to Z.D.).

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Figure 1.

A) The Control EBNA System. 1- Biotin-EBNA Control DNA, 2 - Biotin-EBNA Control DNA + EBNA Extract, 3 - Biotin-EBNA Control DNA + EBNA Extract + 200-fold molar excess of unlabeled EBNA DNA.
B) Binding of in vitro translated hPXR to DR3 motif of human CYP3A4 promoter. In vitro translated hPXR and RXR-α were incubated with a biotin-labeled CYP3A4-DR3 probe and electrophoresed on 5 % polyacrylamide gel as described under 'Materials and methods section.' There are no shifted bands corresponding to PXR-DR3 interaction because of the shifts of a biotinylated proteins present in reticulocyte lysate

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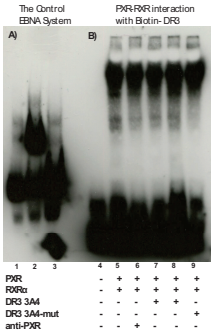

Figure 1

Figure 2. Identification of biotinylated proteins. Presence of biotinylated proteins in reticulocyte lysate were showed by SDS-PAGE and western blot using streptavidin-horseradish peroxidase conjugate

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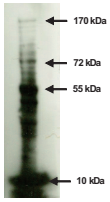

Figure 2

Figure 3. Binding of hPXR isolated from HeLa cells to DR3 motif of human CYP3A4 promoter. Nuclear extracts from HeLa cells transfected with PXR or RXR-α were incubated with a biotin-labeled CYP3A4-DR3 probe and electrophoresed on 5 % polyacrylamide gel as described under 'Materials and methods section.' The complex formation of DR3 response element with hPXR alone or hPXR-RXR-α heterodimer was examined. **A)** In competitive reaction, a biotin-labeled probe was incubated with nuclear extract containing hPXR and RXR-α proteins and 1-, 20- and 200-fold excess of unlabeled oligonucleotides in the binding reaction. The last two lines represent supershift experiment with antibody against PXR or RXR-α, respectively **B)** biotin-labeled CYP3A4-DR3 probe was incubated with nuclear extract from non-transfected HeLa cells, hPXR alone or together with RXR-α; The last line represents the control experiment with mutated biotin-labeled CYP3A4-DR3 probe that was incubated with nuclear extract containing hPXR and RXR-α proteins;

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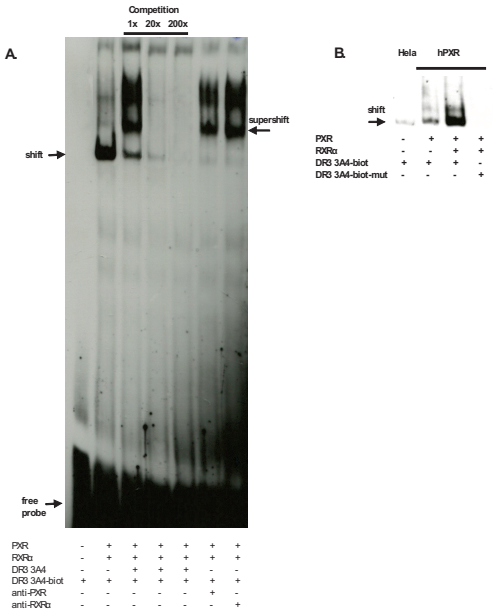

Table 1. Binding Reaction set-up for study of hPXR/RXR-α interaction with DR3 motif.

	Final Amount	1. a) (uL)	2. b) (uL)	3. c) (uL)	4. d) (uL)	5. e) (uL)
Ultrapure water	-	13	5	3	4	5
10 x binding buffer	1x	2	2	2	2	2
50 % glycerol	2,5 %	1	1	1	1	1
1 ug/uL Poly (dI.dC)	50 ng/uL	1	1	1	1	1
1% NP-40	0,05 %	1	1	1	1	1
CYP3A4-DR3 (2pmol/uL)	4 pmol	-	-	2	-	-
PXR/RXR nuclear extract (1:1)	system-dependent	-	8	8	8	8
CYP3A4-DR3-biot (10 fmol/uL)	20 fmol	2	2	2	2	-
CYP3A4-DR3-biot-mut (10 fmol/uL)	20 fmol	-	-	-	-	2
Antibody	0,1 ug/uL	-	-	-	1	-
Total reaction volume	-	20	20	20	20	20

[a] **1. Negative control** containing only biotin labeled CYP3A4-DR3 probe; [b] **2. Positive control** containing sufficient target protein to effect binding and shift of biotin labeled CYP3A4-DR3 probe; [c] **3. Competitive reaction control** to prevent shift by competition from excess non-labeled CYP3A4-DR3 probe (200x molar excess); [d] **4. Supershift reaction** containing sufficient antibody which recognizes the protein; [e] **5.** Nuclear protein extract is incubated with mutant biotin-DR3 probe