Identification and functional characterization of potential isoforms and post-translational modification of Pregnane & Xenobiotic Receptor

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CERTIFICATE

The research work embodied in this thesis entitled **'Identification and functional characterization of potential isoforms and post-translational modification of Pregnane & Xenobiotic Receptor'** has been carried out at the Special Centre for Molecular Medicine, Jawaharlal Nehru University, New Delhi-110067, India. The work presented here is original and has not been submitted in part or full for any degree or diploma of any University/Institution elsewhere.

Date: 25 June 2013

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Dedicated to my parents and my family

CONTENTS

Acknowledgement

Behind every researcher, there exists a support network and I take this privilege to acknowledge all those people who in their small and big ways helped me in this journey.

I dedicate my thesis to my parents with due mention of their patience, support and never-ending faith in me during this long journey of Ph.D. My brother Dipankar , my sisters Pallavi & Niharika and my niece Manaswi supported me whole heartedly. I'm also very thankful for good wishes from Vikas ji, Aunty, Uncle and Niti. I am so fortunate that my Grand Pa and Grand Ma had a big dream for me and because of their blessings I was able to complete my work. I'm indebted to my mentor Dr. Daisaku Ikeda and the philosophy of Nichiren Daishonin, whose prayers and constant encouragements helped me break through the limitations of my potential.

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Priyanka.

ABBREVIATIONS

µg	Microgram
µl	Microlitre
µM	Micromolar
aa	Amino Acid
ABC	ATP Binding Cassette
ACTR	Activator of Thyroid hormone and Retinoid Receptor
ACC-1	Acetyl CoA Carboxylase-1
AF	Activation Function
APS	Ammonium Persulphate
AR	Androgen Receptor
ATP	Adenosine Tri Phosphate
ATI	Alternative Translation Initiation
Bp	Base Pair
CAR	Constitutive Androstane Receptor
CCRP	Cytoplasmic CAR Retention Protein
CBP	cAMP response element-Binding Protein
CDK	Cyclin Dependent Kinase
cDNA	Complementary DNA
CK2	Casein Kinase2
CNS	Central Nervous System
COX2	Cyclooxygenase-2
CYP	Cytochrome P450
DBD	DNA Binding Domain
DEPC	Diethyl Pyrocarbonate
DMEM	Dulbecco's Modified Eagle's Medium
DME	Drug Metabolizing Enzyme
DMSO	Dimethylsulfoxide
CPT1A	Carnitine Palmitoyltransferase 1A
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
DR1	Direct Repeat 1
DTT	Dithiothreitol
ECL	Enhance Chemi-Luminescence
EDTA	Ethylene Diamine Tetra Acetic acid
ER	Estrogen Receptor
FAE	Fatty Acid Elongase

FAS	Fatty Acid Synthase
FBS	Fetal Bovine Serum
FoxO1	Forkhead box protein O1
FoxA2	Forkhead box protein A2
g	Gram
GFP	Green Fluorescent Protein
G6Pase	Glucose-6-Phosphatase
GR	Glucocorticoid Receptor
GRIP1	Glucocorticoid Receptor Interacting Protein 1
GSK3	Glycogen Synthase Kinase 3
GST	Glutathione S-Transferase
h	Hours
HAT	Histone Acetyl Transferase
HDAC	Histone Deacetylase
HEPES	4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid
HMGCS2	3-Hydroxy-3-Methylglutaryl-Coenzyme A Synthase 2
HMT	Histone Methyl Transferase
HNF-4	Hepatocyte Nuclear Factor-4
HRP	Horse Radish Peroxidase
HSP	Heat Shock Protein
IL-6	Interleukin-6
iNOS	inducible Nitric Oxide Synthase
kDa	Kilodalton
LB	Luria-Bertani
LBD	Ligand Binding Domain
LCA	Lithocholic Acid
Luc	Luciferase
LXR	Liver X Receptor
M	Molar
MAPK	Mitogen-Activated Protein Kinase
MDR2	Multidrug Resistance Protein 2
MEKK	Mitogen-Activated Kinase Kinase
min	Minutes
ml	Millilitre
mM	Millimolar
MR	Mineralocorticoid Receptor
mRNA	messenger RNA
MRP2	Multidrug Resistance Protein 2

Msx2	Msh Homeobox 2
Mw	Molecular Weight
NCoR	Nuclear Receptor Co-Repressor
Ng	Nanogram
Ni-NTA	Nickel-Nitriloacetic Acid
NLS	Nuclear Localization Signal
NES	Nuclear Export Signal
NR	Nuclear Receptor
NTD	N-Terminal Domain
^{o}C	Degree Centigrade
OATP2	Organic Anion Transporter 2
O.D.	Optical Density
ORF	Open Reading Frame
PAGE	Polyacrylamide Gel Electrophoresis
PEPCK	Phosphoenolpyruvate Carboxykinase
PBS	Phosphate Buffered Saline
PCN	Pregnenolone-16α-Carbonitrile
PCR	Polymerase Chain Reaction
PGC-1α	Peroxisome proliferator activated receptor γ Coactivator-1α
pH	Power of Hydrogen
PKA	Protein Kinase A
PKC	Protein Kinase C
PMA	Phorbol 12-Myristate 13-Acetate
PMSF	Phenyl Methyl Sulphonyl Fluoride
PPAR	Peroxisome Proliferator Activated Receptor
PP 1/2A	Protein Phosphatase 1and 2A
PR	Progesterone Receptor
PRMT	Protein Arginine Methyltransferase
PTM	Post-translational modification
PVDF	Poly-Vinyl-Di-Fluoride
PXR	Pregnane and Xenobiotic Receptor
RAR	Retinoic Acid Receptor
RFP	Red Fluorescent Protein
RNA	Ribonucleic acid
Rpm	Revolution per minute
RT	Room Temparature
RXR	Retinoid and Xenobiotic Receptor
SDS	Sodium Dodecyl Sulphate

sec	Seconds
SENP	SUMO specific Protease
SHP	Small Heterodimer Partner
SIRT1	Sirtuin 1
SMRT	Silencing Mediator for Retinoid and Thyroid-hormone receptors
SNP	Single Nucleotide Polymorphism
SRC-1	Steroid Receptor Coactivator-1
SRC-2	Steroid Receptor Coactivator-2
SRC-3	Steroid Receptor Coactivator-3
SREBP-1C	Sterol Regulatory Element Binding Protein-1C
STAT	Signal Transducer and Activator of Transcription
SUG1	Suppressor for Gal4
SULT	Sulfotransferase
SUMO-1	Small Ubiquitin-related Modifier-1
SUMO-2	Small Ubiquitin-related Modifier-2
SUMO-3	Small Ubiquitin-related Modifier-3
SXR	Steroid and Xenobiotic Receptor
TAE	Tris-Acetate-EDTA
TBS	Tris Buffer Saline
TBST	Tris Buffer Saline with Tween-20
TE	Tris-EDTA
TEMED	Tetramethylenediamine
TNF-α	Tumor Necrosis Factor-α
TR	Thyroid Receptor
U	Unit
UGT	UDP-Glucuronosyltransferase
UTR	Untranslated Region
VDR	Vitamin D Receptor
VDRE	Vitamin D Response Element
XREM	Xenobiotic Response Element

Review of Literature

Nuclear Receptors:a general overview

Nuclear Receptors (NRs) comprise a super-family of 49 members that act as ligand-inducible transcription factors with numerous physiological functions(**Table I**). This super-family of transcription factors includes receptors for a variety of endogenousmetabolites that function as hormones,like cortisol, aldosterone, estrogen, progesterone,testosterone, vitamin D3, thyroid hormone, retinoic acid etc.or exogenoussubstances, such as pharmaceutical agents or environmental substances of natural or man-made origin (Evans, 1998). Members of the NR gene super-family share structural and functional similarities (**Figure 1**).Theamino-terminal domain and carboxy-terminal domain act as docking sitesfor transcriptional co-regulators and designated as trans-Activation Functions-1 (AF-1)(ligand-independent) and trans-Activation Functions-2 (AF-2) (ligand-dependent), respectively (Mangelsdorf et al, 1995). The centrally located conserved DNA Binding Domain (DBD) consists of two zinc finger motifs that are essential fordirect receptor binding to DNA recognition elements in their target genes while the hinge region contains Nuclear Localization Signal (NLS). Nuclear receptors like AR, PR, GR and TR contain distinct Nuclear Export Signal (NES) in DBD however they lack a leucine-rich NES (Tyagi et al, 1998; Tyagi et al, 2000; Black et al, 2001).The Ligand Binding Domain (LBD) locatedat thecarboxy-terminus is composed of twelve α helices surroundinga ligand-binding pocket Ligand activation induces a profound positional shift of α helix 12 and this is critical for co-regulator recruitment (Moras and Gronemeyer, 1998).NRs communicate with other intracellular signalingpathways on a mutual basis, and their functionality may be altered, positively or negatively,by several players(repressors, activators and bridging proteins)that allow for the sophisticated fine-tuning of transcriptional regulation.In the absence of ligands or in the presence of some antagonists, certain NRs are bound todistinct multiprotein complexes through the interaction with co-repressors, such as NR-Corepressor (N-CoR) andSilencing Mediator for Retinoic acid receptor and Thyroid hormone receptor (SMRT). Co-repressor complexes comprises Histone Deacetylases 1/2/3 (HDACs) that have the capacityto condense chromatin over target gene promoters

(Kishimoto et al, 2006). Ligand-dependent interaction of NRs through AF-2 domain with LXXLL motif present in co-activator complexes named as p160/p300 Histone Acetyl Transferase (HAT) complex and the DRIP/TRAP complex with no HAT activity have been studied (Herry et al, 1997).The p160 members includeSteroid Receptor Coactivator-1 (SRC-1) (Onate et al, 1995), Steroid Receptor Coactivator-2 (SRC-2) (Voegel et al, 1996; Hong et al, 1996), Steroid Receptor Coactivator-3 (SRC-3) (Torchia et al, 1997; Takeshita et al, 1997) and CBP/p300 (Ogryzko et al, 1999). The majority of NRs act either as homo-dimers or as hetero-dimers with Retinoid X Receptor (RXR) and bind to their response elementsto regulate the responsive gene expression.Further, post-translational modifications resulting from the crosstalk between different signaling pathways provide additional regulation ofgene transcription by the members of the NRs super-family. Future studies are needed to focus on the variousunderlying molecular mechanismsinvolved in modulation of complex network of NRs signaling.

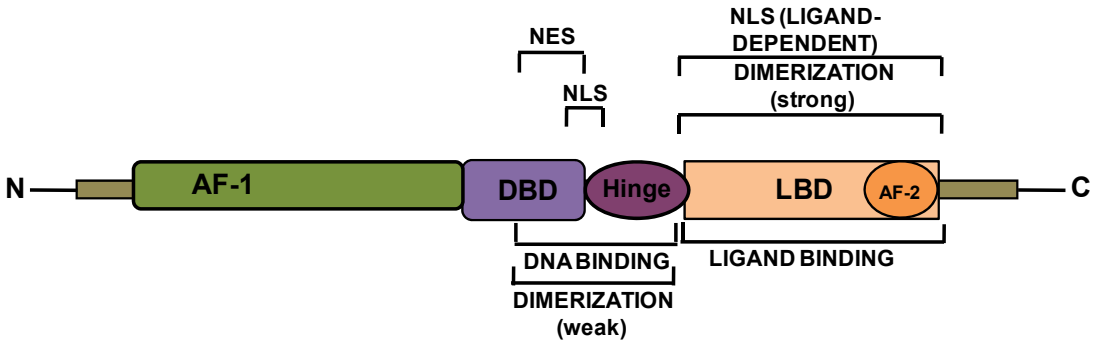


Figure 1: Schematic illustration of the structural and functional organization of NRs. *The amino-terminal region of NRs is highly variable in sequence and length, contains ligand-independent Activation Function-1 (AF-1).The carboxyl-terminal region encompasses the Ligand Binding Domain (LBD) and includes a region termed Activation Function-2 (AF-2), which is an important site for co-regulator proteinbinding. The LBD is connected to a DNA Binding Domain (DBD) by a hinge region.Nuclear Localization Signal (NLS) is present in DBD along with hinge region and Nuclear ExportSignal (NES) present in DBD of NRs. Different structural and their domain functions are depicted in the scheme. (adapted and modified from Germain et al, 2003).*

Table I: Major NRs, ligands, primary functions and disease associations

Receptor/symbol	Ligands	PrimaryFunctions	Disease
Estrogen receptors/ ERα/NR3A1; ERβ/NR3A2	Endogenous: 17β-estradiol Clinical: Mixedagonists(e.g.tamoxifen, raloxifene,andtoremifenein breastcancer) Xenobiotics:BisphenolA,PCBs	Regulationofcellgrowthandproliferationin multipletissues (e.g.,femalereproductive tissues, bone,andCNS)	Cancer,cardiovascular, immune and inflammatory, metabolic,neurological, reproductive
Androgen receptor/ AR/NR3C4	Endogenous: Testosterone, dihydrotestosterone Clinical: Flutamide andbicalutamidefor prostatecancerandalopecia	Keyrolein malereproductiveorgans in additiontoothersystems(e.g.,CNS)	Cancer,cardiovascular, immune, metabolic, neurological, reproductive
Glucocorticoid receptor/ GR/NR3C1	Endogenous: Cortisol(hydrocortisone) Clinical: Fluticasone andprednisolonein inflammatorydisorders	Diversedevelopmentalandphysiological roles(e.g.,antagonismofinflammatory signaling pathways,mediationofthestress response, andgluconeogenesis)	Metabolic, cardiovascular,immune andinflammatory, memory
VitaminD Receptor/ VDR/NR1I1	Endogenous: Calcitriol(1′,25′ dihdroxy vitaminD3) Clinical: Paracalcitolfor2° hyperparathyroidismin renalpatients; Tacalcitolforpsoriasis	Maintenance ofserum calciumand phosphatelevelsforskeletalintegrity; antiproliferativein many tissues	Bone, metabolic,cancer, cardiovascular, immune and inflammatory,renal, neurological
Thyroidhormone receptors/ TRα/NR1A1; TRβ/NR1A2	Endogenous: Thyroxine(T4), Triiodothyronine(T3) Clinical: Levo-thyroxine,triiodothyroacetic acid(TRIAC)in resistance tothyroid hormone	Regulationofoxygenconsumption;protein, carbohydrate,lipid,andvitaminmetabolism	Thyroidconditions, cancer
Progesterone receptor/ PR/NR3C3	Endogenous: Progesterone Clinical: RU486(Mifepristone)asan abortifacient	Diversereproductivefunctions(e.g., establishingandmaintaining pregnancy, developingbreasttissue, andstopping proliferationin theuterus)	Cancer,metabolic, reproductive
Mineralocorticoid receptor/ MR/NR3C2	Endogenous: Aldosterone Clinical: Spironolactonein hypertensive cardiovasculardisease	Regulatingelectrolyteandfluidbalance in thekidney;specificrolesin theCNS	Metabolic
Peroxisome- proliferator-activated receptor-γ/ PPARγ/NR1C3	Endogenous: FAsandFAintermediates Dietary:FAsandPUFAs Clinical: Thiazolidinediones(e.g., rosiglitazone)in typeIIdiabetes	Regulationofadipocytes,insulin sensitivity andlipogenesis, andbroaderintegrationof energy,lipid,andcarbohydratemetabolism	Cardiovascular, metabolic,cancer, neurological
Peroxisome- proliferator-activated receptor-α/ PPARα/ NR1C1	Endogenous: FAsandFAintermediates Clinical: Fibrates(e.g.,fenofibrate)in hyperlipidemia Dietary:FAsandPUFAs Xenobiotics:DEHP,DEHA	Regulatingenergyexpenditure;modulating fattyacid oxidationsystems (mitochondria), peroxisomeβ-oxidation,andmicrosomalω-oxidation	Cardiovascular, metabolic,cancer, neurological
Peroxisome- proliferator-activated receptor-δ(β)/ PPARδ/ NR1C2	Endogenous: FAsandFAintermediates Dietary:FAsandPUFAs	Regulatingcellproliferation,differentiation, andmigrationin woundhealingand inflammatoryprocesses	Cardiovascular, metabolic,cancer, neurological
Retinoic acid receptors/ RARα/NR1B1; RARβ/NR1B2; RARγ/NR1B3	Endogenous: All-*trans*and9-*cis*retinoic acid Clinical: Tretinoinfortreatingacneand acutepromyelocyticleukemia	Pleiotropiccontrolofembryonicpatterning andorganogenesis,cellproliferation, differentiation,apoptosisandhomeostatic control	Neurologicaland psychiatric,cancer
LiverXreceptors/ LXRα/NR1H3 LXRβ/NR1H2	Endogenous: Oxysterols	Cholesterolandsteroidsensorswithrolesin lipidandcarbohydratemetabolism	Metabolic
Retinoid X receptors/ RXRα/NR2B1 RXRβ/NR2B2; RXRγ/NR2B3	Endogenous: 9-*cis*retinoicacid	Embryoniccellpatterningand organogenesis,cellproliferationand differentiation,otherfunctionsas heterodimerswithothernuclearreceptors	Neurologicaland psychiatric,immune
PregnaneX Receptor/ PXR/NR1I2	Endogenous: Bileacids Xenobiotic:St.John'sWort(hyperforin), Taxol,rifampicin,phenobarbital Dietary:VitaminE,sulforaphane, Gugulipid	Metabolismandtransportofpharmaceutical drugs,xenobiotics,andtoxicbileacidsintheliverandGItract	Immune
Constitutive androstane receptor/ CAR/NR1I3	Endogenous: Androstanol,androstenol Xenobiotics:Phenobarbital, DEHP, Meclizine	Metabolismofxenobioticsandendogenous lipidsbyregulating expressionof cytochromeP450genes	Involved in hepatotoxicityof acetaminophen
FarnesoidX Receptor/ FXR/NR1H4	Dietary:Cafestol, guggulsterone	Asensorforbileacidthathelps regulatebile acidhomeostasis	Metabolic

(adapted from McKenna and O'Malley, 2010)

Pregnane & Xenobiotic Receptor (PXR): an introduction

Many key aspects of mammalian physiology involving diverse phenomena ranging from mitosis to apoptosisare regulated by members of NR super-family. At thebeginning of 1998, a new NR was originally isolated from themouse and human sources based upon the sequence homology toother NRs(**Figure 2A**) (Kleiwer et al, 1998; Blumberg et al, 1998). It was first found to respond tovarious natural and synthetic pregnanes, which gave rise toits name, PXR.In other species (rabbit, rat, mouse, chicken and monkey) PXR was latercharacterized with the help of alignment and evolutionarytree analysis (**Figure 2B**)(Jones et al, 2000; Reschly &Krasowski, 2006). Typically, PXR contains a highly variable amino-terminal domain, a central conserved DBDand a carboxy-terminal LBD. At the most amino-terminus of PXR, has a short AF-1region that allows for a ligand-independentregulation. The LBD contains both a ligand binding pocketand a ligand-dependent AF-2 region,which binds to transcriptional co-activators and transcriptional co-repressors. Upon ligand engagement, PXR binds to the promoter regions of its target genes as a hetero-dimer with RXR to initiate gene transcription (Bertilsson et al 1998; Kliewer et al 1998). Primary target genes of PXR include genes for phase I and phase II drug metabolizing enzymes (DMEs) and phase III ATP binding cassette (ABC) drug transporters (**Figure 3**).Like others NRs, PXR also functions as part of a complex with transcriptional co-activator [e.g. SRC-1, Glucocorticoid Receptor Interacting Protein 1 (GRIP1), Activator for Thyroid hormone and Retinoid receptors (ACTR) and Peroxisome proliferatoractivated receptor γ Coactivator-1alpha (PGC-1α)] or transcriptional co-repressors (e.g. NCoR andSMRT) (Kishimoto et al, 2006). Alignment of the PXRsequences among different species revealed interesting differences. Although the DBDs of rabbit, rodent, and human PXR are most conserved, sharing approximately 95% protein identities, the LBDs are much more divergent across different species as shown in **figure 2B**. The PXR sequences were useful in PXR comparative pharmacology and selecting appropriate animal models for preclinical studies predictive of effects in humans. However, subsequent observations made it apparent that species-

specific variations may limit the utility of using general animal models and their tissues for testing purposes.

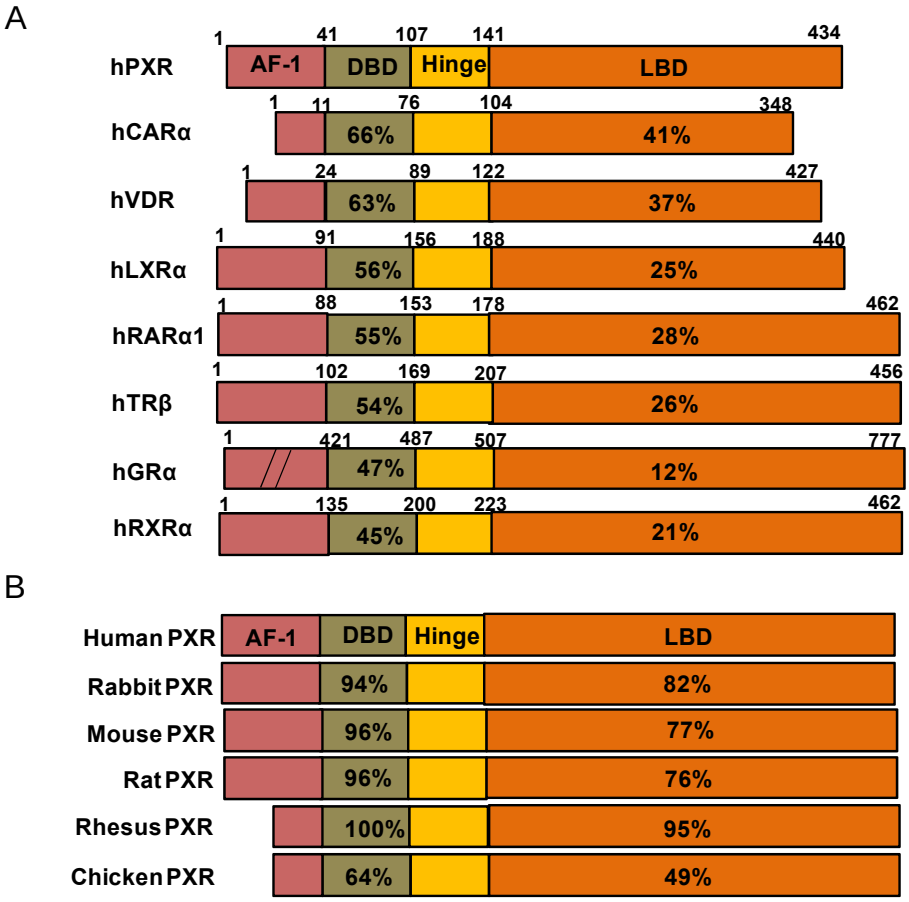


Figure 2: Amino acid sequence comparison between human PXR with other NRs and percentage similarity of PXR among different species.*A.* *Amino acid sequence comparison between human PXR with some major members of nuclear receptor super-family. The similarity is expressed as percentage of amino acid identity in the DNA binding domain (DBD) and ligand binding domain (LBD) of human PXR.* ***B.*** *Comparison of sequence similarity of human PXR with other species. The similarity is expressed as percentage of amino acid identity in the DBD and LBD of human PXR.*

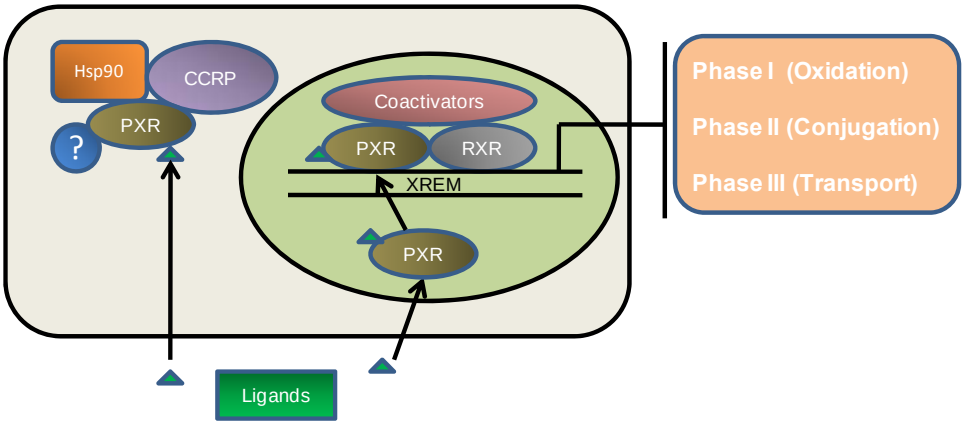


Figure 3: A simplified model depicting PXR activation by its ligands. *Unliganded PXR may be cytoplasmic or nuclear localized under different situations. When localized in cytoplasm prior to ligand binding, it is in complex withcytoplasmic constitutive active/androstane receptor retention protein (CCRP), heat-shock proteins (HSPs) and unknown factors.Ligand binding triggersdissociation from HSPs and translocation to the nucleus.However, some studies have also shownligand-independent nuclear localization of human PXR. PXR, heterodimerize with RXR andbinds with xenobiotic response element. It induces the expression of phase I, phase II drug metabolizing enzymes and phase III drug transporters.*

Role of PXR in health and diseases

Role of PXR in normal physiological controls and patho-physiological situations are recently becoming more apparent (**Figure 4**) (Zhou et al, 2009; Qiao et al, 2013; Koutsounas et al, 2013). PXR as a 'xenosensor' responds to a large range of chemically distinct endobiotics (steroids, bile acids and their derivatives, vitamins etc.) and xenobiotics (synthetic drugs, herbal medicines, endocrine disruptors etc.). PXR primarily functions in maintaining homeostasis that involves rapid and timely elimination of toxic endogenous metabolites and exogenous chemicals. Other involvements of PXR are suggestedin altered metabolic conditions like osteomalacia, bile acid homeostasis, cancer etc. When PXR encounters conditions that are unusual with normal homeostasis, it orchestrates a response by utilizing and modulating the components of the central detoxification defense machinery, i.e. phase I and phase II drug metabolizing enzymes and drug transporters. The presence of PXR in various tissues other than the liver and intestine along with the occurrence of various isoforms attributed much more diverse roles thanwere previously suspected. The presence of PXR isoforms in

different tissues suggests utilization of combinatorial mechanisms to regulate differential sets of genes under various physiological and pathological conditions. Further studies are expected to divulge important roles of PXR in drug-drug interactions, development of several metabolic disorders and in designing safer therapeutic molecules.

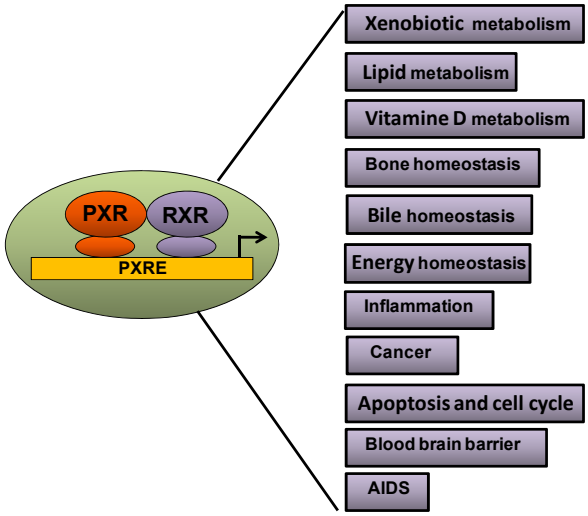


Figure 4:Diverse physiological functions of PXR.*PXR forms hetero-dimer with RXR and binds to its cognate element PXRE in promoters of its target genes. In addition to its established role in xenobiotic metabolism, PXR has additional protective role in various biological functions like lipid metabolism, vitamin D metabolism, bone homeostasis, bile homeostasis, energy homeostasis, inflammation, cancer, apoptosis and cell cycle, blood brain barrier and AIDS. This suggests that PXR has physiological roles beyond xenobiotic metabolism.*

A. Role of PXR in physiological homeostasis and endobiotic metabolism

Although PXR has been recognized as a 'xenobiotic receptor', emerging evidences also implicate PXR as an 'endobiotic receptor' which responds to a wide array of endogenous chemicals and regulate physiological homeostasis which are describe below:

PXR in bile homeostasis:Bile acids are the end products of hepatic cholesterol catabolism and play an important role in the digestion and absorption of lipids in the small intestine. Secondary bile metabolite lithocholic acid (LCA)

and its 3-keto metabolite in presence of PXR ligand can efficiently activate PXR and reduce the liver damage (Staudinger et al, 2001; Xie et al, 2001).Activation of PXR down-regulates expression of CYP7A1, the first and rate limiting step in the metabolism of cholesterol to bile acids (Staudinger et al, 2001; Xie et al, 2001) via squelching with co-activator PGC1α (Bhalla et al, 2004; Li and Chiang, 2005) which is distinct from FXR. Bile acid induced activation of PXR also up-regulates the expression of genes involved in bile acid metabolism and transport, such as MRP2, OATP2 and CYP3A (Kast et al, 2002; Guo et al, 2003; Kullak-Ublick, 2003; Frank et al, 2005).

PXR in bone homeostasis:VitaminK is known as a critical factor required for bone homeostasis and blood coagulation and functions as an effective therapeutic agent for the management of osteoporosis (Tabb et al 2003; Ichikawa et al, 2006). Besides this, Vitamin K2 acts as ligand for PXR and up-regulates expression of PXR target gene CYP3A4 and bone marker genes, such as bone alkaline phosphatase, osteoprotegerin, osteopontin and matrix Gla protein (Tabb et al 2003).Additionally,fourteen vitamin K2 target genesare up-regulated via PXR activation, among which, tsukushi, matrilin-2 and CD14 antigen are shown to be primary PXR target genes that participate in extracellular matrix formation in osteoblastic cells suggesting important role of PXR in bone formation (Ichikawa et al 2006). Another study strengthens the fact that vitamin K-activated PXR stimulates osteoblast differentiation through msh homeobox 2 (Msx2) gene (Igarashi et al, 2007). So, from these studies it is apparent that vitamin K acts via PXR to potentiate osteoprotective function.

PXR in energy homeostasis:PXR functions as a link between drug metabolism and energy metabolism (Buler et al, 2011). The role of energy sensing factors PGC-1α and sirtuin 1 (SIRT1) in control of PXR-mediated transcriptional regulation has been investigated. PGC-1α stimulates PXR expression and transactivation. However, this transactivation is attenuated by the NAD-dependent deacetylase protein, SIRT1 in glucose starvation condition (Buler et al, 2011). In addition to the induction of PXR expression by fasting, other

energy metabolism related factors can also regulate PXR function like insulin regulated Forkhead box protein O1 (FoxO1) and Forkhead box A2 (FoxA2) (Kodama et al, 2004). FoxO1 was found to be a co-activator to CAR and PXR and facilitates their transcriptional activity in starved state of mouse by activating the gluconeogenic genes, such as phosphoenolpyruvate carboxykinase-1 (PEPCK-1), glucose-6-phosphatase (G6Pase) and insulin-like growth factor-binding protein 1. In contrast, CAR and PXR in presence of their ligand, act as co-repressors to down regulate FoxO1-mediated transcription. In addition to inhibiting FoxO1 activity, drug-activated PXR and CAR may also inhibit hepatocyte nuclear factor-4 (HNF-4)activity by binding to PGC-1α and competing for the DR1 (direct repeat spaced by one nucleotide) binding motif in the gluconeogenic enzyme gene promoters (Miao et al, 2006). Further, it has been reported that activated PXR directly interacts with FoxA2and leads to repression of lipid metabolism in fasting mouse livers.This interaction prevents FoxA2 binding to the CPT1A (Carnitine Palmitoyltransferase 1A) and HMGCS2 (Hydroxymethylglutaryl CoA Synthase 2) gene promoters required for fatty acid oxidation and ketogenesis (Nakamura et al, 2007). In other way, to regulate gluconeogenesis process activated PXR directly binds to phosphorylated cAMP Response Element-Binding protein (CREB). So,inhibiting interaction of CREB with cAMP response element results into inhibition of CREB-mediated transcription of G6Pase in fasting liver (Kodama et al, 2007).The crosstalk between PXR and FoxO1 and FoxA2 indicates that PXR not only regulates hepatic drug metabolism, but also plays important roles in glucose and energy homeostasis.

PXR in lipid metabolism:PXR plays an important role in lipid homeostasis by activating genes that facilitate lipogenesis and suppress the oxidative pathways. Liver X Receptor (LXR) is known to promote hepatic lipogenesis by activating the lipogenic transcriptional factor Sterol Regulatory Element-Binding Protein-1c (SREBP-1c) (Kersten et al, 1999; Repa et al 2000). PXR mediates a unique SREBP-independent lipogenic pathway by activating the free fatty acid uptake transporter Cd36 and several accessory lipogenic

enzymes like stearoyl-CoA desaturase-1 (SCD-1) and fatty acid elongase (FAE), which are important for lipogenesis. However,expression of other lipogenic enzymes likes fatty acid synthase (FAS) and acetyl-CoA carboxylase 1 (ACC-1) isnot affected in transgenic mice and in pregnenolone 16α-carbonitrile (PCN)-treated hepatocytes (Zhou et al, 2006; Zhou et al, 2008). CAR and PXR transcriptionally activate Insig-1 by binding to an enhancer sequence of the Insig-1 gene, this leads to reduced levels of active SREBP-1c and consequently to reduced target gene expression including the genes responsible for triglyceride synthesis (Roth et al, 2008). A recent report suggeststhe activation of PXR by PCN in AKR/J mice can prevent the development of high-fat diet-induced obesity and insulin resistance (Ma and Liu, 2012).The activation of PXR is also suggested to be associated with inhibition of pro-β-oxidative genes, such as Peroxisome Proliferator-Activated Receptor α (PPARα) and thiolasewhich leads to up-regulation of PPARγ, a positive regulator of CD36. The cross-regulation of CD36 by PXR and PPARγ suggests that this fatty acid transporter may function as a common target of orphan nuclear receptors in their regulation of lipid homeostasis (Zhou et al, 2006).

PXR in vitamin D metabolism:Vitamin D plays an important role in calcium homeostasis and the development and maintenance of bones through activation of vitamin D receptor (VDR).PXR can also induce vitamin D deficiency and bone disease because of its ability to cross-talk with the vitamin D–responsive gene that catabolizes 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D (1,25-VD3) (Pascussi et al, 2005; Holick, 2005). Due to 60% homology of the amino acid sequence in DNA binding region between PXR and VDR, ligand-induced VDR/RXRα heterodimer bind to PXR response element (distal ER6) for maximal intestinal expression of CYP3A4 (Bertilsson et al, 1998; Pavek et al, 2010). PXR also binds to proximal VDRE (Pascussi et al, 2005) in the same manner to regulate transcription of the vitamin D(3) 24-hydroxylase (CYP24A1) gene. The involvement of VDR in ABCB1 expression (also under the control PXR) in presence of 1,25-VD3 and lithocholic acid (LCA) in human colorectal adenocarcinoma cell line LS174T

has also been reported (Tachibana et al, 2009). Recent reports suggested that induction of CYP3A4 in the intestinal epithelium by PXR agonists can result in a greater metabolic clearance of 1,25-VD3 and reduced effects of the hormone on the intestinal calcium absorption, which may contribute to an increased risk of drug-induced osteomalacia or osteoporosis in patients receiving chronic therapy (Xu et al, 2006; Zheng et al, 2012). The mechanism by which CYP24A1 expression is regulated by PXR involves recruitment of co-repressor SMRT onto the CYP24A1 promoter. In the absence of vitamin D3, PXR locks co-repressor SMRT onto the CYP24A1 promoter to attenuate vitamin D3 activation and in the absence of vitamin D3, PXR activates the CYP24A1 gene (Konno et al, 2009). So, SMRT locking depends upon the ratio between vitamin D3 to the human PXR activating ligand. Several recent reports also indicate that cross-talk between PXR and VDR plays a role in calcium homeostasis, bone metabolism, cell growth and maturation, immunomodulation, renin and insulin production functions (Pascussi et al, 2005; Holick, 2005; Konno et al, 2009).

B. Role of PXR in xenobiotic metabolism

PXR hasnow been established as an important xenosensor that regulates the expression of many detoxifying enzymes and transporters and is crucial for normalphysiological functioning of living organism (**Figure 3**). The implication of this regulation also results in drug metabolism, drug–drug interactions and several human diseases.Subsequent functional analysis has revealed the presence of PXR response elements in the promoter regions of many drug metabolizing enzymes and transporter genes (Kliewer et al, 2002) and is briefly described below:

Phase I:It catalyzes the first step of detoxificationof lipophillic or aliphatic compounds.It includes oxidation, reduction, hydrolysis and hydration.The products of phase I metabolism are generally more polar and more readily excreted than the parent compounds and are often substrates for phase II enzymes. These include several phase I enzymes likeCYP3A4, CYP3A23,

CYP3a11, CYP2B6, Cyp2b9, Cyp2c55, CYP2C8, CYP2C9, CYP2C19 and CYP1A CYP3A7 (Synold et al, 2001).

Phase II:It involves conjugation with endogenous hydrophilic compounds to further increase polarity and water solubility.Phase II enzymes includes i) glutathione S-transferases(GSTs) catalyzes nucleophilic attack via reduced glutathione(GSH) on non-polar compounds containing an electrophilic carbon, rendering them less reactiveand more hydrophilic (Falkner et al, 2001), ii) UDP-glucuronosyltransferases (UGTs) catalyzing the addition of a UDP-glucuronic acid to endobiotics and xenobiotics,enhancing their water solubility and elimination,and iii) sulfotransferases (SULTs) catalyzing the addition of sulfate conjugates on drugmolecules leading to more water soluble compounds (Sonoda et al, 2002).

Phase III: It involves excretion through drug transporters, multidrug resistance protein 1 (MDR1) (Geick et al, 2001), MDR2 (Dussault et al, 2001), multidrug resistance-associated protein 2 (MRP2) (Kast et al, 2002) and the organic anion transporter polypeptide 2 (OATP2) etc. (Staudinger et al, 2001).

C.Role of PXR in metabolic disorders

Evidences emerging from recent reports suggest that other than being the 'master-regulator' of xenobiotic metabolism PXR may have other diverse roles in various metaboloic disorders which remain tobe fully explored (Qiao et al, 2013; Koutsounas et al, 2013).In general, PXR is believed to be expressed primarily in liver and intestine, tissues where maximum detoxification of noxious compounds occurs. However, the expressionand function of PXR in certain malignancies remains somewhat unclear. Also, PXR mediated chemoresistance originating from inducible activity of PXR leading to reduced efficacy of chemotherapy is a major concern that warrents further investigation. Some of the areas where involvement of PXR is implicated are briefly described below:

PXR in Inflammation:Recent evidence revealed a hepatoprotective role for PXR in chronic and acute liver injury, inhibiting liver inflammation through suppression of the NFκB pathway (Zhou et al, 2006). Significant relationships between expression of PXR and enhanced expression of drug transporters like ABCB1, ABCC2, ABCG2 have been reported in peripheral blood mononuclear cells (PBMCs) and small intestine (Albermann et al, 2005). Presence of PXR in B1 cell (Casey and Blumberg, 2012) and T lymphocytes (Dubrac et al, 2010) shed some light on conjunction of PXR with inflammation.PXR is shown to modulate key NFκB target genes in liver during fetal development (Casey and Blumberg, 2012). Upon immune activation PXR inhibits T lymphocyte proliferation and anergizes (lesser immune reactive) T lymphocytes by decreasing the expression of CD25 and IFN-γ and decreasing phosphorylated NFkB and MEK1/2 in mouse and human (Dubrac et al, 2010). Recent evidence supports a hepatoprotective role for PXR in chronic liver injury, inhibiting liver inflammation through suppression of the NFκB pathway (Zhou et al, 2012). However, PXR-mediated induction of CYP3A enhances APAP-induced acute liver injury by generating toxic metabolites (Li et al, 2012). Activation of PXR not only suppresses expression of NFkB target genes, including those encoding IL-1b, IL-10, inducible Nitric Oxide Synthase (iNOS) and Tumor Necrosis Factor α (TNFα), suggesting that PXR dampens the inflammatory response (Cheng et al, 2012).A recent report revealed that SUMOylated PXR directly represses NFkB in liver (Hu et al, 2010). NFkB activation by lipopolysaccharide and TNF-α plays a pivotal role in the suppression of CYP3A4 through interactions of NFkB with the PXR-RXR complex (Gu et al, 2006). PXR is also shown to play a role during inflammation in the down-regulation of several hepatic proteins like hepatic transporters and metabolic enzymes (Teng and Piquette-Miller, 2004).

PXR in cancer:The precise roles of PXR in cancerous tissues remain unclear. Some reports suggest that PXR and its activators suppress cancer proliferation in some tissues however; there are also reports that indicate PXR as an inducer of cell proliferation in some cancer cells (Qiao et al, 2013;

Koutsounas et al, 2013). In addition, there are substantial reports suggestingPXR cross-talk with many other signaling pathways to modulate their function. It has been recently reported that PXR can negatively regulate some distinct signaling pathways like HNF4α-mediated transcription and NFκB (Bhalla et al, 2004; Zhou et al, 2006). In contrast, in a human colon adenocarcinoma LS180 cells, it is suggested to be involved in nullifying the protective effect mediated by vitamin D (Zheng et al, 2012). In addition, a significant correlation between PXR expression and ERα status exist in breast cancer as both direct and inverse relationship with higher PXR expression in ER-positive tumors (Miki et al, 2006). Additionally, higher PXR expression was found to be positively associated with lymph node status, histologic grade, Ki-67 proliferation marker and p450 aromatase (estrogen synthase) expression in ER-positive cases (Miki et al, 2006). In contrast to this,increase in PXR expression down regulates ERα expression was identified in ER-negative breast cancer cell lines (Dotzlaw et al, 1999; Conde et al, 2008).Furthermore, in several cancerous cell types, PXR-mediated induction of xenobiotic metabolizing enzymes especially CYP3A4, as well as certain transporters, i.e. MDR1, leads to chemo-resistance in these cells. A recent study demonstrated that reduced chemo-sensitivity of colorectal cancer cells to irinotecan (a topoisomerase I inhibitor) was reversed by the PXR antagonist sulforaphane (Zhou et al, 2007; Raynal et al, 2010). So, the concept has been proposed to tackle resistance to anticancer drug by pharmacological antagonistof PXR (Raynal et al 2010; Chen et al, 2010). In addition, the emerging evidences point to the involvement of PXR in regulating apoptotic and antiapoptotic as well as growth factor signaling that promote tumor proliferation and metastasis (Pondugula and Mani, 2009).PXR regulates proliferation of both cancerous states like colon (Zhou et al, 2008; Harmsen et al, 2010; Ouyang et al, 2010), ovarian (Gupta et al, 2008; Yue et al, 2010), prostate (Chen, 2007), endometrial (Masuyama et al, 2003; Masuyama et al, 2007), osteosarcoma (Osman et al, 2007), esophageal (Takeyama et al, 2010) and non-cancerous cells like progression of liver regeneration (Dai et al, 2008).Further study demonstrated tumor suppressor protein p53 negatively regulates PXR activity that appears to play a

significant role in carcinogenesis (Elias and Chen, 2013). So, the studiessuggest hitherto unknown but important functionsfor PXR in tumor.Therefore, investigations directed towards exploring the differential transcriptional regulatory mechanisms of PXR in normal and cancerous tissues need to be pursued to gain major insights into the development of chemo-therapeutic resistance during the course of malignancy.

D. Lesser known roles of PXR

PXR in apoptosis and cell cycle:PXR is implicated in sensitizing cells to oxidative cellular damage and in regulation of two crucial apoptosis inhibitor proteins, Bcl-2 and Bcl-xl which permit the cells to propagate with damaged DNA that eventually transforms them into malignant state (Zucchini et al, 2005). In addition, the emerging evidence points to PXR in regulating apoptotic and antiapoptotic as well as growth factor signaling that promote tumor proliferation and metastasis (Zhou et al, 2008; Pondugula and Mani, 2013). PXR induces osteoclast apoptosis (Tabb et al, 2003; Igarashi et al, 2007) and regulates cell growth in a variety of cancer tissues (e.g., colon, ovarian, prostate, endometrial, osteosarcoma etc.) through multiple mechanisms. On the contrary, PXR inhibits the proliferation and tumourigenicity of colon cancer cells by controlling cell cycle at G0/G1 cell phase by regulating p21WAF1/CIP1 and E2F/Rb pathways (Ouyang et al, 2010). Another report has been suggested that rifampicin-activated PXR inhibits the proliferation of HepG2 cells by arresting in G0/G1 phase (Zhuang etal 2011).

PXR in blood brain barrier:P-glycoprotein, an ATP-driven drug export pump which is uniquely regulated by PXR, is expressed at the blood-brain barrier and is a primary ‘gatekeeper’ for poor penetration of many therapeutic drugs into the central nervous system (CNS) (Bauer et al, 2004). Experiments have shown that PXR is indeed expressed in rat brain capillaries and increased p-glycoprotein activity which tightened the blood-brain barrier to methadone (a CNS-acting, P-glycoprotein substrate), reducing the drug's CNS efficacy after

in vitro and *in vivo* (transgenic mouse expressing human PXR) exposure to the PXR ligands PCN and dexamethasone (Bauer et al, 2004; Bauer et al, 2006).Another efflux pump, MRP2 and phase-II drug-metabolizing enzyme, GSTπ expression were increased in rat brain capillaries where both were colocalized to a large extent at the endothelial cell luminal plasma membrane after *in vitro* and *in vivo* exposure to PCN and dexamethasone (Bauer et al, 2008). Due tospecies specific differences rodent models are not suitable to predict xenobiotic interactions with human PXR.To validate this further, study establishedthe expression patterns of the genes encoding ATP-binding cassette in porcine brain capillary endothelial cells, brain microvessels and cortex biopsies isolated from patients with epilepsia or glioma (Dauchy et al, 2008; Ott et al, 2009; Lemmen et al, 2013). These findings indicated that, as in hepatocytes, brain capillaries possess a regulatory network consisting of NRs, metabolizing enzymes and efflux transporters, which in concert may modulate blood–brain barrier functions.

PXR in AIDS:Certain drugsused to treat human immunodeficiency virus (HIV) infectionsinduce cytochrome P450via PXR. A reverse transcriptase inhibitorPNU-142721, which also proved to be efficacious PXR agonist to treat HIV infection, causes strong drug–drug interactions (Cheng and Redinbo, 2011). Atazanavir (ATV) is an HIV protease inhibitor and its plasma concentrations are influenced by PXR-regulated drug metabolizing enzymes and transporters like CYP3A4, ABCB1 etc. The PXR single nucleotide polymorphisms (SNP) 63396C→T alters PXR expression and CYP3A4 activity resulting in reduced plasma concentration of unboosted(without booster) atazanavir (Siccardi et al, 2008; Alessandro et al, 2010). The association of SNP in PXR is likely to be mediated through an effect on hepatic PXR expression and therefore expression of its target genes (e.g., CYP3A4, SLCO1B1 and ABCB1), which are known to be involved in HIV drugs clearance (Schipani et al, 2010).

Nuclear receptor isoforms: basic insight

Protein diversity is generated by different forms of the same protein which may be produced from related genes or may arise from the same gene by using different mechanisms like alternative splicing, alternative promoter usage and alternative translation initiation mechanisms. Generation of isoforms from a single gene greatly increases the total number of receptor isoforms which may be expressed in a cell-dependent and time-dependent manner with diverse roles.This diversityregulates the transcription functions, co-factor recruitment, localization of receptor proteins and their interaction with ligands.

Mechanism of generation of different receptor isoforms

Alternate mRNA processing:The mammalian genes use alternative splicing as a key means to enlarge transcript pool that results in a single gene coding for numerous proteins. In this process, particular exons of a gene may be included within, or excluded from, the final, processed messenger RNA produced from that gene (**Figure 5**). Consequently, the proteins translated from alternatively spliced mRNAs will contain differences in their amino acid sequence and often, in their physiological functions. Notably, alternative splicing allows the human genome to direct synthesis of many more proteins than would be expected from its 20,000 protein-coding genes.Alternative splicing can modulate the biological function of proteins by i) deleting or adding specific domains ii) post-translation modification sites or iii) by causing significant changes in protein structure by changing even just a few residues (Davletov and Jimenez 2004).

There are ample evidences suggesting that NRs undergo alternative splicing examples of which include AR (Dehm et al, 2008; Guo et al, 2009; Hu et al, 2009), GR (Lewis-Tuffin & Cidlowski, 2006; Kino et al, 2009), MR (Laurent Pascual et al, 2005), TR (Pramfalk et al, 2011) etc.Four alternatively spliced CAR variants have been reported in human liver (Auerbach et al,

2003). ERα and ERβ are the products of two geneslocated on different chromosomes, but share significant sequence homology (Gustafsson, 1999).A number of variant isoforms of the wild type ERβ1, have been identified by using of alternative start sites or deletion of exons by alternative splicing of exons encoding the carboxy- terminus which results in formation of several isoforms (ERβ2-β5) (Graeme et al, 2002).

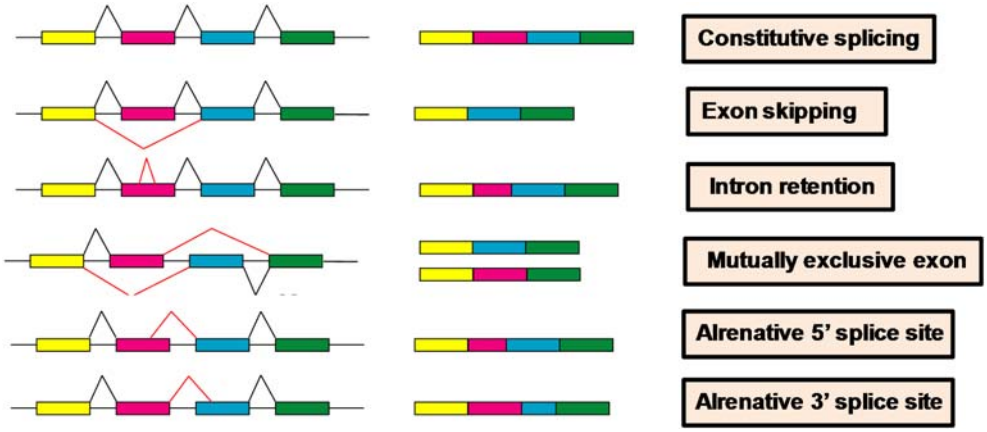


Figure 5: Pre-mRNA alternative splicing generates divergent protein isoforms by different mechanisms.*In a given transcript of pre-mRNA which has been transcribed from one gene can be chopped and reconnected in different ways like exon skipping, intron retention, mutually exclusive exon, alternative 5' splice site and alternative 3' splice site to yield various new mRNAs.*

Differential promoter usage:The mRNA isoforms are usually transcribed by alternative promoters by inclusion of alternative first exons (**Figure 6**). Numerous genes displaying complex transcriptional regulation, because of the use of alternative promoters, have been identified (Landry et al, 2003).

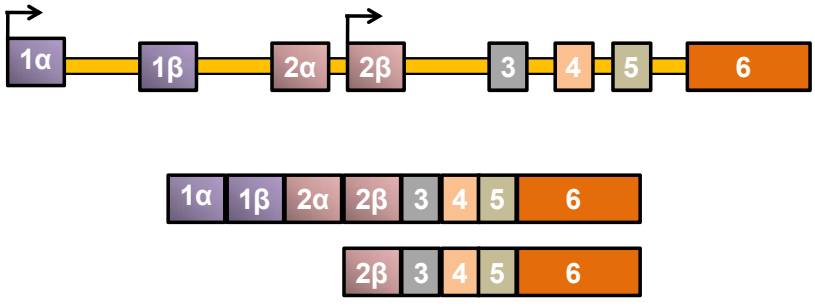


Figure 6:Schematic representation of alternative promoter usage.*Differential promoter usage results in the transcription of either short or long mRNA. Different square boxes represent different exons and arrow represents a alternative promoter usage.*

Alternative first exons of GR, under the control of specific transcription factors, control both the tissue specific expression and transcriptional response (Turner et al, 2006; Davuluri et al, 2008).HNF4Aexists inmultiple isoforms that are generated by alternative promoter use and splicing (Tanaka et al, 2006). Altered expression patterns of these isoforms in gastric, hepatocellular and colorectal carcinomas have been observed and it is proposed that the dysregulation of alternative promoter use of HNF4A is associated with the pathogenesis of certain cancers (Tanaka et al, 2006). Using knock-in mouse models, it is reported that mice that exclusively express either HNF4A1 or HNF4A7 show dyslipidemia and impaired glucose tolerance, respectively, revealing functional specificities of the differential promoter use (Briancon and Weiss, 2006).Nuclear receptor PR transcribed from a single gene by alternate initiation of transcription from two distinct promoters, results in PR-A and PR-B forms (Kastner et al, 1990; Gronemeyer et al, 1991). In case of PXR,alternative transcription initiation sites results in generation of two isoforms, PXR-1 and PXR-2 having altered usage of first exon by transcriptional machinery (Tompkins et al, 2008).

Alternative translation initiation mechanism:Regulation of the translation of eukaryotic mRNA has been shown to be the origin of several isoforms and thus constitutes anadditional mechanism of biological control (Strubin et al, 1986; Kobayashi et al, 2009). Alternative translational initiation (ATI) is one of mechanisms to increase the complexity level of an organism by alternative gene expression pathways. The use of ATI codons in a single mRNA contributes to the generation of protein diversity. The genes produce two or more versions of the encoded proteins and the shorter version, initiated from a downstream in-frame start codon, lacks the amino-terminal amino acids fragment of the full-length isoform version (**Figure 7**).

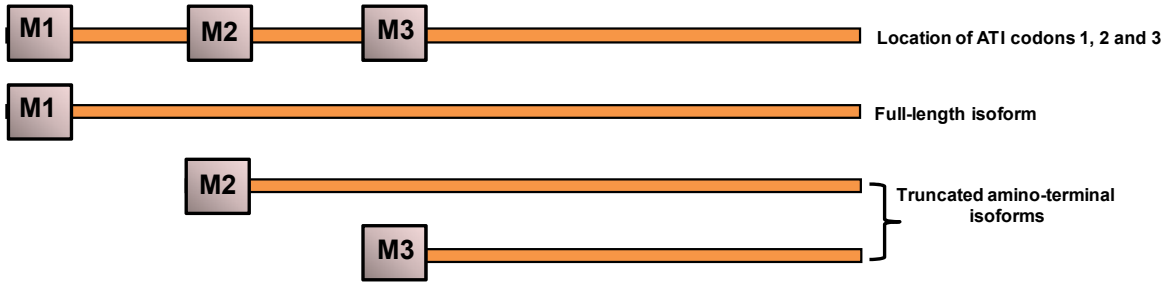


Figure 7:Pictorial representation of alternative translation initiation.*Alternative start AUG codons (for methionine) within a single transcript can contribute to diversity of the proteome.Alternative translation initiation from downstream in-frame methionine (M) results into amino-terminal truncated form of protein isoforms M2 and M3.*

There are several example of alternative translation initiation in nuclear receptor super-family including ERα, MR, AR and GRα (**Table II**) (Wilson & McPhaul, 1994; Barraille et al, 1999; Pascual-Le Tallec et al, 2004; Lu & Cidlowski, 2005).In case of ERβ the use of alternative start sites within the mRNA leading to translation of either a long (530 amino acids, hERβ1L) or a truncated small form (487aa hERβ1s) has been also reported(Graeme et al, 2002).Two forms of AR protein, AR-A and AR-B (87 and 110 kDa respectively) are present in human genital skin fibroblasts (Wilson & McPhaul, 1994). Two major forms of human MR protein result from the utilization of alternative initiation AUG sites located in the extreme NTD, named MR-A and MR-B (Pascual-Le Tallec et al, 2004).The co-expression of both isoforms results into significant reduction of transcriptional activity of MR-A isoform (Pascual-Le Tallec et al, 2004). Although the generation of distinct GR isoforms by alternative splicing has been recognized for many years, only recently was it demonstrated that an additional cohort of receptor proteins is produced by ATI from a single GRα mRNA species (Yudt et al, 2001; Lu & Cidlowski, 2005; Lu et al, 2007). Polymorphisms in the GR gene, as well as heterogeneity in the 5'-untranslated region of the GRα mRNA, have been reported to influence the efficiency of alternative start codon usage (Pedersen et al, 2004; Russcher et al, 2005).

Table II: Reported alternative translation initiation in different NRs

NRs	Methionine sites	Molecular weight	Mechanism	Functional significance	Reference
ERα-67 ERα-45	1 174	67 45	cap independent process which include an internal ribosome entry site	unable to induce cell proliferation, bypasses EGF activation of the unliganded ER	Bunone et al, 1996; Barraille et al, 1999
MR-A MR-B	1 15	107 105.4		Distinct transcriptional properties (MR-A>MR-B)	Pascual-Le Tallec et al, 2004
AR-A AR-B	1 188	110 87		AR-A and AR-B possess similar functional activities	Wilson & McPhaul, 1994; Wilson & McPhaul, 1998
GRα-A GRα-B GRα-C1 GRα-C2 GRα-C3 GRα-D1 GRα-D2 GRα-D3	1 27 86 90 98 316 331 336	94 91 82–84 (C1-C3) 53–56 (D1-D3)	ribosomal leaky scanning and ribosomal shunting	No significant differences in their transcriptional activity. GRα-C isoform is most active while GRα-D isoforms is least active. All the isoforms show cytoplasmic localization in absence of ligand however GRα-D shows ligand-independent retention in the nucleus. All isoforms regulate both common and unique sets of genes	Yudt et al. 2001; Lu & Cidlowski, 2005; Lu et al, 2007

The sequences residingin the NTD may play a previously unappreciated role in nuclear translocation, nuclear export and/or cytoplasmic retention of NRs. Interestingly, it has been reported that amino-terminal variants of steroid receptors play important roles in normal and patho-physiological situations. For example, variation in the ratio between the A and B forms of PR (Kumar et al, 1998) or between the 45 and 67 kDa forms of the ERα have been directly associated with human uterine and breast cancers (Flouriot et al, 2000). These amino-terminal isoforms possessdifferential transcriptional effects that appear to be due to differential recruitment of co-regulators and RNA polymerase II to their target gene promoters.The various unknown *cis*- and *trans*-factors which regulate this process of translation initiation now need to be characterized. Due to the low level of expression of these isoforms and the lack of high-affinity antibodies it might be a difficult taskto detect endogenous expression of these varients in different patho-physiological conditions. So,the generation of very specific antibody against small peptide of amino-terminal domain may facilitate investigation of the relative expression level of these isoforms *in vivo*.

PXR isoforms: an update

Owing to the small coding potential of the genome nature has evolved alternative mechanisms to sustain proteomic diversity. Regulations of these mechanisms were found to occur at the level of receptor expression, ligand availability and more recently, through post-translational modifications of the receptor and interaction with a variety of co-activators/co-repressors. To increase diversity of protein isoforms differing in structural or functional properties, many genes encode several variants of proteins by usage of alternate mRNA processing (alternate splicing, alternate exon usage) [GR (Lu et al, 2004)] or differential promoter usage [PR (Richer et al, 2002), PXR (Kurose et al, 2005), RAR (Katsetos et al, 1998)]. This also allows for targeted tissue-specific expression of varients from a single gene (Tompkins et al, 2008). In this context, spliced PXR mRNAs have been detected in human liver, breast tissue, colon, and small intestine (Dotzlaw et al, 1999; Fukuen et al, 2002; Lamba et al, 2004). The human PXR gene is reported to have alternative splicing in exon 5 (LBD) that yields three transcripts: PXR-1, PXR-2 and PXR-3 (Figure 8) (Bertilsson, 1998; Lamba, 2004). Human PXR-1 which is aptly spliced PXR includes exon 5; PXR-2 is lacking 111 nt, the first 37 amino acids in exon 5 and PXR-3 is lacking 123 nt, the first 41 amino acids in exon 5 (Lamba et al, 2004) (**Figure 8**). A recent study explains the functional significance of PXR-2 isoform that can bind to PXREs but fails to transactivate target genes (Lin et al, 2009). The study further explains that ligands do not bind the LBD of PXR-2 while co-repressors remain tightly bound and hence co-activators are not recruited to PXR-2. However, according to current view another molecular mechanism which potentially allows single mRNA to produce several proteins is 'alternative translation' (Barraille et al, 1999; Lu et al, 2004; Kochetov et al, 2008). Alternative use of amino-terminal initiation codons have been implicated in translation of various NR transcripts like MR (Pascual-Le Tallec et al, 2004), ER (Barraille et al, 1999), GR (Lu et al, 2005) etc. Different isoforms of PXR whether at transcriptional level, translational level or post-translational level, may not only change the co-regulators interaction profile, sub-cellular localization but

can also have a dramatic impact on its functional activity which further expands the signaling capacity of PXR.

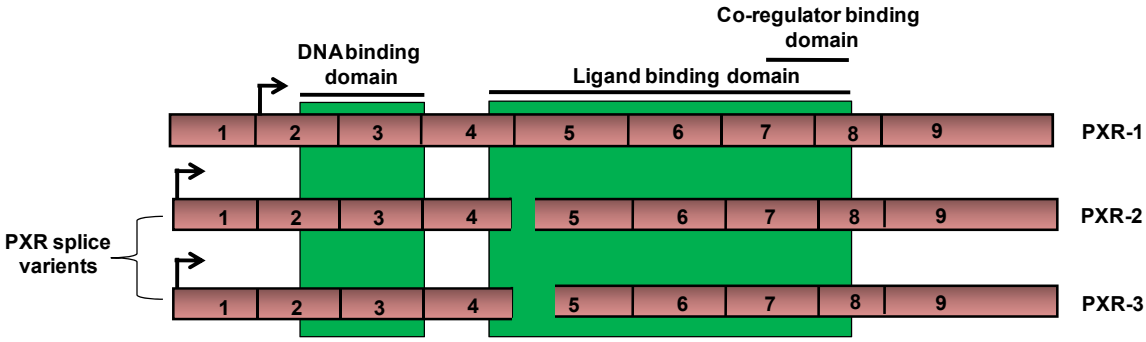


Figure 8: Human PXR gene has alternative splicing in exon 5 that yields three transcripts: *The PXR gene consists of nine exons.Three alternatively spliced transcripts that encode different isoforms of PXR have been described. PXR-1 (appropriately spliced PXR that includes full exon 5)initiatestranslation from an in-frame, downstream non-AUG (CUG) codon, PXR-2 encodes the longest isoform and initiates translation from the standard AUG codon present in its 5' terminal exon (lacks 111 nt, the first 37 amino acids in exon 5), PXR-3 (lacks 123 nt, the first 41 amino acids in exon 5). Arrow signify translational initiation site of PXR transcript (adapted and modified from Lamba et al, 2004).*

Taken together, these results provide convincing evidence that different members of NR super-family may generate isoforms by different mechanismsimparting altered functions towards cell fate. These findings suggest that these mechanisms may be a common strategyby which NRs mediate diverse signaling responses.

Post-translational modifications of PXR

Post-translational modifications (PTMs) of NRs are involved in the regulation of gene transcription and other nuclear processes (Faus and Haendler, 2006; Berrabah et al, 2011; Anbalagan et al, 2012).In case of PXR different post-translational modification like phosphorylation, ubiquitation, acetylation and SUMOylation are being reported (**Figure 9&Table III**) (Masuyama et al, 2000;Pondugula et al, 2009; Lichti, et al, 2009; Hu etal, 2010; Biswas etal, 2011; Staudinger et al, 2011). Future research in the field of oncology is for identification of specific PTMs of nuclear proteins as cancer biomarkers to provide a better view for the development of small molecules to

inhibit their functions for cancer chemotherapy.Recently, many investigations have provided directevidence for NRs PTM in the pathophysiologicalprogression of several diseases including cancers,diabetes, obesityetc. (Anbalagan et al, 2012; Mukherjee et al, 2012; Knutson et al, 2012).

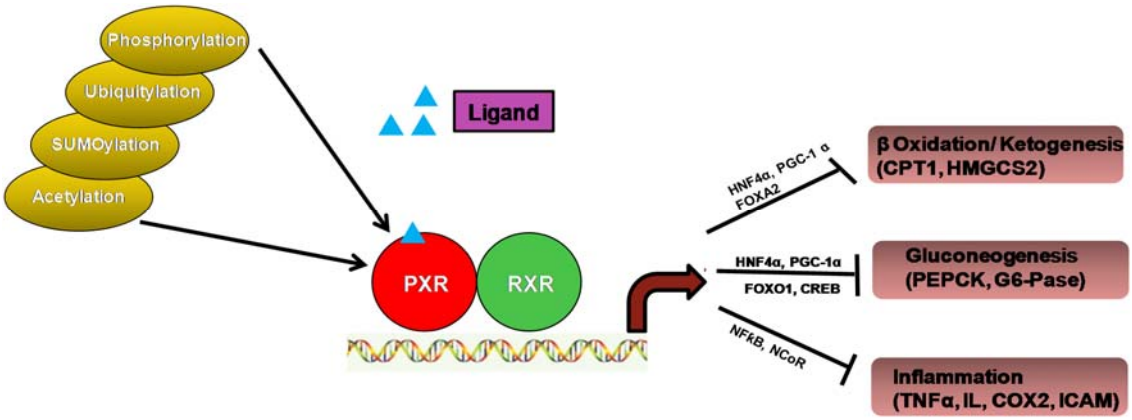


Figure 9:Different post-translational modifications of PXR and negative regulatory roles of PXR.*PXR was originally characterized for its role in xenobiotic and endobiotic detoxification. However, recent evidences have described role of PXR in glucose and lipid homeostasis, as well as in repression of inflammatory programs of gene expression. A central role for post-translational modification of PXR is hypothesized to selectively repress biochemical pathways in liver and intestine. Abbreviations: Forkhead box protein 1(FOXO1), Forkhead box protein A2 (FOXA2), Carnitine Palmitoyltransferase 1 (CPT1), 3-Hydroxy-3-Methylglutaryl-CoA Synthase 2 (HMGCS2), Cyclooxygenase 2 (COX2), Intercellular Adhesion Molecule (ICAM), Nuclear receptor Co-Repressor (NCoR), Phosphoenolpyruvate Carboxykinase (PEPCK) (adapted and modified from Staudinger et al, 2011).*

Phosphorylation

There is ample evidence in literature indicating that phosphorylation plays an important role in modulating NRs function(Ding & Staudinger, 2005a; 2005b; Lichti-Kaiser et al, 2009a; 2009b; Pondugula et al, 2009a; 2009b). Phosphorylation was the first identified covalent modification of PXR (Ding & Staudinger, 2005a). Overall 18 predicted consensus kinase recognition sequences in the human PXR protein have been reported, among which ser-8, thr-57, ser-208, ser-305 and thr-408 are critical for biological activity of PXR (Lichti-Kaiser et al, 2009a). These sites are conserved in

mouse and rat. The role of PXR phosphorylation seems to be sometimes activating and often inhibitory (Pondugula et al, 2009b). For example it has been observed that treatment of primary rat and human hepatocytes with Protein Kinase A (PKA) activator leads to the attenuation of *Cyp3A1* (Cyp3A1 is the rat ortholog of human CYP3A4) and *CYP3A4* mRNA levels, respectively while similar treatment of mouse hepatocytes increases the induction of Cyp3a11 by recruiting co-activator SRC-1 and PPAR-binding protein (Ding & Staudinger, 2005a;Lichti-Kaiser et al, 2009b). Similarly, Protein Kinase C (PKC) activation by Phorbol Myristate Acetate (PMA) leads to repression of PXR activity in reporter gene assays (Ding and Staudinger, 2005b). The mechanism behind this repression is due to the increasein strength of interaction between PXR and NCoR protein and by abolishingthe ligand-dependent interaction between PXR and SRC-1. Interestingly, this finding is further strengthened by the fact that the Protein Phosphatase 1 and 2A (PP1/2A) inhibitor okadaic acid strongly represses PXR-dependent transactivation (Ding and Staudinger, 2005b).

Table III: Reported post-translational modifications of PXR

Name of the PTMs	PTMs site	Pathway involved	Functional consequences of PTMs	Reference
Phosphorylation	ser-8, ser-208, ser-305 and thr-408	Protein Kinase C	Represses PXR activity, PMA increases the strength of interaction between PXR and NCoR and also inhibits ligand-dependent interaction between PXR and SRC-1.	Ding and Staudinger, 2005b
	N/A	Protein Kinase A	Modulates PXR activity in a species-specific manner, potentiating effect on PXR-mediated gene activation in mouse hepatocytes, it serves as a repressive signal in both human and rat hepatocytes.	Ding and Staudinger, 2005a
	ser-350	Cyclin-Dependent Kinase 2	S350D phosphomimetic mutation seems to impair the function of human PXR, whereas a phosphorylation-deficient mutation (S350A) conferred resistance to the repressive effects of Cdk2 on a reporter gene in HepG2 cells.	Lin et al, 2008
	thr-57	p70 S6 kinase	Phosphomimetic mutation T57D that is associated with the loss of function of human PXR	Pondugula et al, 2009a
Ubiquitination	N/A	SUG-1, CHIP	Proteasomal degradation	Masuyama et al, 2002; Masuyama et al, 2005; Rana et al, 2012
SUMOylation	Hinge	SUMO-3	Repress the inflammatory response	Hu et al, 2010
Acetylation	N/A	SIRT1	Reduced the transactivation potential of PXR	Biswas et al, 2011

N/A = information not available

Different cell-cycle regulating kinases are also involved in modulation of PXR activity.For example the treatment of HepG2 cells with flavonoids leads to an increase in CYP expression through modulating the activity of Cyclin-dependent kinase 5 (Cdk5) (Dong et al, 2010). Further investigation showed PXR to be a substrate for Cdk5 in *in vitro* kinase assayssuggesting that Cdk5 may modulate the activity of PXR through inhibitory phosphorylation (Dong et al, 2010). PXR also acts asa suitablesubstrate for the Cdk2 enzyme as observed by *in vitro*study, suggesting that Cdk2 may attenuate the activation of CYP3A4 gene expression through inhibitory phosphorylation (Lin et al, 2008). The functionality of putative Cdk2 phosphorylation site wasrevealed by phosphomimeticmutation at (S350D)that impaired the function of human PXR, whereas a phosphorylationdeficientmutation (S350A) conferred resistance to the repressiveeffects of Cdk2 on a reporter gene in HepG2 cells (Lin et al, 2008; Lichti-Kaiser et al, 2009a).Subsequently, it was reported that mutation ofhighly conserved putative phosphorylation site at thr-57 and less conserved site at thr-408,which is present in ligand binding domain,abolishes ligand-inducible PXR activity whereas different mutations at ser-8, ser-305, ser-350 and thr-408 decreases the ability of PXR to form hetero-dimer with RXRα which in turns affects the gene regulation cascades. Mutation at position ser-208, ser-305, ser-350 and thr-408 appears to alter PXR protein co-factor recruitment implicated in differential transcriptional behavior (Lichti-Kaiser et al, 2009a).

Phosphorylation also modulates the cellular trafficking of the receptor like mutation at thr-408 affects the sub-cellular localization of PXR protein (Lichti-Kaiser et al, 2009a). The major kinases that phosphorylate PXRinclude protein kinase A (PKA) (Ding and Staudinger, 2005a), PKC (Ding and Staudinger, 2005b), CDK2 (Lin et al, 2008), ribosomal protein S6 kinase (p70 S6K) (Pondugula et al, 2009a),Glycogen Synthase Kinase 3 (GSK3) and Casein Kinase 2 (CK2) (Lichti-Kaiser et al, 2009b), further suggesting that PXR may be modulated by a wide range of protein kinases. However, to this end, *in vivo* phosphorylation of PXR remains undetectable. It is possible that

the level of phosphorylation of PXR is below current detection limits. Asystematic approach to mutate serine/threonine (S/T) residues to aspartic acid (D) revealed that S8D, T57D, S208D, and T408D resulted in a decrease in PXR transactivation (Lichti-Kaiser et al, 2009a; Pondugula et al, 2009a). In addition to this, phosphorylation may not be the sole PTM acting at a given site, but it may compete or act in synergy with other modifications such as SUMOylation, methylation and ubiquitinylation which will tag NRs for a distinct fate or function. Collectively, phosphorylation of PXR confers a negative regulatory effect, possibly through altering its pattern of sub-cellular localization or affecting its interaction with co-repressors or co-activators. The results of these studies appear to confirm that the activity of PXR is modulated by changes in its overall phosphorylation status. Determining whether phosphorylation of PXR at specific sites influences the integration between cell-signaling pathways and PXR-mediated repression remains an open and important question for future studies.

Methylation

The protein arginine methyltransferases (PRMTs) including PRMT1, PRMT2, and PRMT4 (CARM1) act as co-activatorsto regulate expression of numerous genes(Chen et al, 1999; Koh et al, 2001; Qi et al, 2002; Rizzo et al, 2005) through methylation of histone and non-histone proteins, and these methylation codes are important for the NR-mediated transcriptional activity like AR and ERα (Ko et al, 2001; Le Romancer et al, 2008; Subramanian et al, 2008). Direct methylation of PXR protein is not yet known, though PRMT1 interacts with PXR (Ying et al, 2009). This suggested that PRMT1 is required for the transcriptional activity of rifampicin activated PXR and recruited to the regulatory region of the PXR target gene like CYP3A4, with a concomitant methylation of arg-3 of histone-H4 (Ying et al, 2009). Interestingly, PXR appears to have a reciprocal effect on the PRMT1 functions by regulating its cellular compartmentalization as well as its substrate specificity. Taken together, these results demonstrated mutual interactions and functional interplays between PXR and PRMT1 and this interaction may be important for the epigenetics of PXR-regulated gene expression.

Acetylation

Acetylation of proteins has emerged as an important regulatory modification which is diverse, context-dependent in nature and differs from protein to protein (Wang et al, 2011). Numerous studies have documented that acetylation of amino-terminal chains of histonesand the modifying enzymes involved were named HAT and HDACbased on their role in regulation of regulated acetylation or deacetylation process, respectively at specific lysine residues.This is an essential step in making chromatin accessible or inaccessible to regulatory proteins (Yang & Seto, 2008). Their substrates are not only limited to histones,but also to a variety of other non-histone proteinsincluding NRs like AR, GR, ER, RXR, LXR, FXR, TR and PXR which have been reported to undergo acetylation (Biswas et al, 2011; Wang et al, 2011).Studies have established that NR acetylation not only affect NR activity but also governs a variety of other cellular functions including DNA binding affinity, ligand sensitivity, receptor stability andsub-cellular distributionimplying that acetylation plays a crucial role in various physiological processes (Wang et al, 2011).The important role of acetylation affecting PXR functions has only recently been revealed (Biswas et al, 2011).It has been shown that activity of PXR is inversely proportional to the acetylation state of the protein. PXR/RXR agonist rifampicin/retinoic acidrespectively stimulates deacetylation of the PXR protein and SIRT1 a protein deacetylase is partially responsible for PXR deacetylation.Further efforts being are being made to investigate the molecular mechanism of acetylation affecting PXR functions.

Ubiquitination

Protein ubiquitination is catalyzed by a three-step enzymatic mechanism involving E1, E2 and E3 enzyme system linking ubiquitinmolecules to the targeted proteins and is traditionally considered to be an importantpathway in protein degradation (**Figure 10**).Polyubiquitylation usually targets a protein for degradation via the proteasome pathway whereas mono- and bi-ubiquitylation may influence

protein–protein interactions or sub-cellular localizationand may serve as a signaling mechanism,especially in DNA synthesis and repair (Haglund &Dikic, 2005).This process can be reverted by a de-ubiquitylating enzyme which allows precise control of key transcriptional events (Geng et al, 2012).

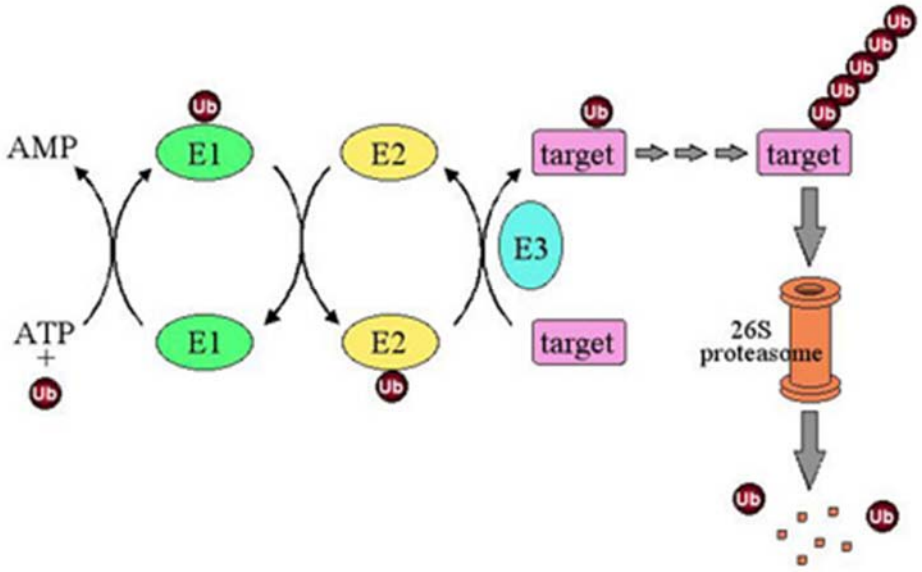


Figure 10:Schematic representation of ubiquitin-dependent proteolysis. This process involves E1-activation enzyme in an ATP dependent manner where *Ubiquitin (Ub) is activated and then transferred to an E2-conjugation enzyme. Finally ubiquitin is transferred from E2 to target substrate with the help of an E3-ligase enzyme. After several cycles of ubiquitination, the polyubiquitinated substrate is recognized by the 26S proteasome and then degraded.*

There are several lines of evidences showing that NRs undergo ubiquitylation thatinclude AR, ER, PR and GR (Faus &Haendler, 2006). Apparently, degradation also plays a pivotal role in precise control of key transcriptional eventsthoughno in-depth study has been performed (Staudinger et al, 2011). Also, there is insufficient evidence to reveal the exact mechanism behind the regulation of PXR protein stability, although a semi-quantitative approach has determined that the half-life of unliganded PXR is less than four hours. PXR ligand binding increases receptor half-life, due to the dissociation of its interaction with a component of the 26S proteasome machinerysuppressor for gal-1 (SUG1)(Masuyama et al 2002; Masuyama et al 2005).PXR was found to interact with SUG-1 and differentially degraded in response to progesterone but not in the presence of endocrine disrupting

chemicals (Masuyama et al, 2000). This finding suggests that proteasomal-mediated PXR degradation may be differentially affected by different ligands. It has been demonstrated that PXR mainly undergoes a lys-48 polyubiquitin linkage, which signals its degradation. Further,dramatic increase in ubiquitinated PXR after treatment with proteasomal inhibitor MG132and activation of the PKA signaling pathway selectively increases the ubiquitination of PXR but itnotably,abolishes PXR transactivation of the CYP3A4 promoter in reporter gene assay (Staudinger et al, 2011). A recent finding suggests that RBCK1, an E3 ubiquitin ligase, interacts with and ubiquinates PXR (Rana et al, 2012).However, it is noteworthy that many co-regulators of PXR are also subject to regulation through the proteasomal pathway (Lonard & O'Malley, 2009).The extent to which interactionbetween PXR and the ubiquitin signaling pathway affects lipid homeostasis, bone metabolism, energy homeostasis and inflammatory responses in humanmay providevaluableinformation.

SUMOylation

Analogous to ubiquitylation, SUMOylation is a three-step process involving an E1-activating enzyme (SAE1/SAE2), an E2-conjugation enzyme (UBCh9) and E3-ligases (**Figure 11**). It leads to reversible covalent attachment of a SUMO chain onto the lysine residues embedded in the consensus ψKxE motif where ψ is hydrophobic residue and X is any amino acid (Yang & Gregoire, 2006; Anckar &Sistonen, 2007). SUMOylation transiently marks the proteins for modulating their functions and specific protease catalyzes the deconjugation of SUMO-modified proteins known as SUMO specific protease(SENP) (Hay, 2005; Hay, 2007).

Even though SUMOylation modifies only a small proportion of a target protein, its effect can be quite dramatic. SUMOylation may influence different biological activities such as sub-cellular localization, DNA-binding or transcriptional potential of its target protein. Concerning transcription factors, in most of the cases a repressive action of SUMOylation has often been detected, but this may vary, depending on the promoter and cell type

studied (Anbalagan et al, 2012). Indeed, subtle differences in the effects of SUMO modification on steroid receptor function have been reported. For example studies with AR, ER, GR, MR, PR etc. indicate that SUMOylation is important for the fine-tuning of the activity of these receptors (Staudinger et al, 2011; Treuter &Venteclef, 2011).

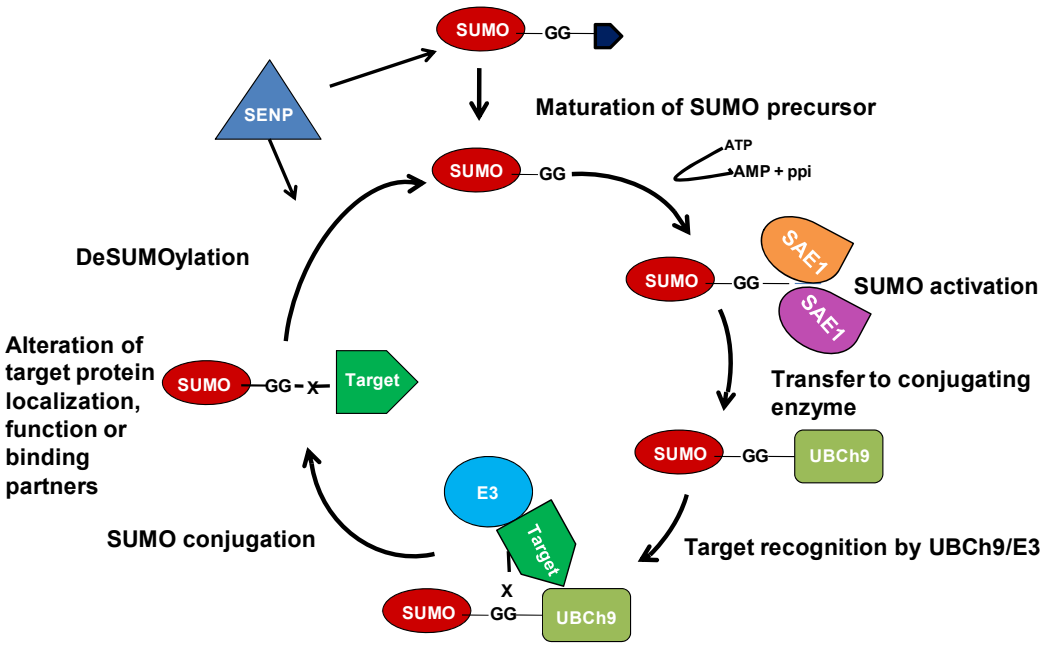


Figure 11:Schematic representation of enzymatic cascades involved in SUMOylation.*SUMOylation is a reversible covalent modification that involves three enzymatic steps, i) activation (E1), ii) conjugation (E2) and iii) ligation (E3).Prior to activation step, it is essential that the carboxy-terminal extension of SUMO is removed by specific proteases to generate a caroxy-terminus that ends in two glycine residues. Subsequently, E1 activation system which is a heterodimer of SAE1 (SUMO-activating enzyme subunit 1) and SAE2, uses ATP to transfer SUMO to E2 conjugating enzyme UBCh9. The SUMO E3 ligase (E3) then promotes the transfer of SUMO from UBCh9 to the target substrate, forming an isopeptide bond between the carboxy-terminus of SUMO and the epsilon-amino group of a lysine residue in the target protein.*

With respect to PXR fewer evidencesexist to explore the effect of SUMOylation on functional activity of PXR. A recent study demonstrated SUMO-dependent PXR transrepression pathways that link metabolism and inflammation (Lu et al, 2011). It has been suggestedthat inflammatory response in the liver significantly attenuates the SUMOylation of ligand-

bound PXR. SUMOylation of PXR was shown to occur mainly through the SUMO-3 chains which in turns down regulate the NFκβ target gene expression. By utilizing promoter-reporter based assaylittle effect of PXR SUMOylation on CYP3A4 gene expression has been seen.Moreover, E1-conjugation enzyme UBCh9 and E3 ligases like PIAS1 and PIAS4 can increase SUMOylation of PXR in individual manner (Hu et al, 2011). Presently,PXR SUMOylation is in early stage of demonstration and the present study shedsfurther light on the role of SUMOylation on PXR functions.

This review of literature summarizes the recent advances to elucidate the roles of PXR as a positive and a negative regulator of hepatic genes including xenobiotic metabolism, apoptosis, cholesterol biosynthesis, lipid metabolism, and cytokine signaling pathways. The purpose of the present review is to provide a comprehensive update of different isoforms and PTM of NRs in general with specific reference to PXR. There are at least three splice isoforms of PXR (i.e. PXR-1, PXR-2 and PXR-3) (Kleiwer et al, 1998; Blumberg et al, 1998). These isoforms exhibit differential expression, ligand binding affinity and transcriptional activity. The observations are tempting to speculate that differential expression of PXR isoforms may influence the functional consequences in normal physiological controls and dysregulation in metabolic disorders.Tumor-specific regulation of different isoforms or splice variants of PXR may serve as cancer prognostic marker. Identifying isoforms in human tumors and non-tumor tissues may be an important step towards defining the role of PXR isoforms in human cancer. Furthermore, emerging literature is indicating that PXR undergoes different PTMs that may alters its response to several metabolic, pathogenic and xenobiotic stresses associated with diseases and infections. In this regard, the study of PXR in terms of its isoforms and PTM is expected to reveal receptor involvement in many unknown physiological functions.

Aims & Objectives

Nuclear Receptors (NRs) are members of super-family of ligand-modulated transcription factors that are involved in diverse physiological phenomena ranging from mitosis to apoptosis. NRs regulate essential biological processes such as reproduction, development, metabolism, differentiation and maintenance of cellular homeostasis (McEwan et al, 2009). NRs are comprised of a ligand-independent transcriptional Activation Function 1 (AF1) domain at the amino-terminus, a highly conserved centrally located DNA Binding Domain (DBD), a hinge region, a moderately conserved carboxy-terminal Ligand Binding Domain (LBD) and a ligand-dependent Activation Function 2 (AF2) region at the carboxy-terminus (Bain et al, 2007). Precise spatial and temporal patterns of gene expression are crucial for the normal development of all organisms. The NRs regulate transcription by binding to response elements in the promoters of specific target genes at specific times and act as scaffolds for the assembly of large co-regulator complexes, coordinating numerous cellular events (Nagy & Schwabe, 2004; Perissi et al, 2005). The biological importance of NRs is further emphasized by the facts that like many other proteins these may also be subjected to various mechanisms such as alternative splicing, alternative promoter usage, alternative translational initiation, post-translational modifications (PTMs), polymorphism generating multiple isoforms which in turn execute differential and distinct biological functions (Breitbart et al, 1987; Ayoubi et al, 1996; Touriol et al, 2003; Lu & Cidlowski, 2004).

Pregnane & Xenobiotic Receptor (PXR; SXR; NR1I2) is one of the important members of the NR super-family and functions as a 'xenosensor' in our body. It is responsible for coordinated regulation of metabolic genes by diverse endogenous and exogenous compounds (Kliewer et al, 2002). For activation, PXR requires hetero-dimerizing partner, Retinoid X Receptor (RXR) and recognizes response elements in the promoter of target genes to alter their expression. Molecular studies have revealed that PXR executes transcription function by modulating a network of genes, including numerous drug metabolism genes *CYP3A*, *CYP2B*, *CYP2C* and *UGT1A1,*

along with several important transporter genes, including *MDR1* (Multidrug Resistance 1), *OATP2* (Na^+-independent Organic Anion Transporter 2) and *MRP2* (Multidrug Resistance-associated Protein 2) which are involved in xenobiotic metabolism and elimination (Kurose et al, 2005). However, the regulation of xenobiotic metabolizing enzymes by its different isoforms remains to be identified. PXR has been detected in various tissues, being relatively higher in the liver, small intestine and colon; and lower levels in the skeletal muscle, stomach, testes, trachea, kidney, adrenal gland and thyroid gland (Blumberg et al, 1998; Nishimura et al, 2004). PXR was also reported to be expressed at higher levels in different cancerous states like endometrial cancer (Masuyama et al, 2003), breast cancer (Dotzlaw et al, 1999), prostate cancer (Chen et al, 2007) and epithelial ovarian carcinoma (Gupta et al, 2008). The role of PXR in bile homeostasis (Kliewer et al, 2005), blood–brain barrier function (Bauer et al, 2008), inflammatory bowel disease, bone metabolism etc. are beginning to emerge. However, with respect to its different isoforms, PXR functions remain to be evaluated (Zhou et al, 2009; Qiao et al, 2013; Koutsounas et al, 2013).

Presently, there is a considerable interest in complex part of proteomics because of the small coding potential of the genome. Nature has evolved various mechanisms to meet proteomic diversity. It is well known that many genes encode several variants of proteins due to the usage of alternate mRNA processing (alternate splicing, alternate exon usage) [GR (Lu & Cidlowski, 2004)] or differential promoter usage [PR (Richer et al, 2002), PXR (Kurose et al, 2005), RAR (Katsetos et al, 1998)] to allow for greater protein diversity and targeted tissue-specific expression from a single gene (Tompkins et al, 2008). The human PXR gene is reported to have alternate splicing in exon 5 (LBD) that yields three transcripts: PXR-1, PXR-2 and PXR-3 (Bertilsson et al, 1998, Lamba et al, 2004). However, according to current view another molecular mechanism which potentially allows single mRNA to produce several proteins is ‘alternative translation’ (Barraille et al, 1999; Lu & Cidlowski, 2004; Kochetov, 2008; Bazykin & Kochetov, 2011; Coldwell et al, 2012). In eukaryotic cells translation initiation occurs

through two alternative mechanisms, i) a 'cap-dependent mechanism' operating in the majority of mRNAs and ii) a 'cap-independent mechanism' driven by internal ribosome entry site (IRES) elements (Komar et al, 2005; Blaszczyk et al, 2007), specific for a subset of mRNAs like insulin-like growth factor 1 receptor (Giraud et al, 2000), ultrabithorax and Antennapedia (Ye et al, 1997) and utrophin A (Miura et al, 2008) which recruit the translation machinery to an internal position in the mRNA through a mechanism involving the IRES structure and several trans-acting factors (Pacheco et al, 2009). A cap-dependent mechanism involves initiation of translation in eukaryotes which is the rate-limiting step of protein synthesis and involves a set of specialized proteins (Marcotrigiano et al, 1999). The recognition of the AUG triplet as translation initiation signal depends on its nucleotide context: if the context is optimal [consensus sequence, (GCC) GCCRCCAUGG, where R = G or A (Kozak, 2005)], most 40S ribosomal subunits will scan along the 5' untranslated region (UTR) until an AUG codon placed in the appropriate context which is recognized by the translation machinery to start protein synthesis. However, if the context is suboptimal, some 40S ribosomal subunits recognize it as translation initiation signal, but others may skip it, continue scanning in 3' direction and initiate translation at a downstream AUG called leaky scanning for e.g. chicken vitamin D receptor (Lu et al, 1997). Another mechanism called 'ribosomal shunting mechanism' involves discontinuous scanning of mRNA for as in case of GR (Lu & Cidlowski, 2005). Alternative use of the amino-terminal initiation codons implicated in translation of various transcripts like basic Fibroblast Growth Factor (bFGF) (Prats et al, 1992), GATA-1 transcription factor (Calligaris et al, 1995), GR (Lu & Cidlowski, 2005), Paired-like homeodomain transcription factor 2 (PITX2) (Lamba et al, 2008) and prion protein (Juanes et al, 2009).

From the functional point of view, proteins need to be directed to their proper cellular compartments in order to perform their necessary functions. The overall observations suggest that about >80% of alternative translation events generated biological diversity. Analysis of isoelectric point values

revealed that most amino-terminal truncated isoforms significantly lowered their isoelectric point values and targeted at different sub-cellular localizations in order to perform their necessary functions and allow regulation of key cellular events in a highly sophisticated manner (Cai et al, 2006). These alternative mechanisms used in cellular context are an economical way implies that there is no need for several genes, promoters or alternatively spliced mRNA variants. Different isoforms of protein whether at transcriptional level, translational level or post-translational level, may not only change the co-regulators interaction profile, sub-cellular localization but can also have a dramatic impact on their activity which further expands their signaling capacity.

In addition to the remarkable complexity of multiple receptor isoforms generated by alternative splicing and alternative translation initiation, each isoform may be subjected to a variety of post-translational modifications including phosphorylation, ubiquitination and SUMOylation that further regulate their transcription behavior of specific receptor (Faus & Haendler, 2006; Anbalagan et al, 2012). Studies with receptors like GR (Lu & Cidlowski, 2005; Lu et al, 2007), AR (Poukka et al, 2000), PR (Chauchereau et al, 2003), ER (Sentis et al, 2005) etc., have indicated that these modifications play important roles in the receptors' sub-cellular distribution, protein turnover and transcription functions (Faus & Haendler, 2006; Wang et al, 2011; Anbalagan et al, 2012). As reported in recent studies PXR also serves as a substrate for a variety of post-translational modifications (PTMs) like, phosphorylation (Ding & Staudinger 2005a; 2005b; Lichti-Kaiser et al, 2009a; 2009b; Pondugula et al, 2009a; 2009b), SUMOylation (Hu et al, 2010), acetylation (Mani et al, 2011) and ubiquitination (Masuyama et al, 2002; Staudinger and Mani, 2011). PXR, as is true with other NRs, mediates gene transcription in a complex and multi-step manner which involves precise sequence of events like ligand-binding, nuclear translocation, DNA-binding, co-regulator interaction and chromatin modification. All these events cooperate to achieve a precise regulation of gene transcription in a spatial-temporal manner. There are adequate numbers of evidences which suggest

that different isoforms of NR possesses diverse dynamic patterns and distinct transcriptional regulatory profile (Pascual-Le Tallec et al, 2004; Lin et al, 2009).

So, in this perspective it is reasonable to speculate that PXR and its potential isoforms may also display differential behavior and transcriptional function. Therefore, a systematic and comparative analysis of intra-cellular functional dynamics of PXR and its potential isoforms need to be studied. In this regard, differential sub-cellular localization, co-factor interaction profile, DNA binding and transcriptional activity of PXR and its isoforms warrant detailed investigation. Based on the literature survey, the current study focuses on two main aspects of PXR, with major emphasis on PXR isoforms and its PTMs. Such studies are expected to impart a better understanding of the functional impact of different isoforms and post-translational modifications on PXR transcription function and stability which could provide additional important clues for the mechanisms involved in gene specific regulation.

In view of the existing literature, the present research proposal is designed with the following four basic objectives:

1) Generation and characterization of monoclonal antibody against human PXR
2) Detection and identification of PXR and its potential isoforms
3) Post-translational modifications and functional analysis of PXR
4) Expression and distribution profile analysis of PXR and its potential isoforms in different cancerous cell lines

The findings from the proposed study are expected to provide basic insights into the mechanistic details of PXR functions under normal and patho-physiological situation.

Materials & Methods

MATERIALS

Mammalian & bacterial cell types and sources

Different mammalian cell lines used in this study were African green monkey kidney cell line COS-1, human hepatocellular carcinoma cell line HepG2, HepXR cell line (HepG2 cells stably integrated with PXR), HepXREM (HepG2 cells stably integrated with PXR and promoter-reporter CYP3A4-Luc), human breast cancer cell line MCF-7, human gastric adenocarcinoma cell line AGS, human prostate cancer cell line DU145, human embryonic kidney cell line HEK293, human colon adenocarcinoma cell line COLO 320DM, human ductal breast epithelial tumor cell line T47D, human adenocarcinomic alveolar basal epithelial cell line A549 and mouse myeloma cell line sp2/0-Ag14. All the cell lines are originally from American Type Culture Collection (ATCC) and were obtained through National Centre for Cell Science repository (NCCS, Pune, India). Human intestinal colon adenocarcinoma cell line LS180 was directly purchased from ATCC.

E. coli strain DH10β was used for the amplification of plasmid DNA, while *BL21 (DE3)* strain was used for the overexpression of recombinant proteins and purchased from invitrogen (Life Technology, USA).

Animal experiments

For generation of polyclonal and monoclonal antibodies mice were required. Immunization was performed on 6-8 week old female *Swiss albino* mice. Ethical clearance for experimental animals was obtained from institutional animal ethical committee (IAEC no. 23/2010), Jawaharlal Nehru University, New Delhi, India. Animals were maintained in the central animal house under standard condition with food and water *ad libitum*, a core facilility for Jawaharlal Nehru University, New Delhi, India.

Bacterial and mammalian cell culture media, supplements and antibiotics

Product name	Company	Cat. No.
8-Azaguanine	Sigma, St. Louis, MO, USA	A5284
Agar	Himedia, INDIA	RM301
Ampicillin	Himedia, INDIA	RM645
Charcoal Stripped FBS	PAN Biotech, GmbH, Germany	P30-2301
DMEM (high glucose)	Sigma, St. Louis, MO, USA	D7777
Escort III	Sigma, St. Louis, MO, USA	L3037
Escort IV	Sigma, St. Louis, MO, USA	L3287
FBS	PAN Biotech, GmbH, Germany	3302
HAT	Sigma, St. Louis, MO, USA	H0262
HEPES	Sigma, St. Louis, MO, USA	H4034
Kanamycin	Himedia, INDIA	RM210
L-Glutathione reduced	Sigma, St. Louis, MO, USA	G4251
Luria Broth Powder	Himedia, India	M575
PBS	Sigma, St. Louis, MO, USA	D-5652
PEG-1450	Sigma, St. Louis, MO, USA	P7181
PSA	Himedia, India	A002A
Rifampicin	Sigma, St. Louis, MO, USA	R-8883
RPMI-1640	Sigma, St. Louis, MO, USA	R8005
Serum free medium	Sigma, St. Louis, MO, USA	14610C
Trypsin-EDTA	Sigma, St. Louis, MO, USA	T3924

Plasticwares

Product name	Company	Cat. No.
0.5 ml microcentrifuge tubes	Tarson, Kolkata, INDIA	500000
1.5 ml microcentrifuge tubes	Tarson, Kolkata, INDIA	500010
2 ml microcentrifuge tubes	Tarson, Kolkata, INDIA	500020
15 ml falcons	Tarson, Kolkata, INDIA	546020
50 ml falcons	Tarson, Kolkata, INDIA	546040
Cell-scrappers	Corning, NY, USA (Sigma)	CLS3020
cell culture flasks surface area 25 cm^2	Corning, NY, USA (Sigma)	CLS430372
cell culture flasks surface area 75 cm^2	Corning, NY, USA (Sigma)	CLS430725
cell culture flasks surface	Corning, NY, USA (Sigma)	CLS431080

area 175 cm^2		
cell culture plates 6 well	Corning, NY, USA (Sigma)	CLS3506
cell culture plates 12 well	Corning, NY, USA (Sigma)	CLS3513
cell culture plates 24 well	Corning, NY, USA (Sigma)	CLS3526
tissue-culture treated culture dishes 35 mm	Corning, NY, USA (Sigma)	CLS3430165
tissue-culture treated culture dishes 60 mm	Corning, NY, USA (Sigma)	CLS3430166
tissue-culture treated culture dishes 100 mm	Corning, NY, USA (Sigma)	CLS3430167
Microtips (0.2-10 µl)	Tarson, Kolkata, INDIA	521000
Microtips (2-200 µl)	Tarson, Kolkata, INDIA	521010
Microtips (200-1000 µl)	Tarson, Kolkata, INDIA	521020
PCR 0.2 ml tubes	Tarson, Kolkata, INDIA	B79001
Petridishes 35 mm	Tarson, Kolkata, INDIA	460035
Petridishes 100 mm	Tarson, Kolkata, INDIA	460095

General laboratory chemicals

Product name	Company	Cat. No.
3-Amino Phthalhydrazide (Luminol)	Biochemika Fluka	73660
Acetic Acid	Merck, INDIA	60006325001730
Acetone	Rankem, INDIA	A0110
Ac-LLnL-CHO (ALLN) (protease inhibitor)	Sigma, St. Louis, MO, USA	A6185
Acrylamide	Sigma, St. Louis, MO, USA	A3553
Agar	Himedia, INDIA	RM 301
Agarose	Sigma, St. Louis, MO, USA	A9539
Ammonium persulphate	Sigma, St. Louis, MO, USA	A3678
AzBTS	Sigma, St. Louis, MO, USA	A3219
β-Mercaptoethanol	Sigma, St. Louis, MO, USA	M7522
Boric Acid	Sigma, St. Louis, MO, USA	B6768
Briliant Blue G 250	Qualigens Fine Chemicals, India	10401
Bromophenol Blue	Himedia, INDIA	RM117
BSA	Himedia, INDIA	RM105
Calcium Chloride	Aldrich	22231-3
Chloroform GR	Merck, INDIA	S13SF53306

Coomasie Briliant Blue R-250	Himedia, INDIA	RM344
CPTH2 (HAT inhibitor)	Sigma, St. Louis, MO, USA	C9873
Dextrose	Himedia, INDIA	RM077
Diethyl pyrocarbonate	Sigma, St. Louis, MO, USA	D5758
Di-Sodium Hydrogen Phosphate	Himedia, INDIA	RM1416
DMSO	Sigma, St. Louis, MO, USA	D2650
DTT	Sigma, St. Louis, MO, USA	D9163
EDTA disodium salt	Sigma, St. Louis, MO, USA	E5513
Equilibrated Phenol	Sigma, St. Louis, MO, USA	P4557
Ethanol	Merck, Germany	1009830511
Ethidium bromide	Himedia, INDIA	RM813
Formaldehyde	Ranbaxy, INDIA	F0070
Formamide	Qualigens Fine Chemicals, India	24015
Freund's adjuvant complete	Sigma, St. Louis, MO, USA	F-5881
Freund's adjuvant incomplete	Sigma, St. Louis, MO, USA	F5506
Glycerol	Qualigens Fine Chemicals, India	15455
Glycine	Sigma, St. Louis, MO, USA	G8898
Glycogen	Fermentas Interanational Inc., Canada	R0561
Guanidine hydrochloride	Sigma, St. Louis, MO, USA	G3272
HEPES	Sigma, St. Louis, MO, USA	H4034
Hoechst 33258	Sigma, St. Louis, MO, USA	86140-5
Hydrochloric Acid	Rankem, INDIA	H0070
Hydrogen Peroxide	Rankem, INDIA	H0120
IPTG	Sigma, St. Louis, MO, USA	I6758
Isopropanol	Rankem, INDIA	P0790
Lauryl Sulfate (SDS)	Sigma, St. Louis, MO, USA	L3771
Lipofectamine 2000	Invitrogen Life Tech., Carlsbad CA	11668019
Lithium chloride	Himedia, INDIA	MB038
Magnesium chloride	Sigma, St. Louis, MO, USA	M8266
Methanol	Qualigens Fine Chemicals, India	43607
MG132 (protease inhibitor)	Sigma, St. Louis, MO, USA	C2211
MOPS	Sigma, St. Louis, MO, USA	M1254
N, N'-Methylene-Bis-Acrylamide	Sigma, St. Louis, MO, USA	M7279
N-ethylmaleimide	Sigma, St. Louis, MO, USA	04259

N-Lauroyl Sarcosine sodium salt	Sigma, St. Louis, MO, USA	L9150
NP40	Himedia, INDIA	RM 2352
Okadaic acid (PP 1/2A inhibitor)	Sigma, St. Louis, MO, USA	07885
Orthophosphoric acid	Qualigens Fine Chemicals, India	29905
p-Coumaric Acid	Sigma, St. Louis, MO, USA	C9008
PMA (Protein kinase C agonist)	Sigma, St. Louis, MO, USA	P8139
PMSF	Sigma, St. Louis, MO, USA	P7626
Potassium acetate	Himedia, INDIA	RM3930
Potassium chloride	Rankem, INDIA	P0240
Potassium dihydrogen orthophosphate	Rankem, INDIA	P0320
Potassium hydroxide	Rankem, INDIA	P0390
Protease inhibitor cocktail	Sigma, St. Louis, MO, USA	P8340
Ribonuclease A	Sigma, St. Louis, MO, USA	R6513
Rifampicin	Sigma, St. Louis, MO, USA	R8883
Saponin	Fluka BioChemika, Germany	47036
Skim milk powder	Titan Biotech Ltd., INDIA	651
Sodium Acetate	Sigma, St. Louis, MO, USA	S-2889
Sodium azide	Sigma, St. Louis, MO, USA	S2002
Sodium bicarbonate	Sigma, St. Louis, MO, USA	S5761
Sodium chloride	Sigma, St. Louis, MO, USA	S5886
Sodium deoxycholate	Sigma, St. Louis, MO, USA	D6750
Sodium hydroxide	Rankem, INDIA	S0270
Sucrose	Sigma, St. Louis, MO, USA	S1888
TEMED	Sigma, St. Louis, MO, USA	T9281
TRI reagent	Sigma, St. Louis, MO, USA	T9424
Trichostatin A (HDAC inhibitor)	Sigma, St. Louis, MO, USA	T8552
Triton X-100	Sigma, St. Louis, MO, USA	T8787
Trizma base	Sigma, St. Louis, MO, USA	T6066
Trypan blue	Sigma, St. Louis, MO, USA	T6146
Tween-20	Sigma, St. Louis, MO, USA	P5927
Urea	Sigma, St. Louis, MO, USA	U5378

Enzymes

Product name	Company	Cat. No.
BamHI	NEB, England	R0136S
Calf Intestinal Phosphatase (CIP)	NEB, England	M0290S
dNTP set	Fermentas Interanational Inc., Canada	R0181
Dpn1	Fermentas Interanational Inc., Canada	ER1701
EcoRI	NEB, England	R0101S
Lysozyme	Sigma, St. Louis, MO, USA	L-6876
Pfu Polymerase	Fermentas Interanational Inc., Canada	EP0571
Proteinase K	Sigma, St. Louis, MO, USA	P-2308
RNase A	Sigma, St. Louis, MO, USA	R6513
RNasin ribonuclease inhibitor	Promega, Madison, WI, USA	N21111
T4 DNA Ligase	Fermentas Interanational Inc., Canada	EL0015
T4 PNK	Fermentas Interanational Inc., Canada	EK0031
Taq DNA Polymerase	NEB, England	M0273L

Primary and secondary antibodies

Product name	Company	Cat. No.
Anti-mouse IgG	Sigma, St. Louis, MO, USA	M6898
Anti-mouse IgG1	Sigma, St. Louis, MO, USA	M5284
Anti-rabbit IgG HRP	Sigma, St. Louis, MO, USA	A0545
Anti-rabbit IgG-cy3	Sigma, St. Louis, MO, USA	C2306
Anti-mouse IgG-cy3	Sigma, St. Louis, MO, USA	C2181
Anti-mouse IgG HRP (adsorbed against human sera)	Immunology Consultants Laboratory, Inc., Portland, Oregon.	CGHL-90XP
Mouse anti-HIS (monoclonal)	This study	-
Mouse anti-hPXR (polyclonal)	This study	-
Rabbit anti-hPXR (polyclonal)	(Saradhi et al, 2005)	
PXR-D8 (monoclonal)	This study	-
PXR-L6 (monoclonal)	This study	-
PXR-L12 (monoclonal)	This study	-
PXR-L13 (monoclonal)	This study	-
Mouse anti-hSUMO-1	This study	-
Rabbit anti-Glutathione-S-Transferase	Sigma, St. Louis, MO, USA	G7781

Protein and DNA standard size markers for SDS-PAGE

Product name	Company	Cat. No.
Prestained Protein Marker	Fermentas Interanational Inc., Canada	SM0671
Unstained Protein Marker	Sigma, St. Louis, MO, USA	SDS7
1Kb DNA Size Standard	Fermentas Interanational Inc., Canada	SM0311
100bp DNA Size Standard	Fermentas Interanational Inc., Canada	SM0241

Kits

Product name	Company	Cat. No.
GenElute™ Gel Extraction Kit	Sigma, St. Louis, MO, USA	NA1111
Luciferase assay kit	Promega, Madison, WI, USA	E1501
Mouse isotypic kit	Sigma, St. Louis, MO, USA	ISOQ5
Plasmid DNA extraction midi prep	MDI Ambala, INDIA	QDPK10
Plasmid DNA extraction mini prep	MDI Ambala, INDIA	MIPK50
Super Sensitive Polymer-HRP Detection Kit	Biogenex, USA	QD400-60K

Chromatographic beads

Product name	Company	Cat. No.
Protein A-Sepharose	Bangalore Genei, India	LIA1M
Protein G- Agarose	Sigma, St. Louis, MO, USA	P7700
Nickel NTA Sepharose	Genotech, USA	786-281
Glutathione-Agarose	Sigma, St. Louis, MO, USA	G4510

Miscellaneous materials

Product name	Company	Cat. No.
Developer	Kodak, INDIA	4908216
Disposable filter paper (0.22µ and 0.45µ)	MDI Ambala, INDIA	CN
Fixer	Kodak, INDIA	4908232
Nylone membrane	MDI Ambala, India	SNNPZ
pDs Red-Express-C1vector	BD Biosciences, USA	8331-1632430
PVDF Membrane	MDI Ambala, India	SVF
Salmon sperm DNA (SS DNA)	Agilent Tech., USA	201190
Whatman Filter Paper 3 MM	Whatman, England	3030917
Whatman Filter Paper No.1	Whatman, England	100125
X-Ray Film	Kodak, INDIA	4910022
Parafilm	Tarson, Kolkata, INDIA	380020

Summary of plasmids used in the present study

Plasmid name	Nature of the plasmid	Source
Human PXR-2	Mammalian expression vector encoding PXR-2 gene sequences cloned in pCDNA vector	Oliver Burk, Dr. Margarete Fischer-Bosch - Institute of Clinical Pharmacology, Stuttgart, Germany
Human PXR	Mammalian expression vector encoding hPXR-1 gene sequences cloned in pSG5 vector	S.A. Kliewer, University of Texas Southwestern Medical Center,Dallas, USA
XREM-Luc	Promoter-reporter expression plasmidencompassing a distal xenobiotic responsive enhancer module from CYP3A4 gene	C. Liddle, University of Sydney at Westmead Hospital, Australia
GFP-SUMO-1, GST-SUMO-1	Mammalian expression and bacterial expression vectors for SUMO modification	Jorma J. Palvimo, University of Kuopio, Finland
GST-SUMO1-1GG	Bacterial expression vectors for SUMO modification	Ronald T. Hay, University of Dundee, U.K.
RFP-SUMO-1	mammalian expression vector encoding full-length SUMO-1GG cloned at BamHI site of pDs Red-Express-C1 vector	This study
GAL-SRC-1	Mammalian two-hybrid expression vector for protein-protein interaction study	Barry Marc Forman, The Beckman Research Institute, Duarte, CA
VP-hPXR, VP vector pM vector	Mammalian two-hybrid expression vector for protein-protein interaction study	Jeff Staudinger, Department Of Pharmacology and Toxicology, University of Kansas, USA
FR-Luc	Mammalian two-hybrid expression vector for protein-protein interaction study	S. Stoney Simons, Jr. NIDDK, NIH,Batheseda, USA
Gal4DBD-PXR	Mammalian two-hybrid expression vector encoding full-length PXR	Jongsook Kim Kemper, University of Illinois, Urbana-Champaign, Illinois, USA
VP-PXR-M2	Mammalian two-hybrid expression	This study

	vector encoding PXR-M2 translational isoforms of PXR	
VP-PXR-M3	Mammalian two-hybrid expression vector encoding PXR-M3 translational isoforms of PXR	This study
VP-PXR-M4	Mammalian two-hybrid expression vector encoding PXR-M4 translational isoforms of PXR	This study
VP-PXR-M6	Mammalian two-hybrid expression vector encoding PXR-M6 translational isoforms of PXR	This study
Human PXR mutant m1	Site-directed mutagenesis of methionine to alanine at position 1 of hPXR protein	This study
Human PXR mutant m2	Site-directed mutagenesis of methionine to alanine at position 1 of hPXR protein	This study
Human PXR mutant m3	Site-directed mutagenesis of methionine to alanine at position 3 of hPXR protein	This study
Human PXR mutant m4	Site-directed mutagenesis of methionine to alanine at position 4 of hPXR protein	This study
Human PXR mutant m6	Site-directed mutagenesis of methionine to alanine at position 6 of hPXR protein	This study
Human PXR mutant m23	Site-directed mutagenesis of methionine to alanine at positions 2 & 3 of hPXR protein	This study
Human PXR mutant m123	Site-directed mutagenesis of methionine to alanine at positions 1, 2 & 3 of hPXR protein	This study
Human PXR mutant m12	Site-directed mutagenesis of methionine to alanine at positions 1 & 2 of hPXR protein	This study
M2-PXR	Mammalian expression vector encoding gene sequences from methionine 2 of hPXR cloned at EcoRI & BamHI site of pSG5 vector	This study
M3-PXR	Mammalian expression vector encoding gene sequences from methionine 3 of hPXR cloned at EcoRI & BamHI site of pSG5 vector	This study

M4-PXR	mammalian expression vector encoding gene sequences from methionine 4 of hPXR cloned at EcoRI & BamHI site of pSG5 vector	This study
M6-PXR	Mammalian expression vector encoding gene sequences from methionine 6 of hPXR cloned at EcoRI & BamHI site of pSG5 vector	This study
PXRwt	mammalian expression vector encoding full-length hPXR cloned at EcoRI & BamHI site of GFP-C2 vector	Saradhi et al, 2005a
PXR_{NTD}	mammalian expression vector encoding NTD of hPXR cloned at EcoRI & BamHI site of GFP-C2 vector	This study
$PXR_{NTD+DBD}$	mammalian expression vector encoding NTD & DBD of hPXR cloned at EcoRI & BamHI site of GFP-C2 vector	This study
PXR_{DBD}	mammalian expression vector encoding DBD of hPXR cloned at EcoRI & BamHI site of GFP-C2 vector	This study
$PXR_{DBD+LBD}$	mammalian expression vector encoding DBD & LBD of hPXR cloned at EcoRI & BamHI site of GFP-C2 vector	This study
PXR_{LBD}	mammalian expression vector encoding LBD of hPXR cloned at EcoRI & BamHI site of GFP-C2 vector	This study
PXR_{K108R}	Site-directed mutagenesis of lysine to arginine at position 108 of hPXR protein	This study
PXR_{K129R}	Site-directed mutagenesis of lysine to arginine at position 129 of hPXR protein	This study
PXR_{K160R}	Site-directed mutagenesis of lysine to arginine at position 160 of hPXR protein	This study
PXR_{K170R}	Site-directed mutagenesis of lysine to arginine at position 170 of hPXR protein	This study
$PXR_{3KR(108-)}$	Site-directed mutagenesis of lysine to arginine at positions 129, 160 & 170 of hPXR protein except at position 108 denoted as (-)	This study

PXR3KR(129-)	Site-directed mutagenesis of lysine to arginine at positions 108, 160 & 170 of hPXR protein except at position 129 denoted as (-)	This study
PXR3KR(160-)	Site-directed mutagenesis of lysine to arginine at positions of 108, 129 & 170 hPXR protein except at position 160 denoted as (-)	This study
PXR3KR(170-)	Site-directed mutagenesis of lysine to arginine at positions of 108, 129 & 160 hPXR protein except at position 170 denoted as (-)	This study
PXR4KR	Site-directed mutagenesis of lysine to arginine at positions 108, 129, 160 & 170 of hPXR protein	This study
PXRD115A	Site-directed mutagenesis of aspartic to alanine at position 115 of hPXR protein	This study
PXRE120A	Site-directed mutagenesis of glutamic to alanine at position 120 of hPXR protein	This study
PXRDE-A	Site-directed mutagenesis of aspartic & glutamic to alanine at positions 115 & 120 of hPXR protein	This study

Summary of oligonucleotides used in the present study

Primer name	Sequence (5'-3')	Used for
F1 R1	CTATAGGGCGAATTCGCGGAGGTGAGACCCAAAG CTTTGGGTCTCACCTCCGCGAATTCGCCCTATAG	Human PXR mutant m1
F2 R2	ATCACTTCAATGTCGCGACATGTGAAGGATGC GCATCCTTCACATGTCGCGACATTGAAGTGAT	Human PXR mutant m2
F3 R3	TTTTTCAGGAGGGCCGCGAAACGCAACGCCCGG CCGGGCGTTGCGTTTCGCGGCCCTCCTGAAAAAG	Human PXR mutant m3
m4F m4R	TGCCTGGAGAGCGGCGCGAAGAAGGAGATGATC GATCATCTCCTTCTTCGCGCCGCTCTCCAGGCA	Human PXR mutant m4
m6F m6R	AAGAAGGAGATGATCGCGTCCGACGAGGCCGTG CACGGCCTCGTCGGACGCGATCATCTCCTTCTT	Human PXR mutant m6
Fm2 Rm	CGGAATTCACCACCATGACATGTGAAGGATGCAAG CGGGATCCTCAGCTACCTGTGATGCCG	PXR-M2

Fm3 Rm	CGGAATTCACCACCATGAAACGCAACGCCCGGCTG CGGGATCCTCAGCTACCTGTGATGCCG	PXR-M3
Fm456 Rm	CGGAATTCACCACCATGAAGAAGGAGATGATCATG CGGGATCCTCAGCTACCTGTGATGCCG	PXR-M4
Fm6 Rm	CGGAATTCACCACCATGTCCGACGAGGCCGTGGAG CGGGATCCTCAGCTACCTGTGATGCCG	PXR-M6
Fn Rm	CGGAATTCACCACCATGGAGGTGAG CGGGATCCTCAGCTACCTGTGATGCCG	PXR^{WT}
Fn Rm	CGGAATTCACCACCATGGAGGTGAG CGGGATCCGATTTGGGGACCTCCGACTTC	PXR^{NTD}
Fn Rn	CGGAATTCACCACCATGGAGGTGAG CGGGATCCCATGCCGCTCTCCAGGC	PXR$^{NTD+DBD}$
Fd Rd	CGGAATTCTGCCGTGTATGTGGGGACAAG CGGGATCCCATGCCGCTCTCCAGGC	PXR^{DBD}
Fd Rm	CGGAATTCTGCCGTGTATGTGGGGACAAG CGGGATCCTCAGCTACCTGTGATGCCG	PXR$^{DBD+LBD}$
Fhl Rm	CGGAATTCAAGAAGGAGATGATCATG CGGGATCCTCAGCTACCTGTGATGCCG	PXR^{LBD}
Flbdsumo Rlbdsumo	CTGGAGAGCGGCATGAGGAAGGAGATGATCATG CATGATCATCTCCTTCCTCATGCCGCTCTCCAG	PXR^{K108R}
F R	TTGATCAAGCGGAAGAGAAGTGAACGGACAGGG CCCTGTCCGTTCACTTCTCTTCCGCTTGATCAA	PXR^{K129R}
F160 K-R R160 K-R	ATGGACGCTCAGATGAGAACCTTTGACACTACC GGTAGTGTCAAAGGTTCTCATCTGAGCGTCCAT	PXR^{K160R}
F170 K-R R170 K-R	ACCTTCTCCCATTTCAGGAATTTCCGGCTGCCA TGGCAGCCGGAAATTCCTGAAATGGGAGAAGGT	PXR^{K170R}
FD115A RD115A	ATGATCATGTCCGCCGAGGCCGTGGAGGAG CTCCTCCACGGCCTCGGCGGACATGATCAT	PXR^{D115A}
FE120A RE120A	GCCGTGGAGGCGAGGCGGGCCTTGATC GATCAAGGCCCGCCTCGCCTCCACGGC	PXR^{E120A}
Proximal ER6 F Proximal ER6 R	ATGCCAATGGCTCCACTTGAG 5'CTGGAGCTGCAGCCAGTAGCAG	Used for ChIP assay

METHODS

Bacterial strains and growth conditions

E. coli strain DH10β was used for the amplification of plasmid DNA, while BL21 (DE3) strain was used for the overexpression of recombinant proteins. Luria-Bertani (LB) medium with appropriate antibiotics (50 µg/ml of kanamycin or 100 µg/ml of ampicillin) was used for growing the *E. coli* cells harboring the plasmids.

Maintenance of mammalian cell lines

Different cell lines used in this study were routinely cultured in DMEM supplemented with 10% FBS, 100 µg/ml penicillin and 100 µg/ml streptomycin (complete medium) and maintained according to ATCC recommendations. All cells were grown in DMEM supplemented with 10% FBS while COLO 320DM cells were grown in RPMI-1640 with 10% FBS. The cultures were maintained in a humidified incubator maintained at 5% CO_2 and 95% air atmosphere at 37°C. The cells were routinely maintained in monolayer culture.

For regular subculturing, cells were detached from culture plates by trypsinization with trypsin–EDTA. During cell trypsinization, medium was discarded and cells were washed with sterile PBS. Then 1 ml of trypsin-EDTA solution/100 mm plate was added for about a minute and removed. The cells were then allowed to stand for a few minutes in a CO_2 incubator and then observed under phase contrast microscope to check if the cells have rounded up and detached. Once trypsinized, complete medium was added to re-suspend and recover the cells by gentle pipetting and further sub-cultured in fresh plates.

For cryopreservation, trypsinized scraped cells were collected in a centrifuge tube and pelleted by centrifugation at 1,000 rpm for 5 min. The supernatant was discarded and the cell pellet was gently suspended in complete medium. Depending on the cell line, 5% or 10% DMSO was added to medium containing the cells as per recommendation from ATCC. The cell

suspension was then transferred to 1 ml cryovials and incubated at -80°C overnight. After 24 h the vials were transferred to the liquid nitrogen container (-196°C) for long term storage.

Preparation and transformation of bacterial competent cells

Competent cells for different *E. coli* strains *(DH10*β *and BL21)* were prepared by $CaCl_2$ method as described in Sambrook et al, 1989. In brief, a primary culture of 5 ml of LB media was inoculated with a single colony of DH10β or BL21 (DE3) *E. coli* strains and incubated overnight at 37°C with vigorous shaking at 250 rpm. A secondary culture of 200 ml of LB was inoculated with 2 ml of overnight culture and incubated at 37°C with vigorous shaking till OD_{600} reached to 0.4-0.6. The culture was chilled on ice for 15 min and cells were harvested by centrifugation at 6,000 rpm for 10 min at 4°C. The pellet was resuspended in 15 ml of sterile ice-cold 0.1 M $CaCl_2$ (freshly prepared) by gentle swirling and cells were again harvested by centrifugation at 6,000 rpm for 10 min at 4°C. The cell pellet was resuspended in 1.5 ml of ice-cold 0.1 M $CaCl_2$ and kept on ice for 2 h, followed by addition of sterile ice-cold glycerol drop by drop with intermittent gentle mixing to a final concentration of 15%, cells were stored in 100 µl aliquots at -80°C till further use.

For transformation, competent *E. coli* cells were thawed on ice for 30 min. An appropriate amount of plasmid DNA (25-50 ng) was added to 100 µl of competent cells and incubated on ice for 30 min. Then cells were given heat shock at 42°C for 90 sec and immediately chilled on ice for 5 min. Cells were grown in 1 ml of LB for 1 h and subsequently plated on LB agar plates containing appropriate antibiotics and grown for 16 h at 37°C until the colonies were appeared.

Preparation of stock solutions for different drugs used in the present study

Stocks for different drugs were prepared according to the manufacturer's protocol. Briefly, okadaic acid was reconstituted in DMSO at

1 mM stock concentration. Similarly, TSA and CPTH2 were dissolved in DMSO at 3.31 mM and 100 mM stock concentration. PMA was dissolved in DMSO to a concentration of 20 mM. MG132 and ALLN inhibitors of proteasomal degradation pathway were reconstituted in DMSO at 10 mM stock concentration. All the constituted drugs were stored at -20°C.

Mini-scale plasmid DNA preparation by alkaline lysis method

Plasmid DNA in small scale was isolated by alkaline lysis method according to Sambrook et al, 1989, with minor modifications. *E. coli* cells were grown overnight (12-16 h) with vigorous shaking at 37°C in 5 ml of LB medium containing appropriate antibiotics. Cells were harvested by centrifugation at 12,000 rpm for 2 min and cell pellet was resuspended in 200 µl of resuspension solution [50 mM glucose, 25 mM Tris.Cl (pH 8.0), 10 mM EDTA (pH 8.0) and 20 µg/ml RNAase] by thorough vortexing. Then 400 µl of lysis solution (0.2 N NaOH and 1% SDS) was added, mixed gently by inversion (6-8 times) and incubated at RT for less than 5 min. To this 150 µl of neutralization solution (3 M potassium acetate and 2 M glacial acetic acid) was added, mixed by gentle inversion and cell debris was cleared by centrifugation at 12,000 rpm for 15 min. The clear supernatant was extracted with same volume of (1:1) phenol and chloroform mixture by centrifugation at 12,000 rpm for 5 min. The upper aqueous phase was separated and re-extracted with chloroform and then mixed with 1/10th volume of 3 M sodium acetate Ph 5.2 and 2 volumes of absolute ethanol and incubated at -80˚C for 1 h. The precipitated DNA was collected by centrifugation at 12,000 rpm for 10 min and washed with 1 ml of 70% ethanol at 12,000 rpm for 10 min. Finally, the DNA pellet was air dried and resupended in Tris-EDTA (TE) buffer [10 mM Tris-Cl (pH 8.0), 1 mM EDTA (pH 8.0)].

Quantification of plasmid DNA

To quantify the eluted DNA, appropriate dilution of the DNA was made and absorbance of the DNA sample was determined by spectrophotometer at 260 nm by setting TE sample as a blank. The

quantification of isolated plasmid DNA was determined by diluting the plasmid to 100-fold and then by measuring the O.D. at 260 nm. The concentration of DNA was calculated with the following equation:

DNA concentration (ng/µl) = O.D. at 260 nm x 50 x dilution factor.

Agarose gel electrophoresis

Agarose gel electrophoresis was performed as described by Sambrook et al, 1989. For plasmid DNA samples, 1% or for ChIP samples 1.6% agarose was melted in TAE [40 mM Tris-acetate, 1.0 mM EDTA (pH 8.0)] buffer by heating, cold to 37°C and ethidium bromide (0.5 µg/ml) was added before the casting of the gel. One-sixth volume of DNA gel loading buffer was mixed with samples and loaded into the wells. The electrophoresis was performed at 5V/cm in TAE buffer and the plasmid DNA was visualized on an UV transilluminator at 260 nm.

Electrophoresis of proteins on Sodium Dodecyl Sulphate-Polyacrylamide Gel (SDS-PAGE)

Polyacrylamde gel electrophoresis under denaturating condition (in the presence of 0.1% SDS) was performed according to Laemmli's method (Laemmli, 1970). The proteins were stacked at pH 6.8 in a stacking gel containing 5% acrylamide, 0.106% N, N'-methylene bisacrylamide, 0.125 M Tris-Cl pH 6.8, 0.01% TEMED and 0.1% ammonium persulfate. The separating gel consisted of 10% or 12% or 16% acrylamide, 0.33% N, N'-methylene bisacrylamide, 0.375 M Tris-Cl, pH 8.8, 0.01% TEMED and 0.1% ammonium persulfate. The protein samples were electrophoresed in a running buffer composed of [0.025 M Tris-base, 0.192 M glycine (pH 8.3), 0.1% SDS]. Protein samples for electrophoresis were prepared in Laemmli's buffer containing 0.0625 M Tris-HCl, pH 6.8; 2% SDS, 10% glycerol and 5% β-mercaptoethanol (Laemmli, 1970) and boiled at 95°C on heating block for 10 min. After a brief spin, the samples were loaded directly onto the gel.

Standard molecular weight markers were electrophoresed alongside the protein samples to determine the subunit molecular size of protein(s).

Liposome-mediated transient transfections

A day before transfection, exponentially growing cells were seeded in 35 mm, 60 mm, 12-well or 24-well plates as per experiment requirement in complete DMEM with 10% FBS to reach ~70% confluence. Next day, just before the transfection complete medium was removed and serum-free antibiotic-free medium was added according to the culture plate used (1 ml in 35 mm dishes, 2 ml in 60 mm dishes, 500 µl for each well of 12-well plates and 250 µl for each well of 24-well plates.). For transfection, DNA-lipid complex was prepared by mixing the indicated amounts of plasmid DNA with ESCORT-III or ESCORT-IV (4 µl/well for 35 mm plates, 8 µl/well for 60 mm plates, 2 µl/well for 12-well plate or 1 µl/well for 24-well plate) in a final volume of 200 µl and 400 µl (for 35 mm plate and 60 mm plate) or 100 µl and 50 µl (for each well of 12-well plate and 24-well plate) of serum-free medium (DMEM without serum and antibiotics) and incubated for 30 min at RT. Each transfection mixture was then added to the respective cell culture well. After 12 h, the serum-free medium was replaced with 5% charcoal-stripped serum containing medium and further incubated at 37°C for 36-48 h according to the experimental design. Endotoxin-free plasmid DNA, used for transfection studies, was mostly prepared by commercially available kits. The efficiency of transfection with plasmids prepared by these column methods was higher than the plasmids prepared by manual methods.

Preparation of luciferase mammalian cell culture lysis reagent

For preparation of 5X reporter lysis buffer [125 mM Tris-phosphate (pH 7.8), 10 mM DTT, 10 mM 1,2-diaminocyclohexane-N,N,N´,N´-tetraacetic acid, 50% glycerol and 5% Triton X-100] was used to make working luciferase cell culture lysis reagent in autoclaved Milli-Q water.

Luciferase reporter gene assay or transcriptional activity assay

Luciferase assay system is highly sensitive and one of the most widely used promoter-reporter gene assay to study gene expression and other cellular events such as receptor activity and protein-protein interactions. In this assay, light is produced by converting the chemical energy of the luciferin oxidation through an electron transition, forming the product molecule oxyluciferin. Using ATP.Mg2+ as a co-substrate firefly luciferase catalyses luciferin oxidation (**Figure 12**). The light intensity can be measured by luminometer.

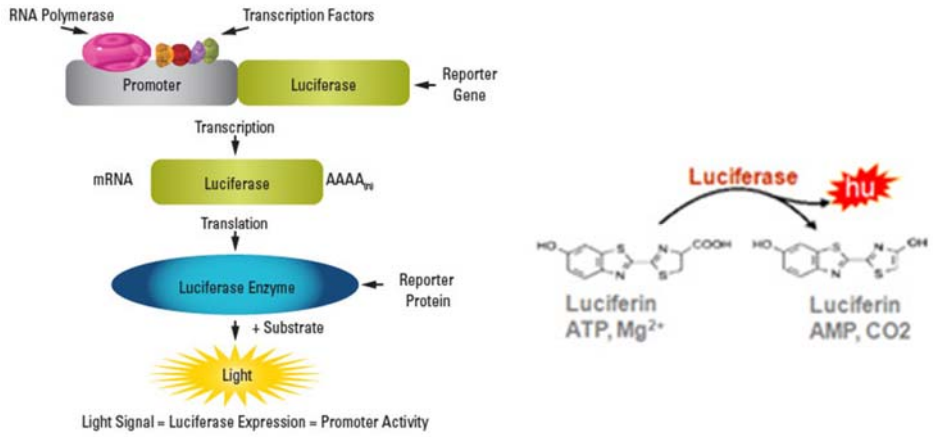


Figure 12: Principle of luciferase assay. *Binding of transcription factor onto the promoter of the construct regulating luciferase gene is shown. Activation of promoter induces luciferase expression in a dose dependent manner. Enzyme luciferase from the cell lysate is measured with substrate luciferin. The reaction produces luminescence which is measured by use of a luminometer.*

For the luciferase assay, cell culture medium was discarded and cells were washed twice with 1 ml of PBS by gentle swirling to remove any residual growth medium. Cells were lysed by adding 100 µl and 50 µl of 1X reporter lysis buffer to each well of 12-well or 24-well culture plates respectively and incubated for 10 min. Then cell lysate was prepared by manually scraping the cells with a rubberpolice man. A homogenous cell lysate was obtained by vortexing the sample for 10-15 sec. To clear the cell extract, lysate was centrifuged at 12,000 rpm for 2 min at 4°C. The clear supernatant was separated and used for the reporter gene assay. If required

the samples were stored at -80°C. Prior to determining the luciferase reporter gene activity, whole cell lysate and luciferase assay reagent were equilibrated to the room temperature and luminometer was programmed to perform a 3 sec pre-measurement delay followed by a 15 sec measurement period for each reporter assay. Then, 25-100 µl of luciferase assay reagent was mixed with 5-20 µl of cell-lysate (5:1 ratio) and luminescence was measured in the TD-20/20 DLReady™ luminometer (Turner Designs).

Isolation of total RNA from TRIzol method

Isolation of total RNA from cultured cells was performed with TRI Reagent™ (a mixture of guanidine thiocynate and phenol in a monophase solution), a single-step method reported by Chomczynski and Sacchi et al, 1987, for total RNA isolation. In short, cells from 100 mm plate were lysed with 1 ml TRI Reagent™ (TRI reagent) and total RNA extracted according to manufacturer's protocol.

Uniform probe labeling for Northern blot analysis by random primers

DNA fragments were purified from agarose gel using gel extraction kit, MDI, India and labeled with α^{32} P- dATP by random hexamer labeling using Hexa Label Kit, MBI, Fermentas, Germany according to the manufacturer's protocol. The unincorporared nucleotides were removed by passing through a Sephadex G-25 spun coloumn pre-equilibriated with STE [10 mM Tris-Cl (pH 6.8), 0.1 M NaCl, 0.1 mM EDTA (pH 8.0)]. The purified probe was denatured at 95°C for 10 min and snap chilled on ice for 5 min prior to hybridization.

Northern blot analysis

RNA samples were prepared by mixing the following components:

RNA (3.33 µg/µl)	9 µl (30 µg)
5X MOPS buffer (0.1 M)	4 µl (0.02 M)
Formaldehyde (37%)	7 µl (1.20%)
Formamide (38%)	20 µl (3.20%)

Samples were incubated at 65°C for 15 min. Chilled on ice and briefly centrifuged. Then 2 µl of loading dye and 1 µl ethidium bromide was added and loaded onto a 1.2% formaldehyde agarose gel prepared as described by Sambrook et al, 1989. Electrophoresis was carried out at 80V for 3 h in 1X MOPS buffer (0.02 M MOPS, 8 mM sodium acetate, 1 mM EDTA). Following electrophoresis, quality of RNA was ascertained by visualizing under UV transilluminator and the gel was rinsed in autoclaved water twice for 10 min each to facilitate the removal of formaldehyde. The gel was then equilibrated in 10X SSC for 10 min followed by transferring the RNA on to Nylon membrane by overnight capillary transfer in 10X SSC. Following transfer, the membrane was cross-linked in a Stratagene UV cross linker. Pre hybridization was done in 0.5 M sodium phosphate (pH 7.2), 7% SDS, 1 mM EDTA (pH 8.0), 100 µg/ml sheared denatured Salmon sperm DNA at 60°C for 4 h following which denatured uniformly labeled DNA probe (random labeled, 10^6 cpm/ml) was added to the pre-hybridization solution. Hybridization was then carried out overnight at 60°C. Filters were washed sequentially in [2X SSC, 0.1% SDS; 1X SSC, 0.1% SDS], [0.5X SSC, 0.1% SDS] and [0.2X SSC, 0.1% SDS] 10 min each at 60°C. Filters were air-dried and exposed to X-ray film. The image was developed by X-ray film developer.

Preparation of whole cell lysate from the mammalian cultured cells

From 35 mm plate, the cells were washed twice with PBS to remove culture media and then mechanically detached from the surface using a cell scraper. The cells suspended in PBS were collected in a microcentrifuge tube and centrifuged at 2,000 rpm for 2 min to pellet the cells. The supernatant was removed and the cell pellet was resuspended in ice-cold water (75 µl) by pipetting. Then equal volume (75 µl) 2X Laemmli's buffer was added, mixed and boiled directly for 10-15 min at 95°C. The supernatant containing lysate proteins was recovered by centrifugation at 12,000 rpm for 5 min and stored at -20°C till further use.

Prokaryotic expression and purification of human SUMO-1 for immunization

The prokaryotic expression vector, pGEX-SUMO-1GG transformed *E. coli* BL21 (DE3) cells were induced with 0.3 mM IPTG for 4 h. *E. coli BL21* (DE3) cells from IPTG-induced cultures were lysed in phosphate buffer [20 mM Na_2HPO_4 (pH 7.6), 300 mM NaCl, 10 mM imidazole, 100 µg/ml lysozyme, 1 mM phenylmethyl sulfonyl fluoride (PMSF) and 1 µg/ml each of pepstatin, leupeptin and aprotinin], and further incubated at 4°C for 1 h. The lysate were sonicated and centrifuged and the proteins were found in both soluble phase and the inclusion bodies. The, soluble fraction containing SUMO-1, protein was purified by GST beads. When electophoresed on SDS-PAGE gel the purified protein yielded a near single band corresponding to expected molecular weight. Though a homogeneous preparation of SUMO-1 protein was clearly evident we preferred to inject the protein after excising the KCl 100 mM, ice-cold) stained band from polyacrylamide gel. Electrophoresed bands of SUMO-1 corresponding to to 30 kDa of the proteins was excised from the gel and homogenized for use. The purity and recovery of gel-excised band was always reconfirmed. Therefore, homogenized preparation containing purified proteins could be confidently used for immunizing the *Swiss albino* mouse. For primary injection 50 µg of antigen in Freund's complete adjuvant and corresponding booster injections of 25 µg in Freund's incomplete adjuvant was used for the immunization at ten days intervals. The blood was collected after 3-4 subsequent boosters. From the blood, serum was collected by centrifugation at 5,000 rpm in a swinging bucket rotor. The antiserum was characterized by western blot assays and stored at -20°C. The antibody raised was successfully able to detect SUMO-1 overexpressed in COS-1 cells in our western blot analysis.

GENERATION OF MONOCLONAL ANTIBODIES AGAINST HUMAN PXR

Prokaryotic expression and purification of full-length hPXR protein

pET30b-hPXR transformed bacterial cells were inoculated in 5 ml LB liquid medium containing kanamycin (50 µg/ml), and grown overnight at

37°C with vigorous shaking. The culture was then transferred to 1,000 ml fresh LB medium and incubated for another 2-3 h at 37°C until the optical density at 600 nm (OD600) of the cultured bacteria reached 0.4–0.6. The expression of the fusion protein was induced with 0.1 mM isopropyl-1-thio-D-galactopyranoside (IPTG) and incubation was continued for another 4 h at 37°C. Cells were harvested by centrifugation at 5,000 rpm for 15 min at 4°C in a Beckman (model J2-21) centrifuge. The cell pellets were re-suspended in lysis buffer [20 mM Na_2HPO_4 (pH 7.6), 300 mM NaCl, 10 mM imidazole, 100 µg/ml lysozyme, 1 mM PMSF and 1 µg/ml each of pepstatin, leupeptin and aprotinin], and further incubated at 4°C for 1 h. Thereafter the cells were sonicated and centrifuged at 11,000 rpm for 15 min, and the pelleted fractions containing inclusion bodies were incubated with urea extraction buffer buffer [20 mM Na_2HPO_4 (pH 7.6), 300 mM NaCl, 8 M Urea, 10 mM imidazole, 1 mM PMSF, 1 mM β-mercaptoethanol and protease inhibitors as mentioned above] at RT for 1 h and centrifuged at 11,000 rpm for 15 min. Urea extracted pellet was incubated with sarcosine extraction buffer [20 mM Na_2HPO_4 (pH 7.6), 300 mM NaCl, 1.0% N-lauroylsarcosine, 10 mM imidazole, 1 mM PMSF, 1 mM β-mercaptoethanol and protease inhibitors as mentioned above] at RT for 1 h and again centrifuged at 11,000 rpm for 15 min. In the supernatant sarcosine concentration was readjusted to 0.3% and incubated with 0.5 ml of Ni-NTA His-Bind matrix with gentle agitation at 4°C for 2-3 h according to the manufacturer's instructions. The matrix was then loaded on to a column and washed with washing buffer [20 mM Na_2HPO_4 (pH 7.6), 300 mM NaCl, 0.3% N-lauroylsarcosine, 20 mM imidazole, 1 mM PMSF, 1 mM β-mercaptoethanol and protease inhibitors]. The matrix-bound protein was then eluted with elution buffer [20 mM Na_2HPO_4 (pH 7.6), 300 mM NaCl, 0.3% N-lauroylsarcosine, 250 mM Imidazole, 1 mM PMSF, 1 mM β-mercaptoethanol and protease inhibitors], and five fractions of 0.5 ml each were collected. The eluted proteins recovered in different fractions were analyzed by SDS-PAGE and coomassie brilliant blue staining. The concentration of the purified protein was measured by Bradford's method and the protein used for immunization and ELISA assays.

Immunization of mice

Eight-week-old five *Swiss albino* female mice were immunized with 25–50 μg purified PXR protein. At the first immunization, the antigen was mixed with an equal volume of Freund's complete adjuvant and subcutaneously injected into the mice. Subsequently 4-5 booster immunizations were given at ten days interval in Freund's incomplete adjuvant. After each booster dose test bleeds were assayed for immunoreativity/binding to PXR by western blot analysis and ELISA.

Isolation of splenocytes from spleen of the mouse

Mice that showed high immunoreactivity to the antigen were selected for the preparation of hybridoma cell line partner. Cervical dislocation of mouse was carried out in a sterile hood. Incision was made through the peritoneal layer at the left side of the abdomen and whole spleen was removed. Then spleen was placed in 100 mm sterile petri dish with 10-15 ml of PBS and extra tissue or connective tissues were trimmed out by forcep. Using sterile forceps, spleen was placed on a sterile wire mesh screen (95 micron pore size). Spleen was pushed through the mesh screen with the plunger of a 2 ml syringe into a fresh 100 mm petri dish with RPMI-1640 devoid of FBS and antibiotics. Subsequently screen was rinsed with the 3 ml of media. The capsule of the spleen was retained by the screen. It is important to minimize the amount of capsule in the media as this interferes with subsequent purification. Splenocytes mixture contains a variety of cell types (red blood cells, splenocytes, etc.). Splenocytes mixtures were then slowly transfered in 15 ml falcon with the help of 3 ml pasteur pipette and centrifuged at 1,200 rpm for 10 min. The supernatant was slowly removed and pellet containing the splenocytes with RBC was recovered. It is optional to remove RBC because this cleaning step is associated with loss of splenocytes. The pellet was then resuspended in 2 ml of RPMI-1640 medium devoid of FBS and antibiotics. Then 1:4 dilution with cell suspension and trypan blue was made in final volume of 50 μL. And the viable cells were counted using a hemocytometer.

Generation of PXR hybridoma by cell-fusion

Spleen cells retrieved from immunized mice were fused to Sp2/0-Ag14 mouse myeloma cells with polyethylene glycol (PEG 1450) to produce monoclonal antibodies according to established techniques (Kohler G, and Milstein C 1975). Hybridomas were then selected with hypoxanthine, aminopterin and thymidine (HAT) supplemented medium. Later, the clones were screened by ELISA. The reactivity of monoclonal antibodies to recombinant PXR protein was determined by direct ELISA.

ELISA

ELISA screening and identification of monoclonal antibodies was done in Nunc MaxiSorp® flat-bottom 96 well plate from Nunc Inc. (Roskilde, Denmark). ELISA plate was coated with 50-100 ng of recombinant PXR protein per well diluted in 50 µl of PBS overnight at 4°C. Plates were washed two times with 200 µl of PBS, and then blocked for 3 h with 100 µl of 5% non-fat milk in PBS to reduce nonspecific adsorption. Supernatants (50 µl) from hybridoma cultures were added to wells and incubated overnight at 4°C. Plates were first washed two times with PBS containing 0.1% tween-20 followed by two times washing with PBS and then incubated with 50 µl of 1:2,000 dilution of HRP conjugated chicken anti-mouse IgG antibody. Wells of ELISA plate were washed two times with 0.1% PBST buffer, then two times with PBS and subsequently incubated with ELISA substrate 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) for 15-30 min. The O.D. at 405 nm was measured with an ELISA plate reader (Microplate Reader 450/550; Bio-Rad, Hercules, CA).

Cloning and expansion of positive hybridoma clones

Hybridoma clones were subcloned 2–3 times in 96-well plates by limiting dilution (calculated as 10 cells/96 well to ensure single cell clone). Clonal cells were retrieved, retested and expanded. Subsequently, monoclonal antibody supernatants were generated in 175cm^2 flask. Selected hybridoma lines were later cultured in serum-free medium for purification of monoclonal antibody.

Maintenance and cryopreservation of hybridoma clones

Hybridoma cell lines expressing and secreting monoclonal antibodies against PXR were thawed in RPMI-1640 medium containing 10% FBS and antibiotics (PSA). Cells were incubated at 37°C with 5% CO_2, 95% air atmosphere. Once cells attain normal growth phase, they were passaged every third day. Once near confluent, a subcultivation ratio of 1:3 was maintained during passaging. It is important to note that hybridoma cells are semi-adherent, therefore, once they are completely confluent, they may begin to undergo cell death. Log phase cells were frozen for stock preparation with at least 2 $X10^6$ cells/ml in RPMI-1640 medium containing 10% FBS and 10% DMSO. Cryovials were frozen at -70°C for one day and then transferred to liquid nitrogen tank.

Isotype determination

In mouse, there are six heavy chain isotypes (IgG1, IgG2a, IgG2b, IgG3, IgM, and IgA)) and two light chain isotypes (κ and λ). In order to determine the isotypes of monoclonal antibodies secreted by the various hybridoma clones, isotyping was performed by using the mouse isotypic kit from Sigma Chemicals Co. (St. Louis, MO, USA).

Purification of monoclonal antibody from culture supernatants

The IgG fraction of mouse monoclonal antibodies raised against human PXR were purified by using Protein A-Sepharose beads or Protein G-Agarose beads and then analyzed with SDS–PAGE. For affinity column procedure, Protein G-Agarose beads for mouse IgG1 and Protein A-Sepharose beads for mouse IgG2a, IgG2b, IgG3 were used. The pH and molarity of cell culture supernatant was adjusted to 100 mm Tris-HCl (pH 8.0) by adding 1M Tris-HCl (pH 8.0). According to general experience hybridoma culture supernatants contain approximately 20-30 µg/ml IgG. One ml of swollen beads binds approximately 10-20 mg of immunoglobulins. So, in this study 250 µl of Protein A-Sepharose or Protein G-Agarose beads were equilibrated with 100 mM Tris-HCl (pH 8.0). Hybridoma culture supernatant and beads were mixed in 50 ml falcon and kept overnight on rotor at 4°C for binding.

After that beads were collected by centrifugation at 3,000 rpm. Supernatant was carefully removed and beads were washed at least two times with 1.5 ml volume of 100 mM Tris-HCl (pH 8.0) followed by one wash with 10 mM Tris-HCl (pH 8.0). Bound antibody was eluted from the beads with 300 µl of 100 mM Tris-glycine (pH 3.0). Eluted fractions were collected into 1.5 ml microfuge tubes containing 30 µl of 1 M Tris-HCl (pH 8.0) for immediate neutralization of antibody solution. From each fraction of purified antibody, 15-20 µl was run on 10% SDS-PAGE gel to examine the purity of antibody and subsequetly quantification was done prior to storage at -20°C.

Preparation of hybridoma supernatant for storage

Supernatant from hybridoma cultures were collected and centrifuged at 2,000 rpm for 10 min to remove cell-debris and filtered by 0.2 µm pore size membrane. After filtration supernatant was collected into sterile tubes, sodium azide was added to 0.1% (w/v) to prevent microbial growth. For short term storage supernatant was kept at 4°C, otherwise, stored at -20°C in 15 ml aliquots till further use.

Analysis of PXR antibodies

In western blot analysis supernatant for PXR-L6, PXR-D8, PXR-L12 and PXR-L13 were used at 2:1 dilution in blocking buffer whereas for flow cytometry, immunohistochemistry and immunocytochemistry supernatants were directly used without any dilution. In ChIP, EMSA and IP analyses purified monoclonal antibodies and polyclonal antibodies were used. Polyclonal mice anti-PXR and rabbit anti-PXR were used at 1:2,000 dilution in western blot analysis.

Western blot analysis

For western blotting equal amount of proteins were resolved by 10% SDS-PAGE. Proteins were transferred to polyvinyldifluoridine (PVDF) membrane using semi-dry transfer system (Amersham Biosciences, USA) or wet-transfer system (Life Tech., Invitrogen, USA). Following transfer, the membrane was blocked with 5% fat-free milk powder dissolved in Tris buffer

saline (pH 7.6) with 0.1% Tween-20 (TBST) for 1 h at RT. Followed by washing 4X with TBST for 15 min each and then incubated with appropriate antiserum as per the requirement and in appropriate dilutions according to the experiment for overnight at 4°C. Further 4X washed with TBST for 15 min each. The PVDF membrane was then incubated for 1 h with 1:10,000 dilution of HRP conjugated anti-rabbit/anti-mouse IgG secondary antibody. The bound antibody complexes were detected using the Enhanced Chemi-Luminescence (ECL) system.

Immunoprecipitation assay

Immunoprecipitation assays were performed with COS-1 extract transfected with GFP-hPXR. After 48 h of transfection, cells were harvested with lysis buffer [20 mM Tris–HCl (PH 7.4), 300 mM NaCl, 0.5% NP 40, protease inhibitors, 100 µM PMSF]. Whole cell lysate was incubated with 3 µg of PXR polyclonal antibody, pre-immune serum, PXR-L6, PXR-L8, PXR-L12 and PXR-L13 and isotype control overnight on rotor at 4°C. Each sample was further incubated for additional 2 h after addition of 20 µl of Protein A-Sepharose or Protein G-Agarose beads slurry on rotor. Beads were washed three times each with lysis buffer at 4°C and bound proteins were dissolved in SDS-PAGE Laemmli's buffer. Proteins were separated by 10% SDS-PAGE. Proteins on gel were transferred to PVDF membrane and subjected to western blot analysis with PXR-L13 monoclonal antibody. Signals were then detected with ECL method.

Co-immunoprecipitation assay

In vivo coimmunoprecipitation assays were performed with COS-1 cells transfected with 2 µg pSG5-PXR and 2 µg GFP-SUMO1 plasmids alone or in combination as per experimental requirement in 60 mm plate. The cells were treated with or without rifampicin for 36-40 h post transfection and harvested with lysis buffer [20 mM Tris–HCl (pH 7.4), 300 mM NaCl, 0.5% NP 40, protease inhibitors, 100 µM PMSF]. Whole cell lysate was incubated with 4-5 µg/ml of rabbit PXR polyclonal antibody or preimmune serum overnight at 4°C. Each portion was further incubated for additional 2 h after addition

of 20 µl of Protein A-Sepharose bead slurry. Sepharose beads were washed three times each with lysis buffer at 4°C and bound proteins were dissolved in SDS-PAGE Laemmli's buffer. Proteins were separated by 10% SDS-PAGE. Proteins on gel were transferred to PVDF membrane and subjected to western blot with mice anti-SUMO-1 antibodies at 1:2,000 dilutions. Signals were then detected with ECL method.

Immunocytochemistry

For indirect immunodetection, HepXR cell line was cultured for 48 h over sterile glass cover slips. The cells were washed thrice with PBS and fixed with chilled methanol (at -20°C) for 20 min on ice and washed 2 times with PBS. Following fixation, cells were permeabilized with Triton X-100 (0.25%) for 10 min at RT, the cover slips were washed twice with PBS, air dried and kept at -20°C for 1 h or stored till further use. For immunodetection, the cells from -20°C were incubated in a humidified chamber for 10 min and then blocked with 5% BSA in PBS for 1 h. The cells were subsequently incubated with PXR polyclonal antiserum or pre-immune serum (1:300 dilution) or PXR monoclonal supernatant at 4°C for 12-16 h. Following three washes with PBS, the cells were further incubated with anti-mice Cy3-conjugated IgG (1:500 dilution) for 1 h at room temperature. To remove unbound antibodies, the cells were washed three times with PBS and the cover slips were mounted with 20% glycerol in PBS on glass slides and visualized by fluorescence microscopy. Hoechst 33342 was co-incubated with the secondary antibody to facilitate the visualization of the nuclei. The fluorescent cells were viewed through a Nikon upright fluorescence microscope model 80i equipped with water immersion objectives and connected to cold CCD digital camera (model Evolution VF, Media cybernatics, USA). Images were captured and analyzed with Image ProPlus version 5.0 software (Media cybernatics, USA). The images were processed using standard image processing techniques.

Immunohistochemistry

Immunohistochemistry was done with Super Sensitive Polymer-HRP Detection Kit from Biogenex Laboratories. Human colon and liver tissue sample were embedded in paraffin and sections (5 µm) were dewaxed in a xylene bath and rehydrated in graded alcohols. Endogenous peroxidase activity was quenched with 0.5% H_2O_2 in methanol for 20 min and washed in running water. For fixing the cells, the cells were incubated in a 4% paraformaldehyde for 10 min and followed by washing twice with PBS. Formaldehyde fixed samples were subjected to antigen retrieval using citrate 0.01 M, pH 6.0 for 30 min at 80°C and washed with running water. Now 2X washing with TBS (pH 7.6). Non-specific binding sites were blocked with power block for 30 min at RT. Subsequently the coverslips were incubated with PXR monoclonal antibodies like PXR-L6, PXR-L12 and PXR-L13 supernatant and hexa histidine tag monoclonal supernatant as negative control. The coverslips were put in a humidified atmosphere at RT for 1 h at RT and then washed with three changes of TBST over 5 min each. The ready to use secondary antibody acts as super enhancer for increasing the strength between primary and tertiary antibody. After 3X time washing with TBST ready to use HRP conjugated tertiary antibody was added and incubated in dark for 30 min at RT. Subsequently another three changes for washing with TBST were performed and staining was developed by using brown chromogen 3, 3′-diaminobenzidine (DAB) for 20 min. Followed by washing in running water, after that counterstained in hematoxylin. After dehydration, sections were mounted with DPX and finally, the images were taken on BX51 microscope (Olympus). This part of the work was done at pathology laboratory ILBS, New Delhi.

Flow cytometry

Flow cytometry was used to characterize monoclonal antibody in HepG2 and HepXR cell line. Hybridoma supernatants were screened by flow cytometry approximately after three weeks following fusion for the presence of antibodies. Briefly, HepG2 and HepXR cells were washed 2 times with PBS and then fixed with ice-cold methanol for 20 min on ice. Subsequently,

the cells were washed with PBS and permeabilized with 0.25% Triton X-100 and 0.1% NP40 at RT for 10 min followed by blocking with 5% BSA, FcR (Fc receptor) blocker at 1:50 dilution and 1% FBS for 1h. Further, cells were incubated with undiluted mAb supernatants for 1h at RT, centrifuged, washed 3 times with 0.1% saponin in PBS and incubated with cy3-conjugated anti-mouse IgG for 1 h at RT. Cells were then washed 3 times with 0.1% saponin in PBS and fixed in 3% paraformaldehyde in PBS prior to analysis by flow cytometry. Different cell populations were gated out on the basis of forward scatter (FSC) and side scatter (SSC) characteristics and a minimum of 5,000 events were acquired for each condition using a FACSCaliber™ flow cytometer (Becton-Dickinson, Mountain View, California, USA) and *CellQuest*™ software (Becton-Dickinson).

Chromatin Immunoprecipitation (ChIP) Assay

HepXR cells were treated with different drugs at different concentration for 12 h. Cross-linking was achieved by adding formaldehyde to a final concentration of 1% at room temperature for 10 min. Glycine (0.125 M) was then added for 5 min at room temperature. Cells were washed twice with ice-cold PBS and collected in 1 ml of ice-cold PBS. Cells were pelleted at 5,000 rpm at 4°C and resuspended in 0.3 ml of cell lysis buffer [50 mM Tris-HCl (pH 8.1), 10 mM EDTA, 1% SDS, complete protease inhibitor mixture] and incubated on ice for 10 min. Cell lysates were sonicated to give a DNA size range from 200 to 900 bp. Samples were centrifuged for 10 min at 4°C. Supernatants were adjusted to give a final solution of 15 mM Tris-HCl (pH 8.1), 1% Triton X-100, 1 mM EDTA, 150 mM NaCl, 0.1% SDS and complete protease inhibitor mixture. The solutions were pre-cleared with 50 µl of 50% slurry of Protein A-Sepharose containing 2.5 µg of sheared salmon sperm DNA for 2 h at 4°C, and then treated with antibody against PXR overnight at 4°C. 50 µl of Protein A-Sepharose containing 2.5 µg of salmon sperm DNA and 2 mg/ml bovine serum albumin were then added to the solution for 1 h. The beads were pelleted and washed sequentially in the following buffers: Buffer A [20 mM Tris-HCl (pH 8.1), 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100]; buffer B [20 mM Tris-

HCl (pH 8.1), 500 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100), and LiCl-detergent buffer (10 mM Tris-HCl (pH 8.1), 0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA] and TE buffer (twice). Immuno-complexes were extracted from the beads with 1% SDS and 0.1 M $NaHCO_3$ at RT. Crosslinking was reversed by heating elutes at 65°C overnight. Elutes were then digested with proteinase K at 45°C for 1 h. The DNA was extracted with phenol-chloroform-isoamyl alcohol and purified by ethanol precipitation, and the region over the everted repeats as ER6 of the CYP3A4 promoter was amplified by PCR. The ChIP primer sequences used are shown below:

ER6 F - 5'ATGCCAATGGCTCCACTTGAG 3'

ER6 R - 5'CTGGAGCTGCAGCCAGTAGCAG 3'

Fluorescence microscopy for analysis of intracellular localization

COS-1 cells were seeded on glass coverslips in 35 mm cell culture plates and grown in DMEM supplemented with 10% FBS for about 12 h. The cells were transfected for 10-12 h with 0.5-0.8 µg of each plasmid as per the requirement. The cells were incubated further for 24 h after transfection. Following the transfection period, the cells were supplemented with fresh DMEM containing 5% charcoal-stripped or FBS and treated with or without 10 µM rifampicin and incubated further for 2-24 h. To facilitate the visualization of the nucleus, Hoechst was added to the live cells at least one hour prior to imaging. For stable cell line HepXR, cells transfection step was not required and cells were directly immunodetected and visualized after the treatment with indicated ligands. The fluorescent cells were viewed under fluorescence microscope model 80i equipped with water immersion objectives with water immersion objectives and connected to cold CCD digital camera (model Evolution VF, Media cybernatics, USA). Images were captured and analyzed with Image ProPlus version 5.0 software (Media Cybernatics, USA).

Mammalian two-hybrid assay

HepG2 cells were seeded in 12-well/24-well plates and transiently co-transfected (with Escort IV) with the indicated amounts of plasmids VP16-hPXR; VP16-UBCh9; VP16-PXR-M2; VP16-PXR-M3; VP16-PXR-M4; VP16-

PXR-M6 (encoding VP16 transactivation domain fusion protein with hPXR; UBCh9; PXR-M2; PXR-M3; PXR-M4; PXR-M6) together with a Gal4-DBD-PXR; Gal-SRC1; Gal4-DBD-PXR-M2; Gal4-DBD-PXR-M3; Gal4-DBD-PXR-M4; Gal4-DBD-PXR-M6 (encoding Gal4 DNA binding domain fusion protein with PXR; SRC1; PXR-M2; PXR-M3; PXR-M4; PXR-M6), along with promoter-reporter plasmid FR-Luc (in 2:1:10 ratio), containing the luciferase reporter gene with upstream five tandem repeats of the 17 bp GAL4-binding element, as a read out for interaction. Stimulation in luciferase activity indicated the interaction between the two receptors. Luciferase activities were assayed as described elsehere in 'Methods'.

Site-directed mutagenesis

Specific mutations were carried out by inverse PCR based methodology. A 50 µl PCR reaction containing *Pfu* reaction buffer, 300 µM dNTP mix, 1 ng of template DNA, 10 pmoles of mutated primer pairs and 1 U *Pfu* enzyme was set up and subjected to PCR cycling conditions. As a control same reaction was carried out in the absence of *Pfu* enzyme. Unlike normal PCR, only 18 cycles were used for amplification of desired mutated DNA sequences with following cycling conditions: i) denaturation at 94°C for 30 sec, ii) annealing at 60°C for 1 min, iii) extension for 72°C for 11 min. Initial denaturation was carried out at 95°C for 3 min and final extension was carried out at 72°C for 15 min. Following PCR amplification, 5 µl aliquot was checked on 1% agarose gel. To the rest of the PCR product (40 µl), 1 µl of Dpn1 restriction enzyme (10 U/µl) was added and incubated at 37°C for 1 h to digest the parental supercoiled dsDNA. In the next step, 1 µl of the Dpn1 treated reaction sample and control sample was used for transformation into DH10β competent cells. Following day colonies were observed on bacterial selection plate. The single isolated colonies were then further inoculated in LB medium for isolation of the mutated plasmid. Mutations were confirmed by DNA sequencing.

Statistical analysis

Most of the experiments were done at least 3 times and values represent the means ± SD of three separate experiments. Statistical analyses were done by two way student T-test and asterisks (*) signify values that differed significantly from the control experiment with less than 0.05 ($p<0.05$ in Student's T-test).

CHAPTER I

Generation and characterization of monoclonal antibodies against human Pregnane and Xenobiotic Receptor (PXR) as an immunological tool in biological research and immunodiagnostics

INTRODUCTION

Pregnane X Receptor (PXR) is a member of the nuclear receptor super-family, is also known as steroid and xenobiotic receptor or SXR. PXR plays an important role in modulation of the components of endobiotics and xenobiotic metabolism and clearance cascades. Member of this nuclear receptor super-family share a common structure consisting of ligand-independent transcriptional activation function 1 (AF1) domain at the amino-terminus, a highly conserved centrally located DNA binding domain (DBD), a hinge region, a moderately conserved carboxyl-terminal ligand binding domain (LBD) and a ligand-dependent activation function 2 (AF2) region at the carboxyl-terminus (Bain et al, 2007). PXR protein is about 50 kDa and binds DNA in a sequence-specific manner. PXR gene consists of nine exons encoding 434 amino acids. It is reported that human PXR gene has alternative splicing in exon 5 that yields three transcripts, PXR-1 (appropriately spliced PXR that includes all of exon 5), PXR-2 (lacking 111 nt or the first 37 amino acids in exon 5), PXR-3 (lacking 123 nt or the first 41 amino acids in exon 5). Out of these three, PXR-1 (described as PXR) is the only one that has been studied in some detail.

PXR gene expression analysis has shown a relatively higher level of expression in liver, small intestine and colon and lower expression levels in skeletal muscle, stomach, testes, trachea, kidney, adrenal and thyroid gland etc (Kleiwer et al, 1998). Furthermore, regulated expression of PXR is reported in different cancerous states like endometrial cancer (Masuyama et al, 2003), breast cancer (Dotzlaw et al, 1999), prostate cancer (Chen et al, 2007), epithelial ovarian carcinoma (Gupta et al, 2008) and esophageal squamous cell carcinoma (Takeyama et al, 2009). However, the precise roles of PXR in cancerous tissues remain unclear. Some reports suggest that PXR and its activators suppress the proliferation of some cancers however; some reports also indicate PXR as an inducer of cell proliferation in some cancer cells (Zheng et al, 2012; Pondugula and Mani, 2013; Qiao et al, 2013). These results suggest a critical role for PXR in cancer progression. Expression of PXR in other tissues, which are not primarily involved in drug metabolism,

indicates that PXR may play some distinct function in those tissues. Several new avenues of research have been opened in recent years that have revealed new and mostly unanticipated roles for PXR in bile homeostasis (Kliewer et al, 2005), blood–brain barrier function (Bauer et al, 2008), bone homeostasis (Azuma et al, 2010) and inflammatory bowel disease (Langmann et al, 2004; Dring et al, 2006). Altogether, these reports suggest that other than being the master-regulator of xenobiotic metabolism, PXR may have some other roles in normal and cancerous tissues that remain to be fully explored. In agreement with these data, PXR expression was correlated as a prognostic marker in various types of cancers (Yue et al, 2010; Pondugula and Mani, 2013).

However, due to an apparent lack of a domain specific monoclonal antibody against PXR, the expression of PXR at the protein level in different pathological states could not be explored well. Previously, our laboratory successfully prepared polyclonal antibody against PXR (Saradhi et al, 2005a). However there is some limitation while using polyclonal antibodies, like animal host dependency and increased chance for cross reactivity due to shared epitopes on different proteins. The major advantage of using monoclonal antibodies is their high specificity for the antigen. Lots of polyclonal and a few monoclonal antibodies against PXR protein are commercially available but these antibodies do not work efficiently in all immunological assays.

So, we developed a specific anti-PXR monoclonal antibody for PXR protein which will be useful in various immunological assays. We expressed a recombinant PXR protein in *Escherichia coli (E. coli)* Rosetta (DE3) and prepared anti-PXR monoclonal antibody by previously described conventional hybridoma procedures with some modifications (Kohler G, and Milstein C 1975). To our knowledge, we have developed the monoclonal antibody against full-length human PXR protein for the first time. Immunization with full-length PXR protein resulted in generation of a panel of monoclonal antibodies against both DBD and LBD of PXR protein. The monoclonal antibodies raised herein against human PXR are being

consistently produced from a replenishing cellular source implying reliable, economical and useful immunological tool in biological research with potential use in immunodiagnostics. Since PXR protein is expected to be up- or down-regulated under certain physiological challenges and altered cellular behavior, therefore, these monoclonal antibodies may serve as good immunological tool to detect PXR protein which may serve as a prognostic marker for evaluating the pathological conditions.

RESULTS

Prokaryotic expression and purification of human PXR for immunization

For expression and purification of human PXR, full-length PXR cDNA was cloned into pET vector (Saradhi et al, 2005a). The prokaryotic expression construct pET-PXR transformed *E. coli BL21* cells were induced with 0.1 mM IPTG for 3 h. *E. coli BL21* cells from IPTG-induced cultures were sonicated and the majority of the PXR was found in the inclusion bodies. The fraction containing the inclusion bodies was sequentially extracted with urea extraction buffer and sarcosine extraction buffer. Finally, sarcosine solubilized His-PXR protein was purified by Ni2+ affinity chromatography using Ni-NTA beads. On SDS-PAGE gel the purified protein yielded a band corresponding to the expected molecular weight of 55 kDa (**Figure13**).

Production of polyclonal antiserum against human PXR in mice and selection of mice for splenectomy

Immunization of five *Swiss albino* mice was performed with PXR protein purified by homogenization of polyacrylamide gel slices containing a single band for purified PXR antigen as discussed under 'Materials & Methods'. Briefly, eluted fractions of PXR protein were pooled, electrophoresed through 10% SDS-PAGE and the gel was soaked in ice-cold 0.1 M KCl for 5 to 15 min. Whitish protein band for PXR (corresponding to

~55 kDa) against a clear gel background were excised with a scalpel and homogenized in 500 µl of PBS.

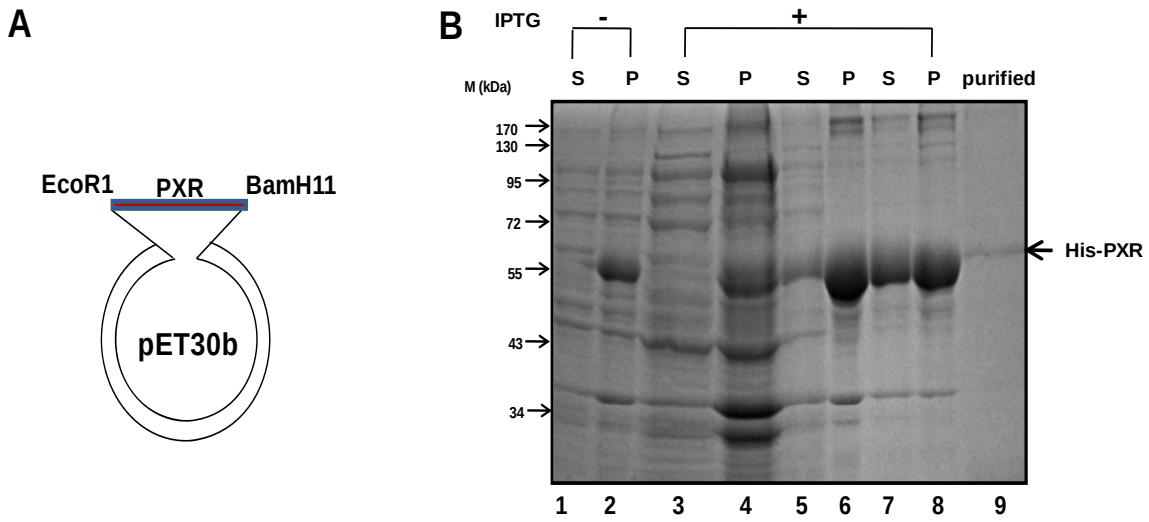
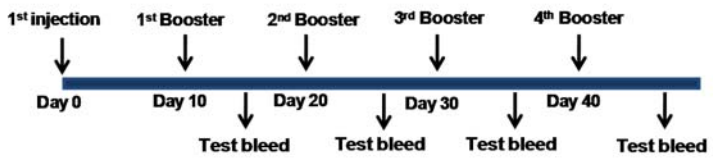


Figure 13: Cloning of PXR into PXR prokaryotic expression vector and its purification. ***A.*** *Schematic representation of the human PXR cDNA encoding sequence in E.coli expression vector pET 30b.* ***B****. A single colony of freshly transformed E.coli BL21 (DE3) cells was used to initiate growth in Luria Broth medium and subsequently used for extraction of PXR protein as described in 'Materials & Methods' section. Lane 1, supernatant fraction from uninduced cells, after sonication; Lane 2, pelleted fraction from uninduced cells, after sonication; Lane 3, supernatant fraction from IPTG induced cells, after sonication; Lane 4, Pelleted fraction from IPTG induced cells, after sonication; Lane 5, supernatant fraction of inclusion bodies after extraction with urea extraction buffer; Lane 6, pelleted fraction of inclusion bodies after extraction with urea extraction buffer; Lane 7, supernatant fraction of urea extracted inclusion bodies after solubulizing in sarcosine extraction buffer; Lane 8, pelleted fraction of urea extracted inclusion bodies after solubulizing in sarcosine extraction buffer; Lane 9, PXR was purified using Ni-NTA-column chromatography of the sample described in lane 7. This purified sample was extracted from homogenized gel slice and used as antigen. 'M' indicates the standard protein markers in kDa. The gel was stained with coomassie blue and different fractions containing recombinant protein was visulised.*

To reconfirm the recovery and purity of PXR protein, a fraction of sample obtained from homogenized gel pieces after overnight incubation in sample buffer was electrophoresed on 10% SDS-PAGE. Mice were immunized subcutaneously with 50µg of PXR antigen after emulsifying in 500 µl of complete Freund's adjuvant. Ten days later, the mice were injected

with 25-50 µg of PXR with Freund's incomplete adjuvant as booster injections. One week after each booster the mice were tail bled and titer of antibody was monitored by ELISA using purified recombinant PXR as coating antigen. Overall 3-4 booster doses were administered at ten days intervals (**Figure 14A**). About 50-100µl of serum was collected prior to each booster. Different titers were tested by ELISA assay (**Figure 14B**). Once titer appeared, the specificity of PXR antibody was further tested by western blot analysis and immunocytochemistry (**Figure 14 C & D**).

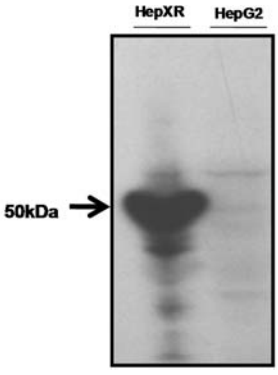
A



B

Pre-immune sera	O.D. (405nm)	Test bleeds	O.D. (405nm)
1 Pre-immune	0.260	1 Mouse anti-hPXR	1.058
2 Pre-immune	0.161	2 Mouse anti-hPXR	0.797
3 Pre-immune	0.280	3 Mouse anti-hPXR	1.039
4 Pre-immune	0.186	4 Mouse anti-hPXR	0.564
5 Pre-immune	**0.260**	5 Mouse anti-hPXR	**1.111**

C



D

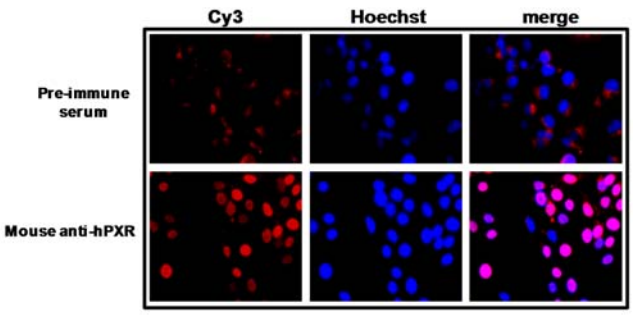


Figure 14: Generation of Mouse anti-hPXR polyclonal antibody specificity tested by Western blot analysis and immunocytochemistry.

A. *Schematic illustration of immunization schedule.* ***B.*** *Test bleeds from five mice were tested by direct ELISA as mentioned in 'Materials & Methods' section. Fifth mouse showed higher O.D. when compare to the rest of the mice.* ***C.*** *Cell extracts were prepared from HepG2 and HepXR cell lines. The protein extracts were electrophoresed through 10% SDS-PAGE, blotted on to PVDF membrane and probed with mouse anti-hPXR polyclonal antibodies at a 1:2,000 dilution. A specific reactivity with a major protein band having molecular weight of 50 kDa was detected.* ***C.*** *HepXR cells were fixed and processed for immunofluorescence study. Cells in upper row and lower row were detected with pre-immune serum and mouse anti-hPXR antibody respectively. Images were recorded using a fluorescence microscope equipped with water–immersion objectives. The left panel shows the predominantly nuclear distribution pattern of PXR in immunodetected cells. The middle panel shows the Hoechst images for visualizing the corresponding nuclei. The right panel shows the merged images for hPXR and nuclei.*

To examine the specificity of PXR polyclonal antibodies western blot analyses were performed. Cell extracts from HepG2 (hepatocellular carcinoma cell line) and HepXR (*HepG2 stably transfected human PXR*) cells were prepared and analysed by western blot (**Figure 14C**). Specific immuno-reactivity towards a major protein band having an expected molecular weight of PXR (~50 kDa) was detected in western blot analysis. Immunocytochemistry was done as discussed in 'Materials and Methods' section (**Figure 14D**). Mouse that showed highest specific immunoreactivity to the antigen was selected for the preparation of splenocytes.

Generation of monoclonal antibody from hybridoma

Splenocytes from mice showing good antibody titer were fused with sp2/0 cells myeloma cells by PEG (1450) as described in 'Materials & Methods' section. The fused cells resuspended in RPMI medium containing hypoxanthine–aminopterin–thymidine (HAT) in 0.5X106/ml dilution and plated in six 96 cell culture flat bottom plates for 10-21 days for selection of surviving fusion clones that produce antibody.

Screening of hybridoma clones by direct ELISA

The early identification of monoclonal antibodies with the desired specificities is the most important step in monoclonal antibody production. Technically simple, sensitive and convenient assay like ELISA is used to

screen large number of hybridoma clones. The initial screening for antibody activity should be done as soon as growth of hybrid cells is seen under the microscope or upon a change in culture medium pH. The media containing the surviving fusion clones were enumerated and screened for specific antibody production by direct ELISA assay using purified PXR protein as coating antigen. After 10 to 21 days of selection, about $1/10^{th}$ volume of well were filled with hybridoma clones and approximately 150 colonies were observed. By ELISA assay, we get 24 positive clones which were shown in **Table IV**. Now these clones were further grown in 24 well cell culture plate. So, the positive immunoreactive clones against purified PXR protein were selected and subjected to further analysis by western blot analysis to confirm specific immunoreactivity to the antigen.

Expansion of cultures for positive clones

After screening procedure with ELISA, 24 positive clones were selected. These clones were one more time rescreened by direct ELISA (**Table V**). Some clones show negative results, might be due to overgrowth of a negative clone or loss of stability of positive clone and also no activity may be detected during the initial assay due to the lesser number of cells of a positive clone. Since purified PXR is tagged with hexa-histidine residues, therefore to confirm that the specific immunoreactivities towards PXR protein and not towards hexa-histidine residue, these clones were examined by ELISA assay with coating of two types of antigen i) his-SUMO-2 ii) his-PXR. Finally four clones (6, 8, 12 and 13) were selected which were found to be reacted positively with PXR protein and six (1, 5, 9, 11, 16, 24) clones were found to produce antibodies specific for hexa histidine tag (**Table VI**). So, by using recombinant PXR protein with hexa-histidine tag we also get monoclonal antibodies against hexa-histidine tag. All positive clones were recloned by a limiting dilution unless all the wells of 96 well plates give same O.D. of all positive clones to assure monoclonality. Subsequently positive hybridoma clones against PXR protein were selected for further expansion in 175 cm^2 flask.

Table IV: First screening of anti-hPXR specific positive clones by direct ELISA

Clones	O.D. (405nm)	Clones	O.D. (405nm)
E1A7	1.118	E3A1	0.251
E1C4	0.792	E3A3	0.237
E1E3	0.799	E3A5	0.231
E1G7	0.551	E3A9	0.693
E1A3	0.139	E3B2	0.230
E1G4	0.189	E3C1	0.649
E1A10	0.109	E3C10	0.235
E1D12	0.145	E3C11	0.371
E2C11	0.379	E3D10	0.583
E2E1	0.408	E3E8	0.372
E2F2	0.335	E3F12	0.292
E2H6	0.129	E3G3	0.260
E2D7	0.146	E3D4	0.173
E2F9	0.151	E3F10	0.123
E4A7	1.118	E4F7	0.799
E49	0.792	E4G9	0.551
E4B1	0.144	Pre-immune	0.200
E4C7	0.135	Mouse anti-hPXR	0.940

Clone no.
Negative clone
Positive clone
Mouse anti-hPXR polyclonal
Pre-immune

The four ELISA plates (numbered as E1, E2, E3 and E4) were coated with purified PXR protein. The higher O.D. (>0.200) clones were considered as positive clones when compared to pre-immune O.D. as negative control. Polyclonal mouse anti-hPXR serves as positive control.

Characterization of PXR monoclonal antibodies for receptor domain specificity

The fragments containing different domain sequences for PXR were used to construct the GFP chimera as described in 'Materials and Methods'. **Figure 15A** shows pictorial representation of organization of structural and functional domains of PXR and different domain sequences of PXR with GFP tag. COS-1 cells were independently transfected with the constructs having different domains of PXR. After 24 h of their expression sub-cellular localization were examined by fluorescence microscopy (**Figure 15B**). Sub-cellular localization reveals the exclusively cytoplasmic localization of PXR_{NTD} and both nuclear and cytoplasmic localization of PXR_{LBD}. Whereas for PXR_{DBD}, $PXR_{NTD+DBD}$ and $PXR_{DBD+LBD}$ plasmid constructs showed

predominantly nuclear localization. Subsequently cell lysates were prepared and western blot analysis was done with different antibodies. A positive control with mouse anti-PXR polyclonal antibody shows the satisfactory expression of all the domains of PXR, since anti-PXR polyclonal antibody have mixture of antibodies reacting against all the epitopes recognizing the different domains of PXR (**Figure 15C**). One more control with commercial rabbit anti-PXR LBD polyclonal antibody obtained from Santa Cruz shows the reactivity against LBD domain of PXR only (**Figure 15D**).

Four selected monoclonal antibodies shows reactivity towards different domains of PXR. We observed that out of four monoclonal antibodies, three monoclonal antibodies (6, 12 and 13) show reactivity towards LBD domain of PXR. So, they were named as PXR-L6 (**Figure 15E**), PXR-L12 (**Figure 15G**) and PXR-L13 (**Figure 15H**) where 'L' signify LBD domain of PXR. While 8th clone shows reactivity toward DBD domain of PXR. Therefore, named as PXR-D8 (**Figure 15F**) where 'D' signify DBD domain of PXR. So, domain-specific monoclonal antibodies may serve as unique tools for structural and functional characterization of PXR and its potential isoforms.

Isotype determination

In order to determine the isotypes of monoclonal antibodies secreted by the various hybridoma clones, isotyping was performed by using the mouse isotypic kit from Sigma Chemicals Co. (St. Louis, MO, USA) as described in 'Materials & Methods'. Out of four monoclonal antibodies PXR-D8, PXR-L12, and PXR-L13 consist of IgG1 and PXR-L6 monoclonal antibody consists of IgG2 heavy chain. All four hybridoma clones had K light chain.

Table V: Rescreening of positive clones by direct ELISA

Clones	O.D. (405nm)	Clones	O.D. (405nm)
1	**0.835**	13	**0.603**
2	0.125	14	**1.036**
3	0.128	15	**0.331**
4	**0.215**	16	0.190
5	**1.008**	17	**0.416**
6	**0.652**	18	**0.341**
7	**0.281**	19	0.119
8	**0.710**	20	0.149
9	**1.029**	21	**0.820**
10	**0.681**	22	0.133
11	**0.257**	23	0.166
12	**0.665**	24	**0.731**
Pre-immune	0.173 0.161	Mouse anti-hPXR	0.848 0.797

Clone no.
Negative clone
Positive clone
Mouse anti-hPXR polyclonal
Pre-immune

Twenty four positive clones were again resreened with direct ELISA assay. Among them only seventeen clones were giving higher O.D. (>0.165) when compared to pre-immune O.D. as negative control. Polyclonal mouse anti-hPXR serves as positive control.

Table VI: Testing the specificity of anti-hPXR specific positive hybridoma clones

Clone	O.D. (405nm) (His-SUMO-2 as coating antigen)	Clone	O.D. (405nm) (His-PXR as coating antigen)
1	**0.799**	1	**0.720**
2	0.197	2	0.153
3	0.167	3	0.179
4	0.214	4	0.147
5	**1.065**	5	**1.036**
6	0.220	6	**0.460**
7	0.178	7	0.208
8	0.232	8	**0.296**
9	**1.094**	9	**1.062**
10	0.282	10	0.255
11	**0.673**	11	**0.680**
12	0.246	12	**0.372**
13	0.171	13	**0.394**
14	0.192	14	0.131
15	0.191	15	0.176
16	**0.379**	16	**0.747**
17	0.179	17	0.211
18	0.203	18	0.177
19	0.196	19	0.178
20	0.213	20	0.138
21	0.185	21	0.101
22	0.155	22	0.136
23	0.228	23	0.225
24	**0.549**	24	**0.728**
Pre-immune	0.173	Pre-immune	0.242
Mice anti-hPXR	0.214	Mice anti-hPXR	0.736

Clone no.
Negative clones
Positive clones for PXR
Positive clones for His
Mouse anti-hPXR polyclonal
Pre-immune

For confirmation of PXR specific hybridoma clones, ELISA plate was coated with two types of antigen i) purified his-SUMO-2 protein (left column) ii)

purified his-PXR protein (right column). The clones which showed higher O.D. (for both antigen) compare to pre-immune were reactive against his tag and clones which showed higher O.D. (only for PXR antigen) compare to primmune were reactive against PXR protein.

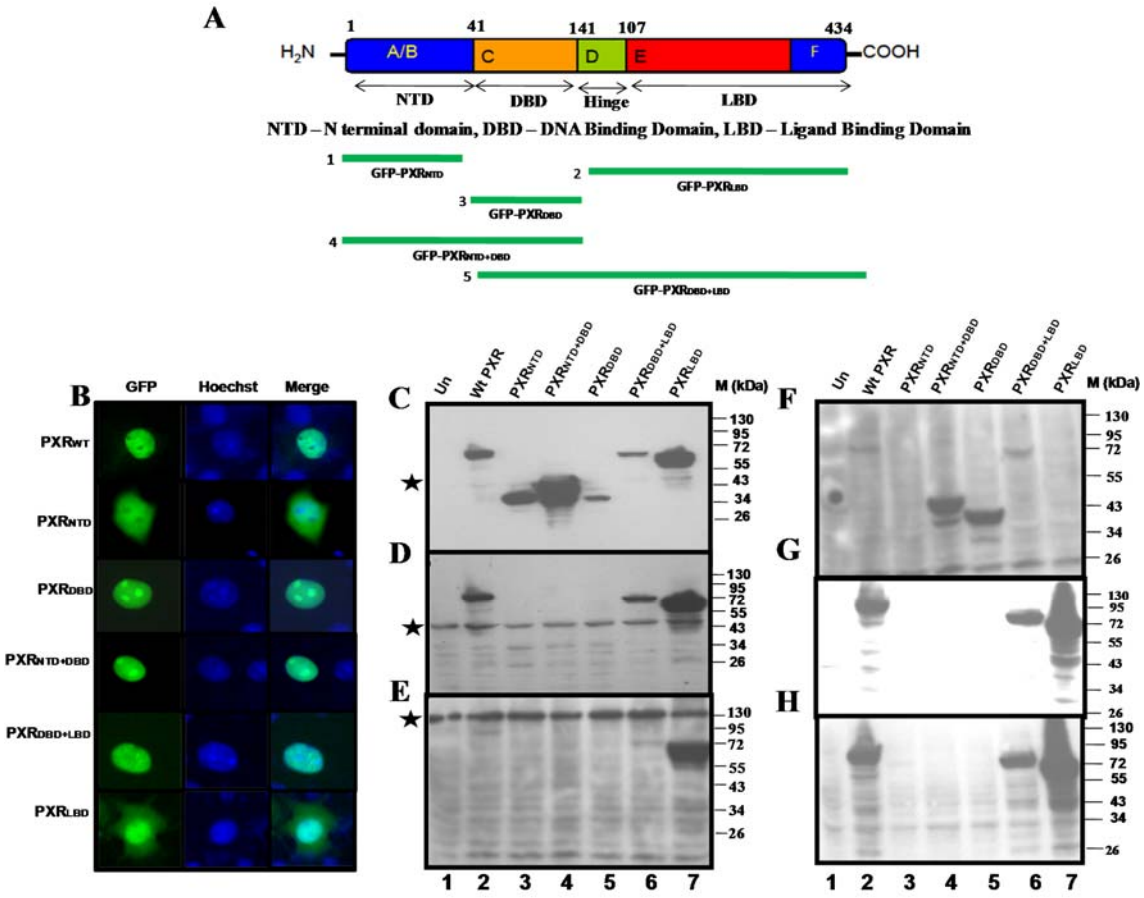


Figure 15: Generation of human PXR monoclonal antibodies recognize epitopes on different domains of receptor. ***A.*** *Organization of structural and functional domains of PXR protein. Different GFP chimera products were shown as different lines indicated as (1) PXR$_{NTD}$, (2) PXR$_{LBD}$, (3) PXR$_{DBD}$, (4) PXR$_{NTD+DBD}$, (5) PXR$_{DBD+LBD}$.* ***B.*** *To determine the domain specificity of different monoclonal antibodies against PXR, GFP-tagged domains of PXR were made as described in 'Materials and Methods' section. The expression patterns of all GFP chimera constructs were examined by transient transfection and immunoflorescence assay in COS-1 cells. Live cell images were recorded using a fluorescence microscope equipped with water-immersion objectives. The left panel shows the predominantly nuclear distribution of PXR$_{Wt}$, PXR$_{DBD}$, PXR$_{NTD+DBD}$ & PXR$_{DBD+LBD}$ and both cytoplasmic and nuclear distribution pattern of PXR$_{NTD}$ & PXR$_{LBD}$ constructs. The middle panel shows the Hoechst stained images for visualizing the corresponding cell nuclei. The right panel shows the merged images of GFP and Hoechst. Subsequently, cell lysates prepared from* ***B*** *were electrophoresed through 10% SDS-PAGE, blotted on to PVDF membrane and probed with different antibodies as* ***C****. mouse polyclonal anti-*

hPXR, ***D****. commercially available anti rabbit PXR LBD antibody H101-260 (Santa Cruz) and different hybridoma clones as* ***E.*** *PXR-L6,* ***F.*** *PXR-D8,* ***G.*** *PXR-L12 &* ***H.*** *PXR-L13. Lane 1, Untransfected (Un) cell extract; Lane 2, GFP-PXR$_{Wt}$ transfected cell extract; Lane 3, GFP-PXR$_{NTD}$ transfected cell extract; Lane 4, GFP-PXR$_{NTD+DBD}$ transfected cell extract; Lane 5, GFP-PXR$_{DBD}$ transfected cell extract; Lane 6, GFP-PXR$_{DBD+LBD}$ transfected cell extract; Lane 7, GFP-PXR$_{LBD}$ transfected cell extract. Astrix (★) signify non-specific band pattern.*

Characterization of monoclonal antibodies by different immunoreactivity tests

Not all antibodies work the same in all immunological assays. For instance an antibody that works well in a western blot may not perform as optimally in flow cytometry, immunocytochemistry, immunoprecipitation assays or other applications. So, we examined the immunoreactivity of different positive hybridoma clones selected by ELISA in other immunological assays. For the characterization of selected monoclonal antibodies, different analyses like western blot, flow cytometry, domain mapping, immunofluorescence, immunoprecipitation, chromatin-immunoprecipitation and immunohistochemistry analyses were performed.

- **Analysis of PXR monoclonal antibodies for substrate specificity by western blot analysis**

Initially, western blot analysis was used to determine the specificity of the anti-human PXR monoclonal antibodies. Whole cell extracts were obtained from HepG2, HepXR, untransfected COS-1, COS-1 cells transfected with human PXR and human intestinal colon adenocarcinoma cells (LS180) by RIPA lysis buffer. In each lane, 20 µg amount of protein were electrophoresed except LS180 cells, 100µg of protein was loaded to detect endogenous expression profile. The immunoblotting study indicated that selected four monoclonal antibodies bound specifically to a 50 kDa protein expressed in COS-1 cells transfected with a PXR expression vector and HepXR cells. Interestingly we also observed the enodogenous expression profile of PXR in LS180 cell line with PXR-L12 and PXR-L13 monoclonal antibodies. Western blot analysis shows reactivity towards PXR protein in

transfected cells but no significant expression of PXR protein in untransfected COS-1 and HepG2 cell-line (**Figure 16A**). We also examined the cross reactivity of these antibodies with splice isoforms PXR-2 derived from PXR-1 isoform, construct having deletion of 37 amino acids [174 to 210 amino acids are deleted from the LBD of PXR (calculated mol. wt. 45.7 KDa) (Dotzlaw et al, 1999; Hustert et al, 2001). We get positive signal with PXR-D8, PXR-L12 and PXR-L13 monoclonal antibodies (**Figure 16B**). So these PXR monoclonal antibodies PXR-D8, PXR-L12 and PXR-L13 are also useful in detecting the PXR-2 isoform.

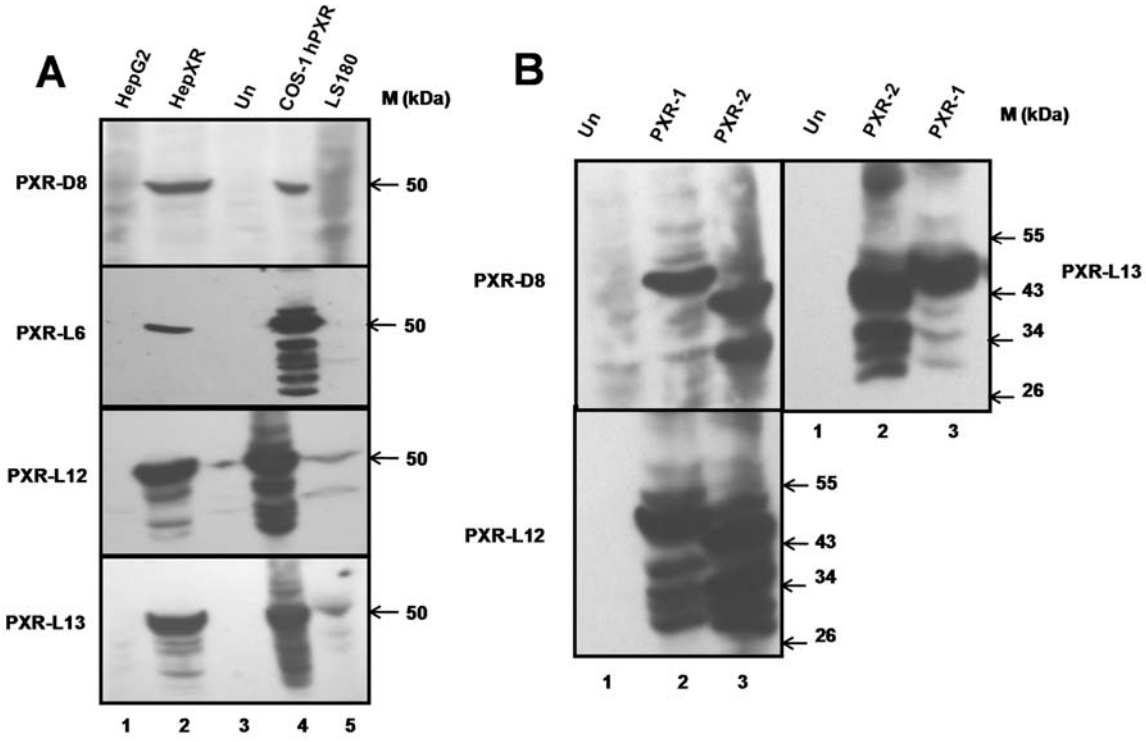


Figure 16: Characterization of four different anti-Hpxr monoclonal antibodies by western blot analysis. ***A.*** *Cell lysates from HepG2, HepXR, COS-1 cells and transiently transfected with human PXR plasmid (PXR) for 48 h and LS180 were electrophoresed through 10% SDS-PAGE, blotted on to PVDF membrane and probed with mouse anti-hPXR monoclonal antibodies supernatants as described in 'Materials & Methods' section.* ***B.*** *COS-1 cells transiently transfected with human PXR-1 and PXR-2 isoforms for 48 h and cell lysate were electrophoresed through 10% SDS-PAGE, blotted on PVDF membrane and probed with mouse anti-hPXR monoclonal antibodies supernatants as mentioned in 'Materials & Methods'.*

- **Analysis of PXR monoclonal antibodies for PXR sub-cellular localization by immunocytochemistry**

Immunocytochemistry serves as a tool to detect the sub-cellular localization of proteins. **Figure 17** depicts the result of immunostaining with different monoclonal antibodies in fixed HepXR cells. HepXR cells exhibited pronounced nuclear staining (left panel) as previously reported from our laboratory for nuclear localization of unliganded and ligand-bound PXR in HepXR cells (Saradhi et al, 2005b). Immunocytochemistry were done as explained under 'Materials and Methods' section. Different monoclonal antibodies which exhibited positive results with ELISA and western blot analysis were chosen for immunocytochemistry tests (**Figure 17**). For this purpose we performed immunocytochemistry with mouse polyclonal serum as a positive control and pre-immune as negative control. We observed that the monoclonal antibodies PXR-L6, PXR-L12 and PXR-L13 were working efficiently with both western blot analysis and immunocytochemistry, though PXR-D8 monoclonal antibody was not able to detect PXR efficiently in immunocytochemistry but was efficient in western blot analysis of PXR protein.

- **Immunoprecipitation analysis with PXR monoclonal antibodies**

Immunoprecipitation (IP) is a technique that enables the purification of a protein antigen using an antibody that physically isolates the protein of interest from the sample containing many thousands of different proteins. The antibody/antigen complex will then be pulled out of the sample using protein A/G-coupled beads. Subsequently, samples were separated by SDS-PAGE for western blot analysis. Immunoprecipitation of intact protein complexes is known as co-immunoprecipitation (Co-IP) which is useful to analyze protein-protein interaction study.

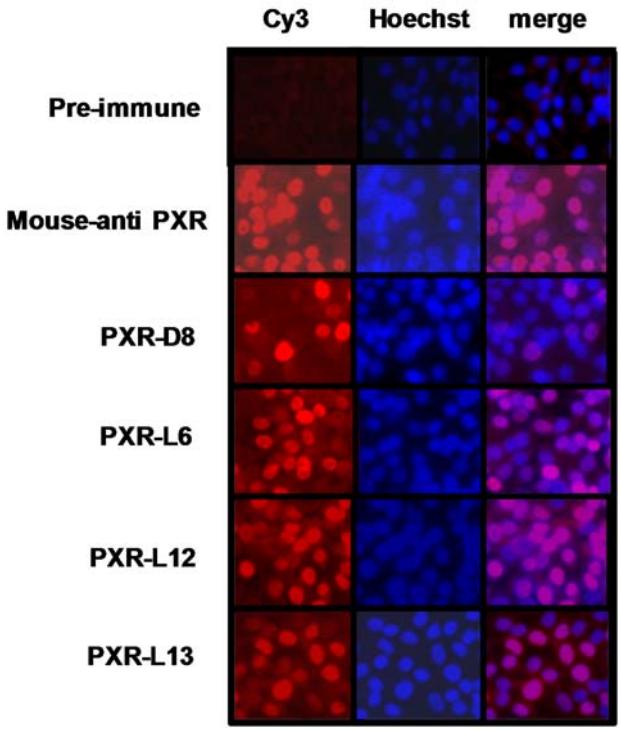


Figure 17: Characterization of four different anti-Hpxr monoclonal antibodies for immunofluorescence analyses. *HepXR cells were fixed and processed for immunofluorescence study. Cells were detected with different monoclonal antibodies against human PXR as indicated in the figure. Cell images were recorded using a fluorescence microscope equipped with water-immersion objectives. The left panel shows the predominantly nuclear distribution pattern of PXR in immunodetected cells. The middle panel shows Hoechst images for visualizing the corresponding nuclei of the same cells. The right panel shows the merged images for visualizing the corresponding nuclei with hPXR protein.*

To assess whether our monoclonal antibodies will be useful in this assay or not, we next studied the utilization of these monoclonal antibodies in immunoprecipitation assay. Immunoprecipitation assays were performed with COS-1 cells transfected with GFP-PXR for 48 h and harvested with lysis buffer (pH-7.6). Equal amount of whole cell lysate (200 µg) were separated and each sample was incubated individually with purified antibodies (3 µg) like PXR polyclonal antibody, pre-immune serum, monoclonal antibodies PXR-L6, PXR-L8, PXR-L12 and PXR-L13 and isotype controls (IgG) overnight at 4°C. Each sample was further incubated for additional 2 h on rotor at 4°C after addition of 20 µl of either Protein A-Sepharose or Protein G-Agarose beads slurry. After incubation period, beads were washed three times each

with lysis buffer (pH-7.4) at 4°C. After complete removal of the wash buffer the bound proteins were extracted in Laemmli's buffer. Recovered proteins were separated by 10% SDS-PAGE. Proteins on gel were transferred to PVDF membrane and subjected to western blot with PXR-L13 monoclonal antibody. Interestingly, all monoclonal antibodies worked with similar efficiency in immunoprecipitation assay (**Figure 18**).

These results confirm that all the four monoclonal antibodies generated will be useful in protein-protein interaction studies of PXR.

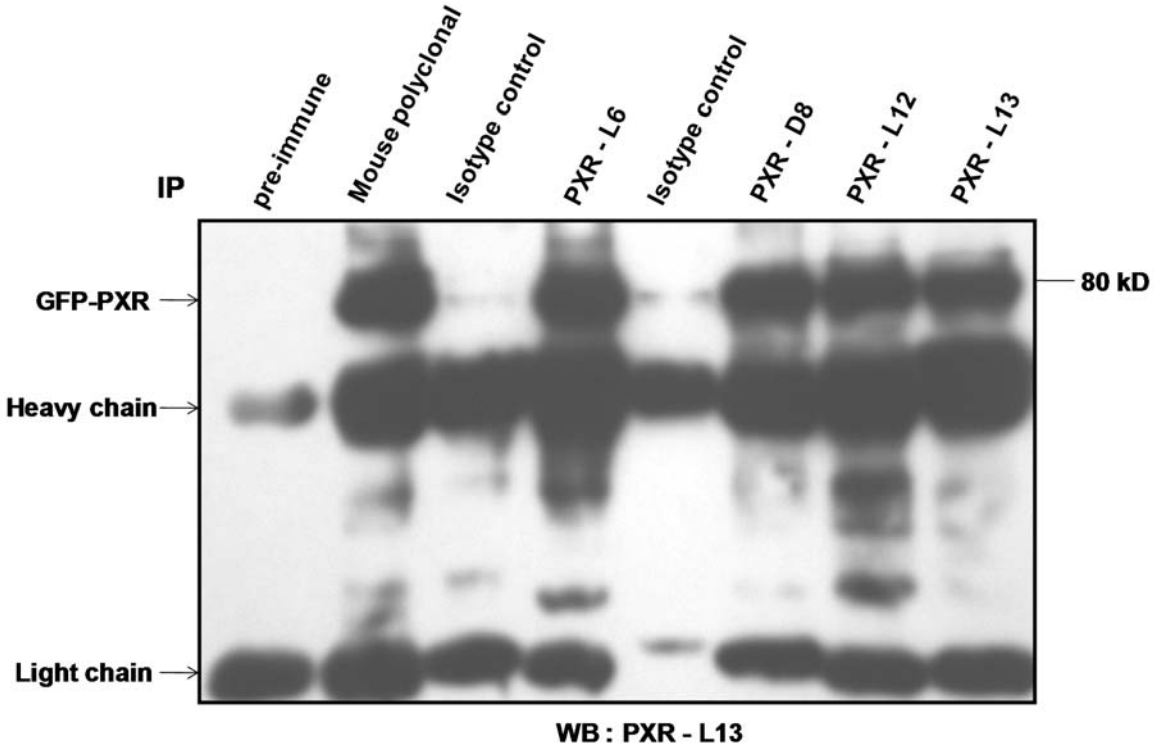


Figure 18: PXR monoclonal antibodies efficiently work in immunoprecipitation assay. *COS-1 cells transfected with GFP-PXR plasmid. After 48 h of transfection period cells were harvested for lysate preparation in RIPA buffer and immunoprecipitated with different antibodies as indicated in the figure. Immunoprecipitated complex were extracted with* Laemmli's *buffer, electrophoresed through 10% SDS-PAGE blotted onto PVDF membrane and probed with PXR-L13 monoclonal antibody. A specific reactivity with a major protein band having molecular weight of 80 kDa was detected which corresponds to GFP-PXR chimeric protein.*

- **Chromatin immunoprecipitation (ChIP) analysis with PXR monoclonal antibodies**

Chromatin Immunoprecipitation (ChIP) is a powerful and versatile technique used to investigate the interaction between proteins and DNA in the natural chromatin context of the cell. To further validate these monoclonal antibodies in context to PXR promoter *in vivo*, association of PXR with the promoter was tested by ChIP. Briefly, HepXR cells are first fixed with 1% formaldehyde, a reversible protein-DNA cross-linking agent that serves to preserve the protein-DNA interactions occurring in the cell. Cells are then lysed and chromatin is harvested and fragmented using sonication. The equal amount of cell lysate (200 µg) were separated and each sample incubated individually with different antibodies subjected to immunoprecipitation, using PXR polyclonal antibody, pre-immune serum, monoclonal antibodies PXR-L6, PXR-L8, PXR-L12 and PXR-L13 and isotype controls (IgG1 and IgG2a). Any DNA sequences that are associated with the protein of interest will co-precipitate as part of the cross-linked chromatin complex and the relative amount of that DNA sequence will be enriched by the immunoselection process. Immunoprecipitated cross-linked chromatin fragments were deproteinized, soluble DNA fragments were purified and desired region of PXR promoter was amplified by PCR using a set of primers specific for 200 bp proximal ER6 promoter region containing binding site for PXR. Using the specific primer sets, a strong amplified PCR product was observed from the PXR polyclonal antibody and light bands appeared from the PXR monoclonal antibodies PXR-L6, PXR-L12 and PXR-L13 precipitated chromatin fragments (**Figure 19**). However, no such PCR product was seen in the case of PXR-D8 monoclonal antibody, pre-immune and isotypic controls.

The inability to observe association of PXR with ER6 with PXR-D8 monoclonal antibody under *in vivo* conditions was attributed to the nature of antibody which was raised against the DNA binding domain of PXR protein. So these result indicate that monoclonal antibodies PXR-L6, PXR-L12 and PXR-L13 working in ChIP assay.

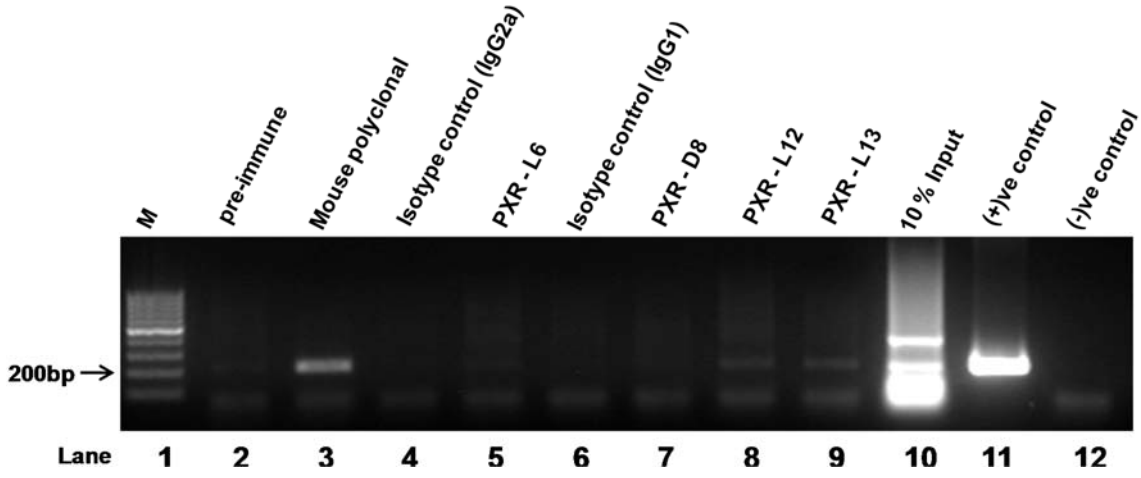


Figure 19: ChIP analysis with PXR monoclonal antibodies. *Cell lysate prepare from HepXR cells were examined by ChIP assay as indicated in 'Materials & Methods' using antibodies as indicated in figure. The sonicated DNA fragments present in the immunoprecipitates were amplified in PCR using primer pairs corresponding to 200bp promoter region of the ER6 region. Results of PCR amplification from control whole cell lysate (input) and the immunoprecipitates are shown. Lane 1, 100bp ladder; Lane 2, pre-immune immunoprecipitate as a negative control; Lane 3, PCR amplification of DNA immunoprecipitated by mouse anti-PXR polyclonal; Lane 4, isotype IgG2a immunoprecipitate as a negative control; Lane 5, PCR amplification of DNA immunoprecipitated by PXR-L6; Lane 6, isotype IgG1 immunoprecipitate as a negative control; Lane 7, no PCR amplification of DNA immunoprecipitated by PXR-D8; Lane 8, PCR amplification of DNA immunoprecipitated by PXR-L12; Lane 9, PCR amplification of DNA immunoprecipitated by PXR-L13; Lane 10, PCR amplification of input DNA; Lane 11, PCR amplification of XREM-Luc plasmid as positive control; Lane 12, PCR amplification of without any DNA template as negative control.*

- **Characterization of PXR monoclonal antibodies by flow cytometry analysis**

Flow cytometry has emerged as a key technology in clinical laboratories and offers the possibility of analyzing a high number of samples in a short time. So, we examined our panel of monoclonal antibodies by flow cytometry analysis in HepG2 and HepXR cell lines. Out of the four positive clones only PXR-L12 and PXR-L13 were found to be positive by flow cytometry analysis (**Figure 20**). So, the utility of these monoclonal antibodies by flow cytometry are expected to provide further insights into the involvement of PXR in cell-cycle regulation.

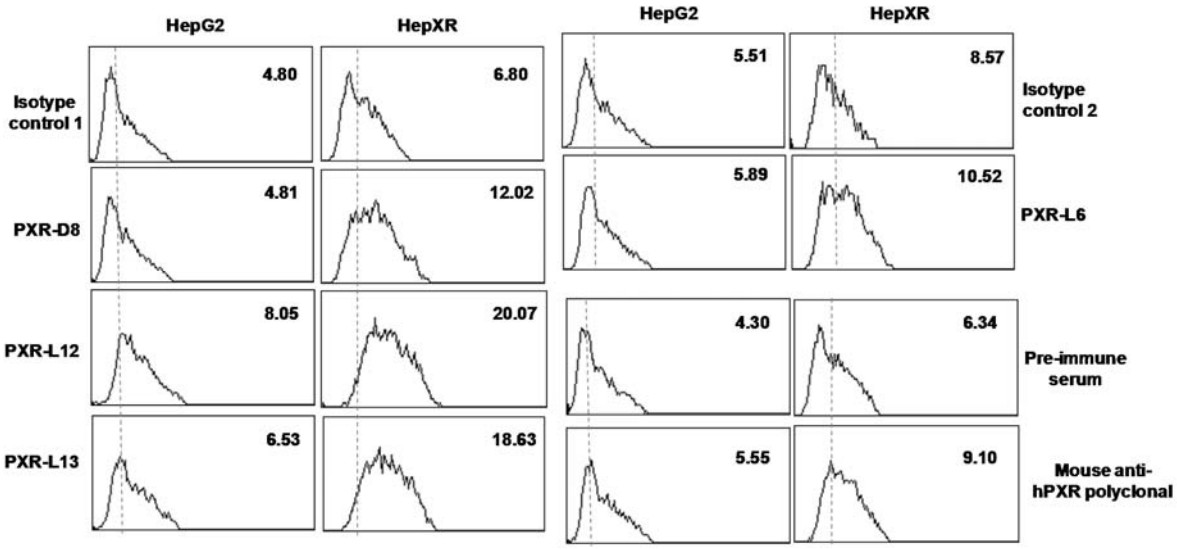


Figure 20: Characterization of monoclonal antibodies by flow cytometry analysis. *HepG2 cells (human hepatocellular carcinoma cell line) and HepXR (HepG2 stably integrated with human PXR) were trypsinized, washed twice with ice-cold PBS, fixed with methanol for 20 min on ice followed by permeabilization in 0.25% (Triton X100) and 0.1% (NP40) for 10 min. Subsequently, blocking was done in 1% FBS and 1:50 dilution of human serum in 5% BSA for 1 h. Cell samples were then incubated with different PXR monoclonal antibodies as PXR-L6, PXR-L12, PXR-L13, PXR-D8 and mouse anti-hPXR polyclonal antibody for 2 h followed by 4 times washing with 0.1% saponin in PBS. Then anti-mouse cy-3 conjugated secondary antibody (1:200) incubation for 1 h followed by 4 times washing with 0.1% saponin in PBS. Four set of controls were included i) unstained cells, ii) incubation with secondary antibody only, iii) preimmune and iv) isotypic controls. After fixation all the steps were carried out at RT. Cells were then fixed in 2% (w/v) paraformaldehyde in PBS. Fixed and stained cells were analysed by flow cytometry using FACSCalibur (Becton Dickinson, San Jose, CA, USA) with Cell Quest software for acquisition and analysis. Mean Fluorescence Intensity (MFI) of each sample were given in the figure. Cells were selected by gating on forward (size) and side (granularity) scatter. A minimum of 5,000 events were recorded for each sample.*

- **Immunohistochemistry analysis with PXR monoclonal antibodies**

Previous report from immunohistochemistry analysis have shown contradictory pattern of staining for PXR in different tissues, cytoplasmic localization of PXR is seen in human colon mucosae (Ouyang et al, 2010) and nuclear is seen in ovarian cancer tissue (Gupta et al, 2008). It may be due to different epitope specific antibody bound to different site of the

protein and gave different results. In addition, these observations may be due to tissue specific differential localization of PXR protein. We examined the reactivity of PXR monoclonal antibodies with human liver and colon tissue samples. In case of human liver sample both non-tumor and tumor cell were analysed with immunohistochemistry assay. We used different controls as negative control (supernatant without reactivity with PXR) and pre-immune and different positive controls as mouse PXR polyclonal antibody and monoclonal antibodies PXR-L12 and PXR-L13 supernatants as mentioned in 'Materials & Methods'. Our results imply that only PXR-L12 and PXR-L13 monoclonal antibodies positively stained the liver and colon samples whereas no significant staining with negative control. Previous study suggests PXR up-regulation in different cancerous condition leads to chemo-resistence. In contrary to this, in human liver sample PXR seems to be overexpressed in non-tumor condition compare to tumor condition (**Figure 21A**). Our results imply from immunohistochemistry, that PXR monoclonal antibodies named PXR-L12 and PXR-L13 with normal colon tissue sample shows strong immunoreactivity with lymphoid tissue. PXR has been observed to be primarily cytoplasmatic in lymphoid tissue whereas in colon mucosae it showed light staining in cytoplasm (**Figure 21B**). A recent report suggests an exclusively cytoplasmic localization of PXR in human T lymphocytes (Dubrac et al, 2012). On the contrary, immunocytochemistry results exhibit specific nuclear staining with these antibodies where PXR is overexpressed. More work is needed to establish these monoclonal antibodies to explore its immunoreactivity in immunohistochemistry analysis. So these antibodies may have the potential to be helpful in immunohistochemistry analysis.

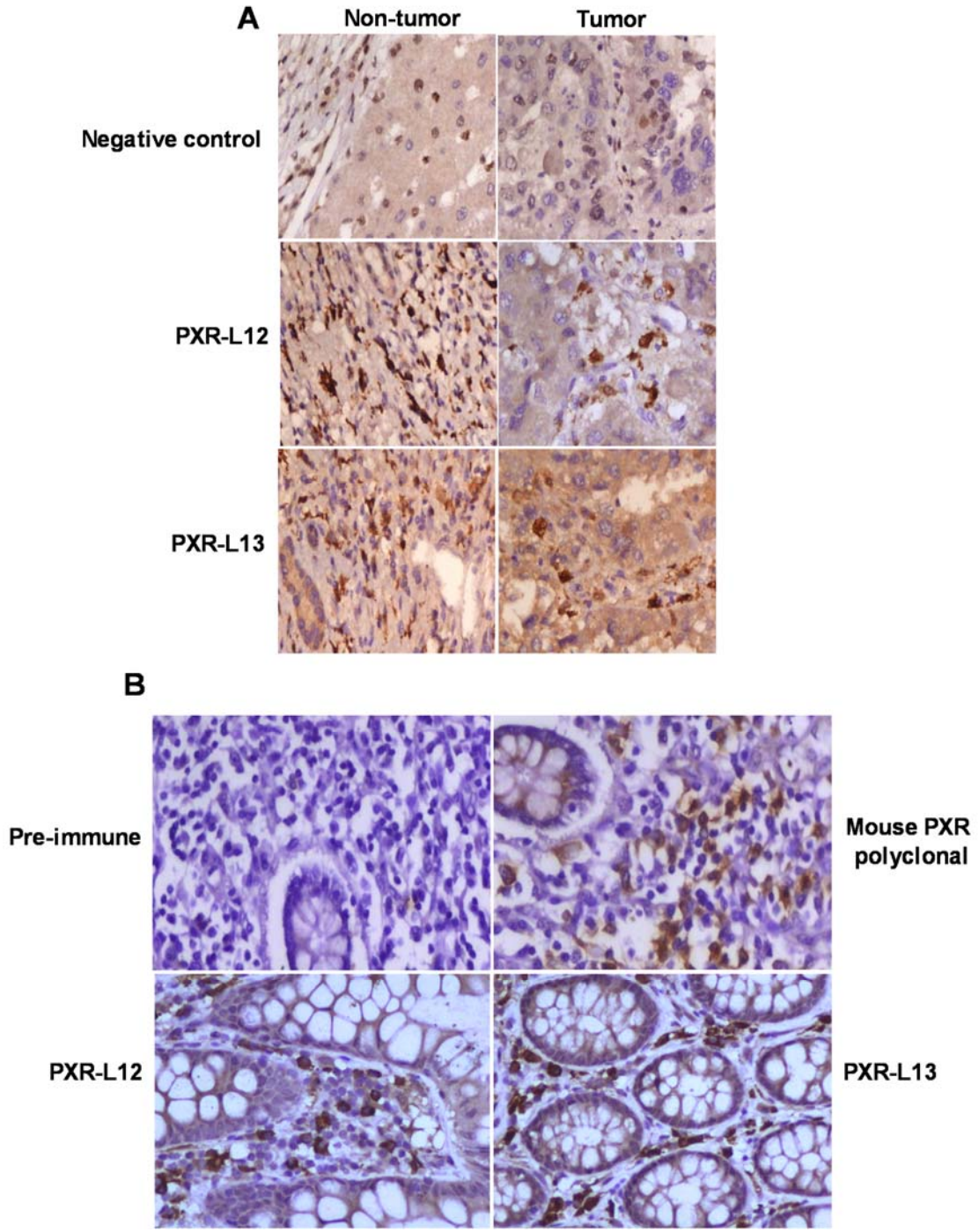


Figure 21: Characterization of human PXR monoclonal antibodies with immunohistochemistry analysis. ***A.*** *Human liver (tumor and non-tumor) and* ***B.*** *colon tissue sample were embedded in paraffin and sections (5 µm) were dewaxed in a xylene bath and rehydrated in graded alcohol as described in 'Materials & Methods'. Endogenous peroxidase activity was quenched with 1.5% H_2O_2 in methanol for 20 min and washed in PBS. Subsequently tissue sections were subjected to antigen retrieval using citrate 0.01 M, pH 6.0 for 30 min at 80˚C. Non-specific binding sites were blocked*

with power block (provided in Super Sensitive Polymer-HRP Detection Kit, Biogenex, USA) for 30 min at RT. Subsequently, the glass slides were incubated with negative control (supernatant without reactivity with PXR), pre-immune, mouse PXR polyclonal antibody and monoclonal antibodies like PXR-L6, PXR-L12 and PXR-L13 supernatant. The coverslips were incubated in a humidified atmosphere at room temperature for 30 min and then washed with three changes of PBS over 5 min. After washing, secondary antibody was added and sections were incubated for 30 min at RT. Subsequently another three changes for washing were performed and incubated with tertiary antibody conjugated with HRP. Stainings were initiated with DAB (brown chromagen) and hematoxylin counterstain was used. After dehydration, sections were mounted with DPX on a glass slide and the images were taken on BX51 microscope (Olympus).

Table VII: A summary of reactivity of PXR-specific monoclonal antibodies by different immunological methods

Monoclonal antibody	Isotype	Western blot	Flow cytometry	Immuno-cytochemistry	Immuno-histochemistry	Immuno-precipitation	Chromatin-immuno-precipitation	Domain reactivity
PXR-D8	IgG1, κ	+	+	+	-	+++	-	DBD
PXR-L6	IgG2a, κ	++	+	++	-	+++	-	LBD
PXR-L12	IgG1, κ	+++	++	+++	++	+++	++	LBD
PXR-L13	IgG1, κ	+++	++	+++	++	+++	++	LBD

- no reactivity; +very less activity; ++ moderate reactivity; +++ strong reactivity

DISCUSSION

In this study, we have successfully generated a panel of specific mouse monoclonal antibodies against full-length human PXR. It is for the first time that PXR monoclonal antibodies have been generated by using the purified full-length PXR protein for immunization purpose that resulted in generation of panel of monoclonal antibodies against PXR. Using different specific monoclonal antibodies within our panel, we showed the reactivity of antibodies towards different domains of PXR. These four positive clones were designated as PXR-L6, PXR-L12, PXR-L13 detecting LBD of PXR and PXR-D8 detecting DBD of PXR. The sensitivity and specificity of the monoclonal antibodies against human PXR were tested in a variety of immunoassays and found to be useful in ELISA, western blot, immunoprecipitation,

immunofluorescence, immunohistochemistry, ChIP and flow cytometry analyses to varying degree. It has been previously demonstrated that PXR expression levels are mostly up-regulated in different cancerous conditions and are influenced by various factors. Contrary to this, it has also reported that PXR inhibits the proliferation and tumorigenicity of colon cancer cells by controlling cell cycle at G0/G1 cell phase by regulating p21WAF1/CIP1 and E2F/Rb pathways (Ouyang et al, 2010). In other report it has been suggested rifampicin-activated PXR inhibited the proliferation of HepG2 cells through the arrest of G0/G1 phase (Zhuang et al 2011). A recent report suggested the role for PXR in the pathogenesis of inflammatory bowel diseases. A significant down-regulation of PXR resulted in decreased expression of several phase II enzymes and xenobiotic transporters (Langmann et al, 2004). It is highly plausible that the expression of PXR is controlled by various factors under different metabolic disorders and by using these monoclonal antibodies, we may observe up/down regulation of PXR protein utilizing different immunological techniques. Even the function of PXR in the cell cycle regulation may be detected by using monoclonal antibodies for flow cytometry analysis which may further facilitate detailed understanding of the PXR-mediated modulation of cell proliferation and cell cycle regulation.

Due to apparent lack of specific antibody against different isoforms of PXR, it was difficult to characterize different splice varients of PXR. Interestingly our antibodies PXR-D8, PXR-L12 and PXR-L13 also cross react with PXR-2 isoform. So, it would be possible to study and characterize different isoforms of PXR with the help of these monoclonal antibodies. In conclusion, the monoclonal antibody against PXR produced in this study may offer a useful molecular reagent for further studies on PXR forms and their functions coupled with other immunoassays. For example, those antibodies may be used to detect protein–protein interaction by immunoprecipitation and to search for PXR protein–DNA binding sites by chromatin immunoprecipitation studies. Availability of these antibodies will also facilitate molecular elucidation of known PXR functions, as well as

exploration of its many unknown functions including its role in cell-cycle regulation.

Evaluation of correlations between PXR expression and clinical outcomes in lymphoid tissue is in progress (Dubrac et al, 2010). Overexpression of PXR protein in colon lymphoid tissue and lower expression in tumor liver cells, as determined by immunohistochemistry, may be a potentially important tool for pathological evaluation of diseases. Understanding the mechanism behind role of PXR in different cancers may aid in the development of chemotherapeutic regimens specifically designed against PXR and its target genes. Finally, a summary of efficacy validation of these monoclonal antibodies in different immunological applications was given in the form of **Table VII**. The studies reported herein demonstrate the utility of our antibodies for detection of PXR in various immunological assays. It is believed that these antibodies should be useful in investigating the presence of PXR and its isoforms in different normal and cancerous states and in studying the interactions of PXR with other proteins. Taken together, our results demonstrate successful generation and functionality of these mouse anti-human PXR monoclonal antibodies in various immunological tools like ELISA, immunoblotting, immunoprecipitation, immunohistochemistry, immunofluorescence, ChIP and flow cytometry, which may provide a useful tool to elucidate the role of PXR in various diseases. These monoclonal antibodies are expected to serve as an important and useful tool for future investigations of human PXR in different pathological states and assisting in a better understanding of the mechanistic aspects of transcriptional regulation.

CHAPTER II

Detection and identification of PXR and its potential isoforms

INTRODUCTION

Nuclear receptors (NRs)are a class of multi-functional proteins that participate in a variety of critical functions in almost every tissue ofthe body. The ligand-activated nature of NRs has a role in regulating diverse physiological processes such as embryonic-development, cellular physiology metabolism and homeostasis. Therefore, the expression of aNR is one of the determinants of the outcome of any specific receptor signaling pathway. In addition to the relative levels of each isoforms in the target tissue, the expression of receptorisoformscan influence NR-mediated responses. These isoforms demonstrate unique and redundant roles in their combined regulatory function. Some receptors exist as subtypes or isotypes for example, retinoid acid receptor (RAR) exist in three forms RARα, RARβ and RARγ and estrogen receptor (ER)exist in two forms ERα and ERβare the products of related but independent genes. Human genome contains approximately 20,000 protein-coding genes considerably less than previously expected (Venter et al, 2001). Variability in protein functions from such a small fraction of protein-coding genes arises through several biological processes can lead to the production of many more unique proteins.

To increase the variability of protein isoforms differing in structural or functional aspects, products of a single gene can use alternative processing to generate multiple mRNA products through alternative splicing, alternative promoter and exon usage and alternative translation initiation mechanism are key players in this phenomenon. For example, GR is expressed as GRα (Oakley et al, 1999) and GRβ (Oakley et al, 1996), PR is expressed as PR-A and PR-B (Kastner et al, 1990; Gronemeyer et al, 1991)and PXR is expressed as PXR-1, PXR-2 and PXR-3 (Bertilsson et al, 1998; Blumberg et al, 1998; Kliewer et al, 1998; Lehmann et al, 1998). Presently, it is estimated that >50% of all human genes have multiple mRNA products generated via alternate mRNA processing. These alternative mechanismsare now beginning to receive more attention for their ability to generate a variety of protein isoforms from a single gene. The use of alternate promoters may

haveseveral outcome:i) no change in the receptor isoform, based on translation initiation in a common downstream exon, but variable 5' regions allowing for tissue-specific expression or altered translation efficiency ii) alternate 5' exons may contain additional ATGs resulting in a protein isoform that differs by amino-terminal additionand iii) addition of 5' regions may introduce a new open reading frame (ORF) and translate an entirely different protein. Both PR-B and PXR-2 are products of amino-terminal addition (Kastner et al, 1990; Gronemeyer et al, 1991;Bertilsson et al, 1998; Kurose et al, 2004). Studies have demonstrated that PR-B has 164 amino acids of additional amino-terminal sequence, within this region it contained a unique third Activation Function-3, (AF-3) and this unique region causes distinct tissue-specific expression compared with PR-A (Mote et al, 1999; Mote et al, 2002). Discovery of isoform-specific functions, whether for GRβ or PR-B, illustrate the functional importance of alternative receptor isoforms. Similarly, PXR expression consists of multiple transcript isoforms generated by alternative splicing and alternate promoter usage (Bertilsson et al, 1998; Kurose et al, 2004; Tompkins et al, 2008). In large part, the biology of these alternate isoforms is unknown.

Reports on the regulation of PXR has suggested that other nuclear receptors, such as GR, PPAR and HNF-4 may participate in PXR promoteractivation (Pascussi et al, 2000; Aouabdi et al, 2006; Zhou et al, 2006; Iwazaki et al, 2008). The ability of other NRs to alter PXR expression addsadditional complexity to the regulation of this critical signaling pathway. The PXR gene consists of nine exons and spans approximately 35 kb in chromosome 13q12-13.3. Three alternatively spliced transcripts that encode different isoforms of PXR have been described (Bertilsson et al, 1998; Blumberg et al, 1998; Kliewer et al, 1998).PXR-1 encodes protein through the use of CUG codon instead of AUG codon. PXR-2 encodes the longest isoform and initiates translation from the standard AUG codon present in its 5' terminal exon. PXR-3 contains an alternate 5' terminal exon and uses a different acceptor splice site at exon 5 in comparison to PXR-2. It initiates translation from an in-frame, downstream non-AUG (CUG) codon, resulting

in a shorter isoform-3 with a different amino-terminus and also lacks an internal segment, compared to PXR-2. All three isoforms are reported to be present at varying levels in the liver and gut. PXR-3 is largely non-responsive to ligands in transient transfectionassays (Gardner-Stphen et al, 2004). Additional transcript variants originated from alternative promoter usage, alternative splicing and/or alternative polyadenylation may exist, but, they have not been completely characterized (Fukuen et al, 2002; Lamba et al, 2004).In accordance with its participation in regulating metabolism, PXR is predominantly expressed in liver, colonand small intestine and has also beendetected in a number of other tissues including lung, heart, breast, kidney and brain (Dotzlaw et al, 1999; Fukuen et al, 2002; Lamba et al, 2004).In each of these tissues and different patho-physiological states, PXR levels, the variety of expressed isoforms and the regulation of their expression have not been convincingly explored.

Here we propose a hypothesis that the presence of alternative translational isoforms of PXR which are derive from PXR-1 splice isoform. By site-directed mutagenesis, we demonstratedthat PXR isoforms viz. PXR-M2, PXR-M3, PXR-M4 and PXR-M6 are generated by alternative translation initiation at the internal Met55 (methionine55), Met69, Met107 and Met113 respectively. Further, data are presented to suggest that alternative translational initiation receptor isoforms of PXR may regulate transcription function of wild type PXR. Our experimental design were primarily focused on characterizing novel translational initiationvariants for PXR splice isoform 1 (PXR-1) andits functional analysis.

RESULTS

Detection and identification of potential isoforms of PXR

With respect to PXR, the phenomenon of alternative splicing leading to various mRNA isoforms as PXR-1, PXR-2 and PXR-3 has been, previously reported in the literaturethough no significant progress was reported in subsequent years (Bertilsson et al, 1998; Blumberg et al, 1998; Kliewer et al, 1998; Lehmann et al, 1998; Lin et al, 2009). In recent years there are

reports that indicate generation of translational isoforms for various NRs (Barraille et al 1999; Liegibel et al, 2003; Pascual-Le Tellec et al, 2004; Lu & Cidlowski, 2005;Lin et al, 2009). In this context, nothing is known about alternative translation initiation mechanism or any other mechanism that can generate additional isoforms of PXR. In this section of study, we focused our efforts towards exploring the possibility of existence of translation initiation mechanism as ameans of generating other PXR isoforms. At first we investigated the conserved methionine (M) residues of PXR-1 isoform in different species. Comparison of sequences revealed thata number of downstream methionines like M2, M3, M4, M5 and M6 are conserved among various speciesmay acts as potential translational start codon (**Figure 22A**). Kozak context has been previously shown to play a major role in initiation of the translation process (Kozak et al, 1991a; Kozak et al, 1991b). So, in accordance with Kozak context strength, M6 lie in optimal Kozak context while M2, M3, M4 and M5 lie in sub-optimal Kozak context region (**Figure22B**). It has also been reported that leaky scanning mechanism is one of the mechanisms to generate additional translational isoform and is shown by graphical representation (**Figure 22C**). The expected molecular weight (kDa) of these putative isoforms of PXR when alternatively translated from different in-frame methionine is calculated as i) PXR-M1 = 49.77 kDa, ii) PXR-M2 = 43.72 kDa, iii) PXR-M3 = 42.23 kDa, iv) PXR-M4 = 37.72 kDa, v) PXR-M5 = 37.20 kDa and vi) PXR-M6 - 36.96 kDa. In our preliminary studies, when human PXR-1 cDNA was transiently expressed in COS-1 cells, multiple protein bands were observed along with a well-characterized 50 kDa band (**Figure 23A & 2B**).

A

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1   1    ---------------------------MRPEESWSRVGLVQCEEADSALEEP-INVEEE-DGGLQICRVCGDKANGYHF  50
2   1    -------------------------MEVRPKESWNHADFVHCEDTESVPGKPSVNADEEVGGP-QICRVCGDKATGYHF  53
3   1    ---------------------------MRPEERWNHVGLVQREEADSVLEEP-INVDEE-DGGLQICRVCGDKANGYHF  50
4   1    ------------------------------------MQCNETDSTSGNSTTNADEEDEGP-QICRVCGDKATGYHF  39
5   1    MSRL--------YDMCLLQLRMSKEMEELSPLD-------------DSGHGDGSEEETEEDDEP-KICQVCGDKSTGYHF  58
6   1    ------------------------------------MHRGEADSASTNPIIGADEEEECP-QICRVCGDKATGYHF  39
7   1    MG--------------------------------------------GKPTISADEE-EGP-QTCRVCGDKANGYHF  30
8   1    MFKVDPRGEFRAKNLPLSSPRGPEANLEVRPKEGWNHADFVYCEDTEFAPGKPTVNADEEVGGP-QICRVCGDKATGYHF  79
9   1    MSEV----------------------------QEAVVLEEEEEEEDASNSCGTGEDEDDGEPKICRACGDRATGYHF  49
1   51   NVMTCEGCKGFFRRAMKRNVRLRCPFRKGTCEITRKTRRQCQACRLRKCLESGMKKEMIMSDAAVEQRRALIKRKKREKI  130
2   54   NVMTCEGCKGFFRRAMKRNARLRCPFRKGACEITRKTRRQCQACRLRKCLESGMKKEMIMSDEAVEERRALIKRKKSERT  133
3   51   NVMTCEGCKGFFRRAMKRNVRLRCPFRKGTCEITRKTRRQCQACRLRKCLESGMKKEMIMSDAAVEQRRALIKRKKREKI  130
4   40   NVMTCEGCKGFFRRAIKRNARPRCLFRKGACEITRKTRRQCQACRLRKCLESGMRKEMIMSDAAVEQRRALIRRKKREQI  119
5   59   NAMTCEGCKGFFRRAMKRPAQLCCPFQS-ACVITKSNRRQCQSCRLQKCLSIGMKRELIMSDEAVEKRRLQIRRKRMQEE  137
6   40   NVMTCEGCKGFFRRAMKRNAQPRCPFRKGTCEITQKTRRQCQACRLRKCLESGMRKEMIMSDAAVEQRRALIRRKKRERI  119
7   31   NVLTCEGCKGFFRRTVKRNARLRCPFRKGACEITRKTRRQCQACRLRKCLESGMKKEMIMSDVAVAQRRALIKRKKRERM  110
8   80   NVMTCEGCKGFFRRAMKRNARLRCPFRKGACEITRKTRRQCQACRLRKCLESGMKKEMIMSDAAVEERRALIKRKKRERI  159
9   50   NAMTCEGCKGFFRRAMKRKLQLSCPFQN-SCVINKSNRRHCQACRLKKCLDIGMRKELIMSDEAVEQRRALIKRKQRLAE  128
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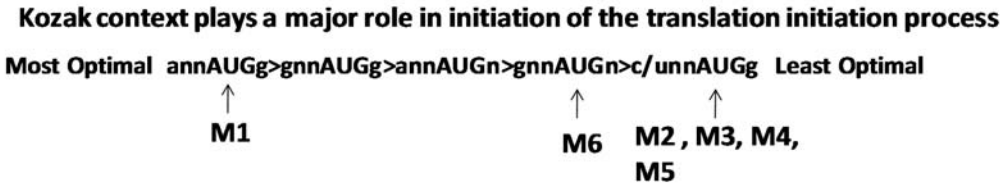
B



C

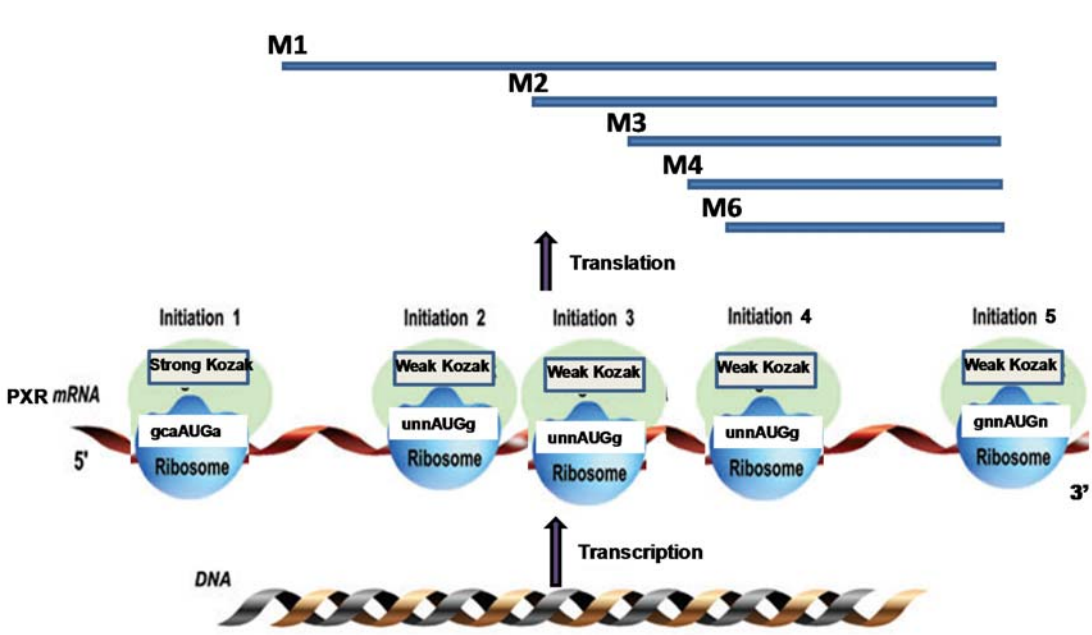


Figure 22: Conserved methionine residues of PXR among various mammalian species and Kozak context in PXR protein.*A.* *Conserved amino-terminal methionines of PXR of different specieswere examinedby using NCBI BLAST programme named 'Cobalt Constraint-based Multiple Protein Alignment Tool'. The amino acid sequences of different species are arranged*

by numbers as (1) Mus musculu – mouse; (2) Homo sapien – human; (3) Rattus norvegicus - rat; (4) Sus scrofa - wild boar; (5) Danio rerio – zebrafish; (6) Bos taurus – cow; (7) Oryctolagus cuniculus – rabbit; (8) Macaca mulatta - rhesus monkey; and (9) Xenopus tropicalis - western clawed frog. ***B.*** *Kozak context play a major role in initiation of the translation process. The figure represents the 'most optimal' to 'least optimal' strength of Kozak context around the different conserved in-frame methionines of PXR.* ***C.*** *The pictorial representation of hypothesis based on reinitiation or leaky scanning mechanisms which enable downstream AUG codons for generation of potential PXR isoforms translated from different in-frame methionines lies in sub-optimal kozak context.*

We used both polyclonal and monoclonal antibodies raised against full-length PXR to confirm the existence of additional bands of PXR. This suggested generation of bands that could be either proteolytic fragments or possible PXR isoforms derived from single mRNA (Kliewer et al, 1998; Saradhi et al, 2005).To rule out the possibility of involvement of proteasomal degradation pathway in generation of multiple bands, we transiently transfected PXR in COS-1 cells.After 12 h of expression period, the cells were treated with different concentration of proteasomal inhibitors (MG132 and LLnL) for 12 h. Even after blocking the proteasomal pathway, we still observed the presence of these additional bands in western blot analysis (**Figure 23C**).

However, no molecular explanation has emerged as to the nature of these bands or suggested isoforms. From this analysis, we ruled out the possibility of proteasomal degradation pathway involved in generation of these multiple bands. Interestingly, the molecular weight of these bands corresponded to the expected molecular weight of alternatively translated isoforms from different in-frame methionines of wild type PXR as was observed in case of GR (Lu & Cidlowski, 2005). These results suggested the existence of the phenomenon of alternative translation initiation resulting in the generation of PXR isoforms.

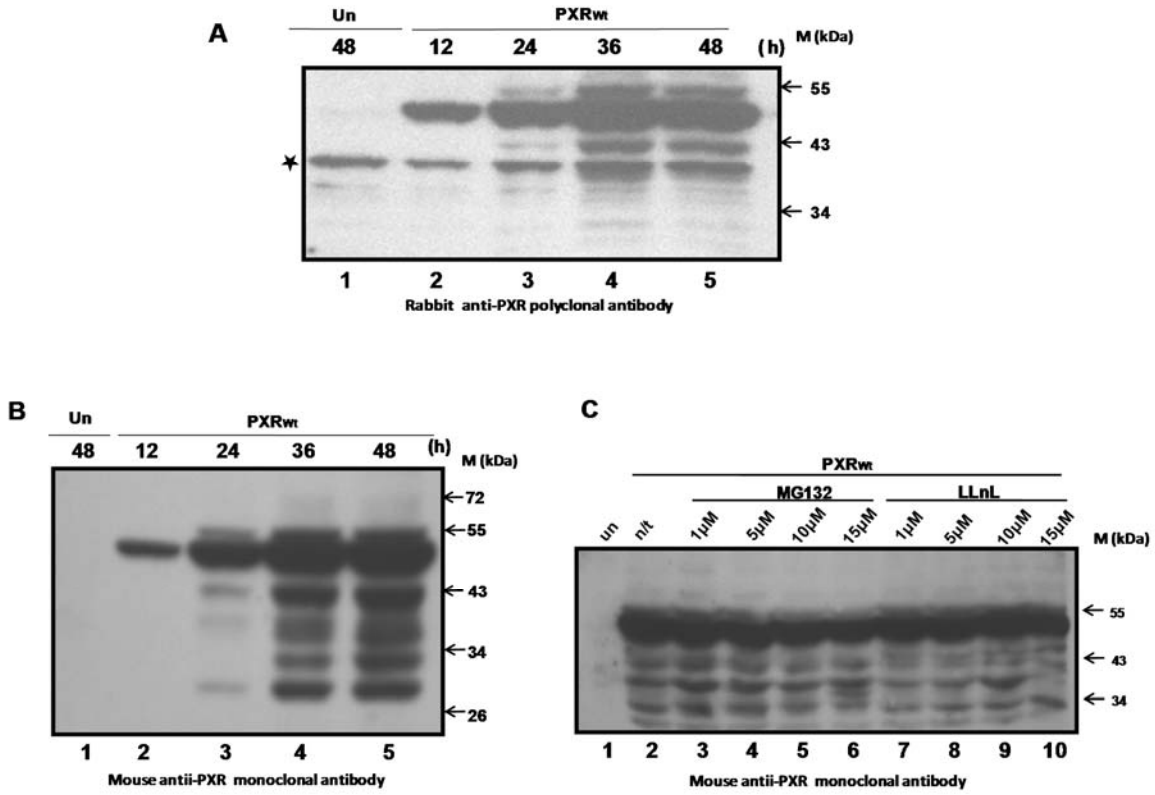


Figure 23: Existence of additional forms of PXR by western blot analysis. A & B. *Western blot analysis of COS-1 cells transfected with wild type PXR and expressing the protein over varied time period. Cell extracts were prepared after 12, 24, 36 and 48 h of expression period and electrophoresed through 10% SDS-PAGE, blotted onto PVDF membrane and probed with rabbit anti-hPXR polyclonal antibody (A) and mouse anti-hPXR monoclonal antibody (B & C) as mentioned in 'Materials & Methods' section respectively. Lane 1, Untransfected COS-1 cell extract; Lane 2, Cell extract prepared at 12 h after transfection; Lane 3, Cell extract prepared at 24 h after transfection; Lane 4, Cell extract prepared at 36 h after transfection; Lane 5, Cell extract prepared at 48 h after transfection; A specific reactivity with a major protein band having molecular weight of 50 kDa was detected. Asterisks (⋆) signify the non-specific band.* **C.** *Western blot analysis of COS-1 cells transfected with PXR in presence of proteasomal inhibitors. COS-1 cells were transfected with PXR plasmid. After 12 h of expression period, cells were treated with different concentration of MG132 (1μM, 5μM, 10μM and 15μM) and LLnL (1μM, 5μM, 10μM and 15μM) for additional 12 h. Cell extracts were prepared after 24 h of transfection and electrophoresed through 10% SDS-PAGE, blotted onto PVDF membrane and probed with mouse anti-hPXR monoclonal antibody as mentioned in 'Materials & Methods' section. A specific reactivity with a major protein band having molecular weight of 50 kDa was detected along with additional bands of PXR. Asterisks (⋆) signify the non-specific band. Un= untransfected cell extract.*

Presence of PXR isoforms in different cancerous cell line

Considering the differential expression of PXR in different cancerous states, reports of PXR expression seemed paradoxical (or inconsistent).Admittedly, it has been noticed that aside from liver and small intestine, PXR expressed in other cells/tissues may have some additional roles in these sites. In the initial reports the highest PXR expression was detected in the small intestine, followed by organs without PXR expression such as lung, adrenal gland, ovary, testis, prostate, and kidney (Dotzlaw et al, 1999; Fukuen et al, 2002; Lamba et al, 2004).

On the basis of key function of PXRin xenobiotic metabolism and elimination, it expression should be restricted in liver and intestine.However, studies indicated it to have broader range of distribution that is fully reflected with endogenous expression profile in different cancerous cell lines.While little work has been done to evaluate its role in different cancerous tissues like endometrial cancer (Masuyama et al, 2003), breast cancer (Dotzlaw et al, 1999), prostate cancer (Chen et al, 2007), epithelial ovarian carcinoma (Gupta, 2008) and esophageal squamous cell carcinoma (Takeyama et al, 2010), nonethelessmore work is needed to understand its role in different cancerous states.This in turn raises questions about additional function and the mechanism of PXR expression outside liver and small intestine. We hypothesized that differential expression of PXR in different tissue would result in a differential response of PXR in these tissue. In the present work we illuminate these issues by demonstrating that the differential expression of PXR and its potential isoforms in different cancerous cell line by using both polyclonal and monoclonal antibodiesagainst human PXR. This was investigated with western blot analysis by using whole cell extract (100µg) of different cancerous cell-line. We examined the endogenous expression profile of PXR in different cancerous cell line and observed expression of PXR in different cancerous cell lines with rabbit anti-hPXR polyclonal antibody and PXR-L12 and PXR-L13 mouse monoclonal antibodies. Different human cancerous cell-lines used in this study like hepatocellular carcinoma cell line HepG2,

breast cancer cell line MCF-7, gastric adenocarcinoma AGS, prostate cancer cell line DU145, embryonic kidney cell HEK293, colon adenocarcinoma COLO 320DM, ductal breast epithelial tumor cell line T47D, intestinal colon adenocarcinoma cell line LS180, HepXR cells (HepG2 cells stably transfected with PXR) and adenocarcinomic alveolar basal epithelial cells A549. When using the polyclonal antibodies, the main 50 kDa PXR form was ubiquitous in all the cell lines tested but some cell lines appeared to differwith respect to the presence of lowerPXR isoforms (**Figure 24**).Whencompared, the results obtained from polyclonal and monoclonal antibodies(PXR-L12& PXR-L13)were not very similar. Between PXR-L12 and PXR-L13 the latter appeared to perform better.Even though the results obtained with both polyclonal and monoclonal antibodies deviated from each other, it was evident that the levels of PXR and its isoforms varied in different cancerous cell lines tested. The observed differential expression of PXR in cancerous cell-lines by different antibodies may exhibit the differential accessibility of the epitope(s) by these polyclonal/monoclonal antibodies.Nonetheless, the results suggestthe absence orpresence (at different expression levels) of additional PXR specific bands in these cancerous cell lines along with the well-characterized 50 kDa band (**Figure 24**). Our preliminary observations with other monoclonal antibodies i.e. PXR-L6 and PXR-D8 revealed poor detection of PXR in different cancerous cell lines (not shown) but efficient detection in transiently/stable overexpressing cell lines (**Figure 16A**).Further work in required to optimize the conditions for utilizing these monoclonals, in single of multiple combinations, to develop an efficient immunological detection system for cell extracts and clinical tissue sections.

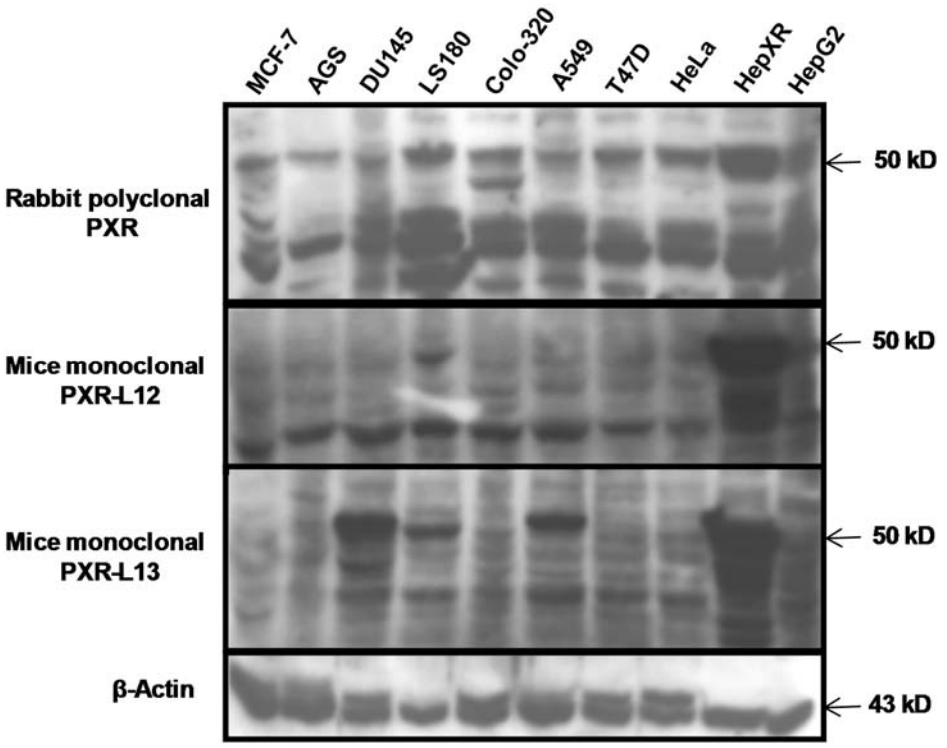


Figure 24: Endogenous expression profile of human PXR and its potential isoforms detected by probing with rabbit polyclonal and mouse monoclonal antibodies in various cancerous cell lines by western blot analysis. *Total cell extracts were prepared from different cancerous cell lines mentioned above by RIPA lysis buffer and total proteins were estimated by Bradford's analysis as described in 'Materials & Methods'. Equal amount of protein (100µg) from different cell lines were electrophoresed through 10% SDS-PAGE, blotted onto PVDF membrane and probed with rabbit polyclonal and also with mouse monoclonal antibodies PXR-L12 & PXR-L13 as described in 'Materials & Methods' section. The last panel shows equal loading control with rabbit anti-β-Actin antibody.*

The observationsmade herein constitute the first description about the existence of additional isoforms of PXR that may be influencing the diverse functionality of PXR in specific cells. Also, differential expression of PXR and its isoforms deserve consideration for subsequent studies for PXR involvement in cancer progression.Theexistence of differential pattern of expression of PXR in diverse cancerous cell lines indicate its potential as prognostic marker where antibodies described in this study could serve as a useful immunological tool.

Confirmation of existence of additional translational isoforms of PXR by site-directed mutagenesis

To examine the existence ofalternative phenomenon for generation of PXR isoforms,whether different in-frame methionines are responsible for generation of possible PXRisoforms. To explore this possibility, we generated variousconstructs by site-directed mutagenesis and inverse PCR involving substitution of methionine 1, 2, 3, 4 and 6 as m1, m2, m3, m4 and m6respectively into alanine.Also combinatorial site-directed mutagenesis was performed to obtain mutant constructs for m12, m23 and m123 in the cDNA coding sequences of PXR. All the mutants were confirmed by sequencing. To examine the expression of PXR in these site-directed mutagenesis constructs at protein level, western blot analysis was performed using rabbit polyclonal antibody raised against full-length PXR (**Figure 25**).

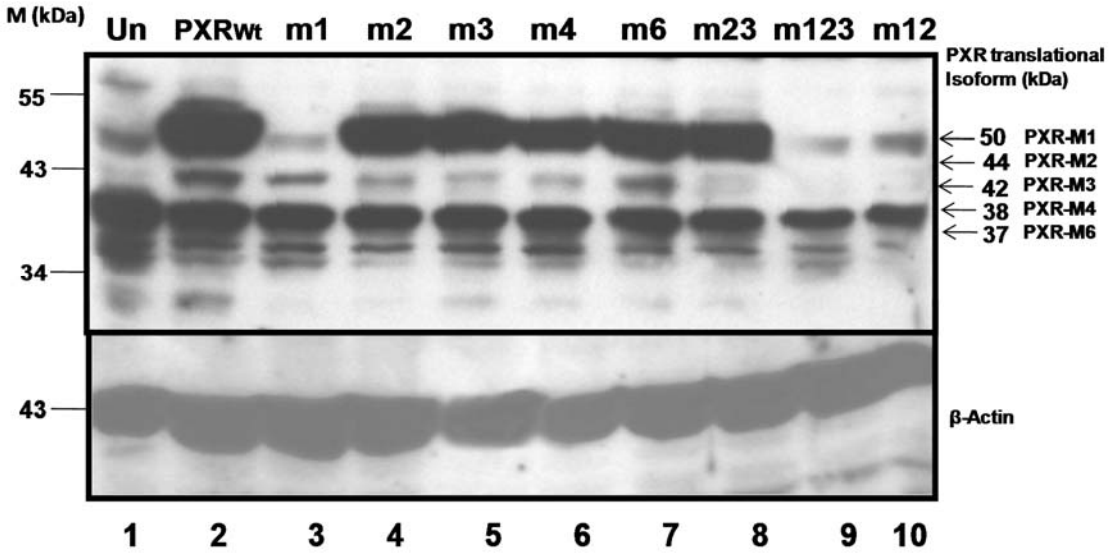


Figure 25: Site-directed mutagenesis reveals existence of PXR isoforms by western blot analysis.*Different in-frame methionine mutation were introduced into the wild type coding sequences of PXR constructs by site-directed mutagenesis and termed as m1, m2, m3, m4, m6, m23, m12 and m123. These constructs then transiently expressed in COS-1 cell line. Total cell lysates were prepared after 48 h of transfection and electrophoresed through 10% SDS-PAGE, blotted onto PVDF membrane and probed with rabbit anti-hPXR polyclonal antibodies at a 1:2,000 dilution. Lane 1, Untransfected cell extract; Lane 2, PXR transfected cell extract; Lane 3, m1 transfected cell extract; Lane 4, m2 transfected cell extract; Lane 5, m3 transfected cell*

extract; Lane 6, m4 transfected cell extract; Lane 7, m6 transfected cell extract; Lane 8, m23 transfected cells extract; Lane 9, m123 transfected cells extract; Lane 10, m12 transfected cells extract. A specific reactivity with a major protein band having molecular weight of 50 kDa was detected along with different translational isoforms of PXR except methionine 1 mutated constructs. Specific methionine mutational constructs resulted in disappearance of specific PXR band generated from corresponding methionines.

We observed that for the respective methionine mutation in different constructs resulted in disappearance of correspondingprotein bands in western blot analysis. This experiment suggests that the additional PXR bands arise by amino-terminal downstream methionines. Hence, it confirmsthat the existence of an alternative translation initiation mechanism for generation of PXR isoforms, may have distinct modulatory effect on wild type PXR functions.

Northern blot analysis to check the presence of PXR mRNA in different mutational constructs

Further we examined the absence of PXR protein or its additional bands in mutational constructs whether due to regulation at transcriptional level or at translational level by northern blot analysis (**Figure 26**). In different mutational construct were methionine 1 is mutated like in m1, m12 and m123, the main form of PXR protein gets hindered. This observation suggests that translation gets hindered due to mutation ofrespective methionines or absence of PXR mRNA resulted in disappearance of PXR protein.Toexamine the presence of PXR mRNA in all these constructs northern blot analysis was performed using PXR cDNA as a probe.Total RNA isolated from transiently transfected COS-1 cell with different mutational constructs like m1, m2, m3, m12, m23, m123 and wild type PXR. Such analysis revealed the existence of PXR transcript in all mutational constructs. Thereby implying that absence of PXR protein in m1, m12 and m123 mutational construct only due to mutation of respective methionines and not due to the absence of respective PXR mRNA.

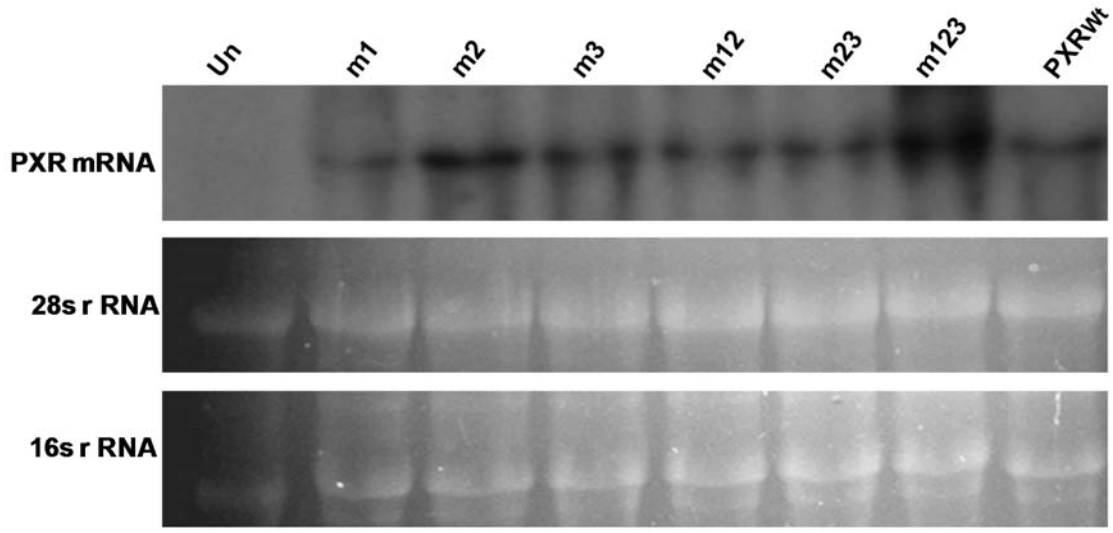


Figure 26: Northern blot analysis shows the presence of specific PXR mRNA in all in-frame methionine mutational constructs.*Different constructs that included wild type PXR expression plasmids, different in-frame methionine mutants of the PXR m1, m2, m3, m12, m23 and m123. Plasmids were independently transfected in COS-1 cells. After 36 h of expression period, the cells were harvested for total RNA isolation by Trizol method and electrophoresed through 1.2% formaldehyde agarose gel. RNA samples were then blotted onto the nylon membrane and probed with 1.3kb fragment of PXR cDNA labeled with radioactive [aP^{32}] dATP. After subsequent washings, membrane was air-dried and exposed to X-ray film. The image was developed by X-ray developer. The input RNA, 28s rRNA and 16s rRNA was shown by ethidium bromide staining.*

Transcriptional activity of different mutants

To investigate and compare the transcriptional activity of all the methionine mutant constructs of PXR, promoter-reporter based luciferase assays were performed in HepG2 cells which were transiently transfected with XREM-Luc reporter gene and different mutants of PXR expression plasmids. Following transfection, cells were incubated with and without rifampicin (**Figure 27**). Our results showed that methionine-1 mutation either alone or in combination (i.e. m1, m23, m12) totally abrogated transcriptional activity of PXR.

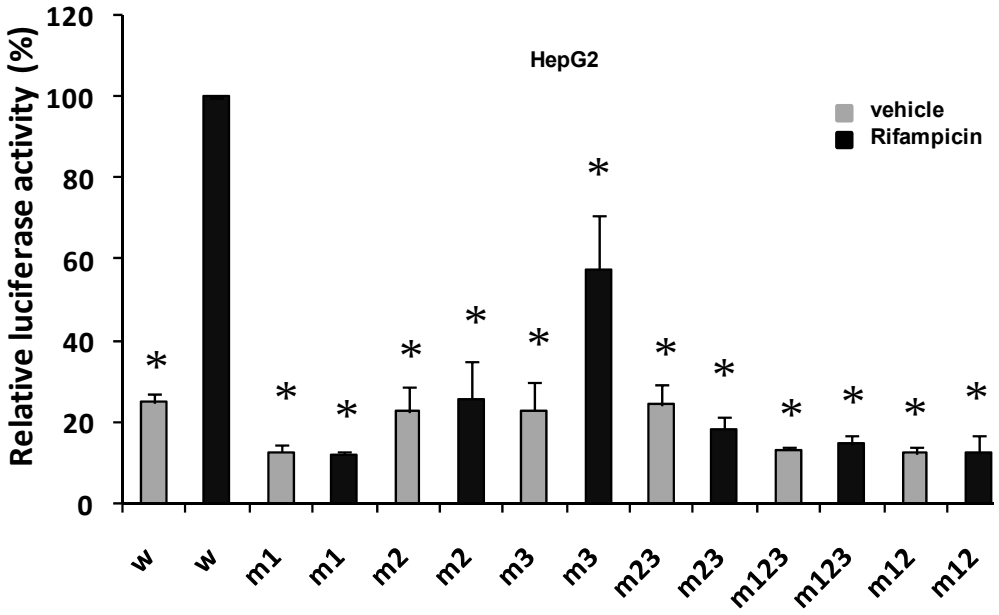


Figure 27: Abrogated transcriptional activity of different in-frame methionine mutants of PXR. *Constructs XREM-Luc, wild type PXR expression plasmids, different in-frame methionine mutants of the PXR like m1, m2, m3, m23, m123 and m12 plasmids were transfected in HepG2 cells according to scheme shown in figure. After plasmid transfection, the cells were treated with or without rifampicin for 36 h and then harvested for luciferase assay. Luciferase activity was calculated in comparison to rifampicin-induced luciferase activity, which was taken as 100%. The value represents the means ± SD of three independent experiments. Asterisks (*) signify luciferase values that differed significantly from rifampicin treated PXR transfected cells (P<0.05 in Student's T-test).*

So,theresultsindicate that the methionine-1 is an important determinant of retention of PXR transcriptional activity. Interestingly, even the retention of methionine-1 in m2 and m23 constructs, showed attenuation in PXR transcriptional activity, suggesting the importance of methionine-2 and methionine-3 in determining the PXR activity upon ligand activation. When compared, mutant constructsm2 and m23exhibited basal transcriptional activity similar to untreated wild type PXR. Among all other mutants, m3 mutant construct retained highest transcriptional activity albeitless thanthe wild type activity (approximately 60% of wild type PXR).

Molecular cloning of putative translational isoforms of PXR

For characterization of potential isoforms of PXR, we sub-cloned amino-terminal deleted PXR starting from different in-frame methionines as PXR-M2 (44kDa), PXR-M3 (42kDa), PXR-M4 (38kDa) and PXR-M6 (37kDa)in pSG5 mammalian vector at EcoR1 and BamH1 restriction sites (**Figure 28A**). In western blot analysis, we observed the expression of all these deletions constructs when transiently transfected in COS-1 cells (**Figure 28B**). In contrastto wild type PXR expression these deletion constructs showed respective expression of truncated protein bands that matched with additional bands present in wild type PXR.Result confirms that these deletion constructs may be putative translational isoforms derived *in vivo*from different in-frame methionine of PXR gene.

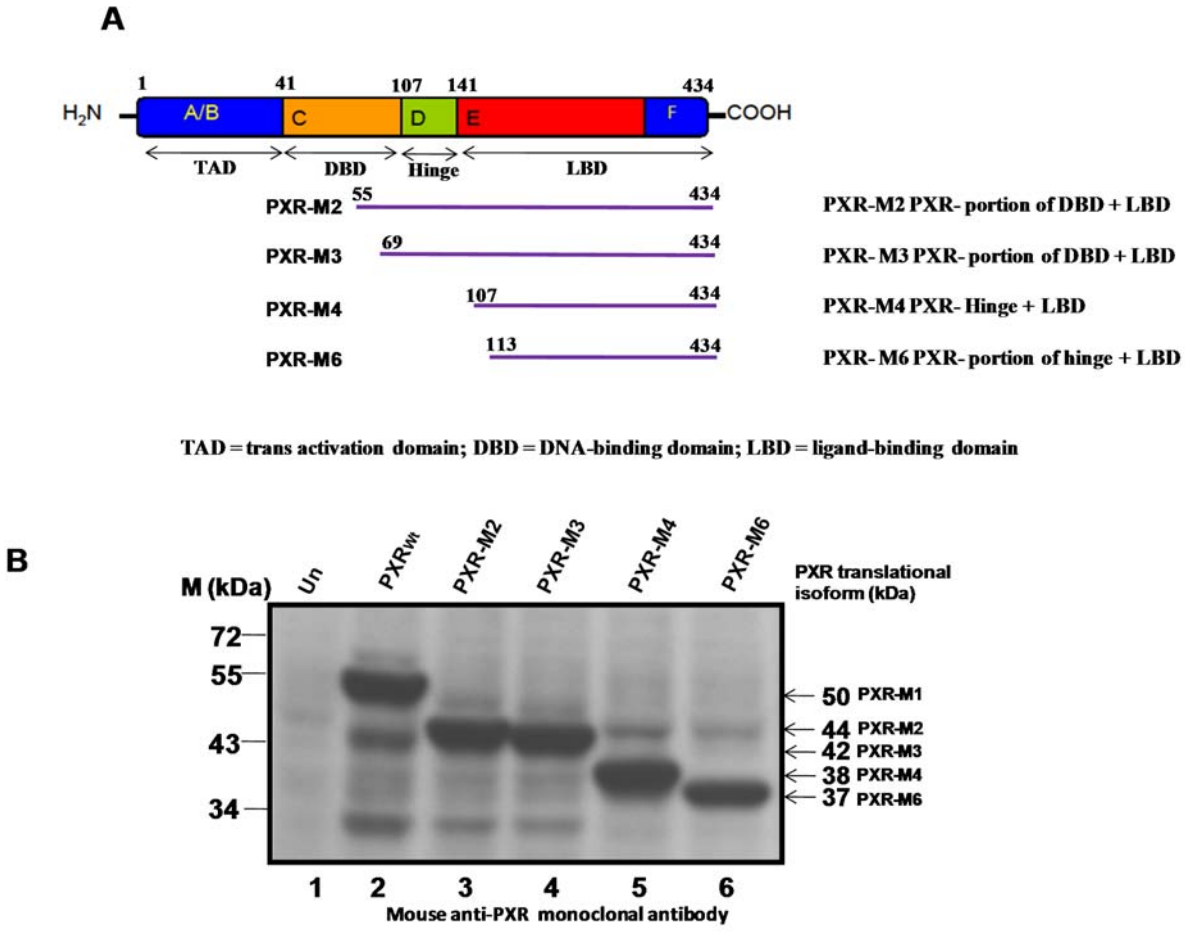


Figure 28: Cloning and expression of potential translational isoforms of PXR starting with different amino-terminal methionines.*A.**Different** domain of PXR comprising, TAD - Trans Activation Domain, DBD - DNA Binding Domain, LBD - Ligand Binding Domain. i) PXR-M2 = portion of DBD +*

*LBD (55-434 a.a.), ii) PXR-M3 = portion of DBD + LBD (69-434 a.a.), iii) PXR-M4 = Hinge + LBD (107-434 a.a.) and iv) PXR-M6 - portion of hinge + LBD (113-434 a.a.) are shown.**B.** Wild type PXR and translational isoforms of PXR constructs as PXR-M2, PXR-M3, PXR-M4 and PXR-M6, were transiently expressed in COS-1 cells. Cell lysate were prepared after 48 h of transfection and electrophoresed through 10% SDS-PAGE, blotted onto PVDF membrane and probed with mouse-anti-hPXR monoclonal antibodysupernatant as mentioned in 'Materials & Methods'. Lane 1, Untransfected cell extract; Lane 2, wild type PXR transfected cell extract; Lane 3, PXR-M2 transfected cell extract; Lane 4, PXR-M3 transfected cell extract; Lane 5, PXR-M4 transfected cell extract; and Lane 6, PXR-M6 transfected cell extract. A specific reactivity towards a major protein bandscorresponding to different isoforms weredetected as PXR-M2, PXR-M3, PXR-M4 and PXR-M6 molecular weight corresponding to 44 kDa, 42 kDa, 38 kDa and 37 kDa respectively.*

Sub-cellular localization of potential translational isoforms of PXR

Sub-cellular localization and dynamic movement of transcription factors have been shown to be one of the major means of regulating their transcriptional activity. To investigate the sub-cellular localization of potential translational isoforms of PXR, different isoforms were cloned intoGFP vector at EcoR1 and BamH1 restriction sites.We determined the GFP expression along with the expression of different translation isoforms of PXR by western blot analysis (**Figure 29A**). We observed the sub-cellular localization of wild type PXR, PXR-M2, PXR-M3, PXR-M4 and PXR-M6 by live cell imaging. We observed that wild type PXR, PXR-M2 and PXR-M3 localized predominantly in the nucleus whereas PXR-M4 and PXR-M6 isoforms showing both cytoplasmic and nuclear localization.Sub-cellular localization pattern of the PXR isoforms remainedunaltered in both rifampicin treated and untreated conditions. When compared to the wild type PXR distribution pattern,PXR-M2 and PXR-M3 showedatypical receptor distributionpattern in nucleoplasm (**Figure 29B**).When analysed 80-90% of cells exhibited this atypical pattern with PXR-M2 and PXR-M3. On the contrary,PXR-M4 and PXR-M6 did not show sucha pattern of receptor distribution (**Figure 29B**).Interestingly, PXR-M4 and PXR-M6 isoforms showed uniformly nuclear and cytoplasmic receptordistribution pattern in 70-80% of cells representing N=C.Subsequently, using the same cell extract and.

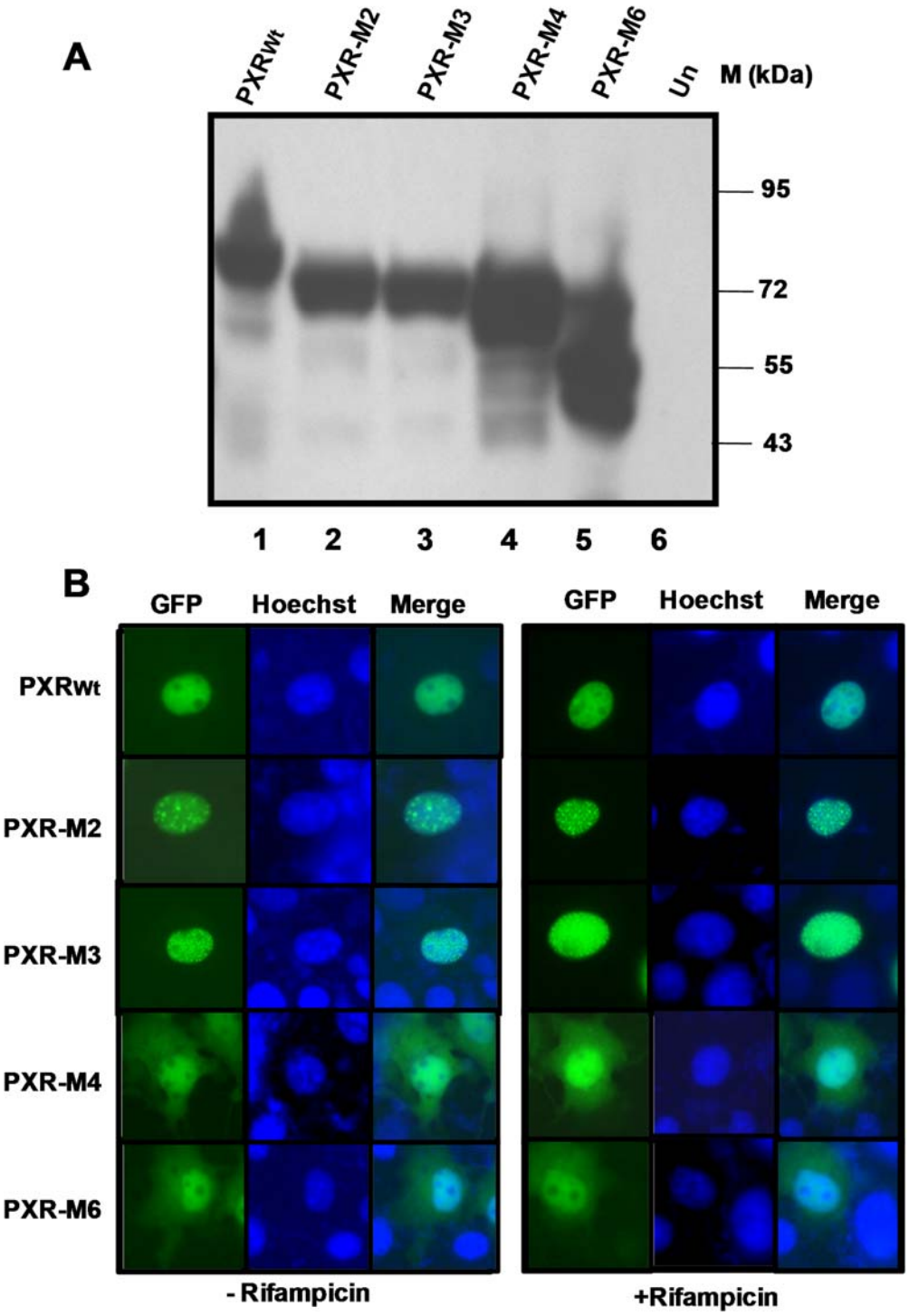


Figure 29: Expression and altered sub-cellular localization of GFP-tagged potential translational isoforms of PXR.*A.* *Western blot analysis of GFP-tagged potential translational isoforms of PXR.GFP-tagged isoforms of PXR were transiently transfected in COS-1 cells. Cell lysate were prepared after 48 h of transfection and electrophoresed through 10% SDS-PAGE, blotted onto PVDF membrane and probed with supernatant of mouse PXR-L12*

monoclonal antibody as mentioned in 'Materials & Methods'. Lane 1, wild type PXR transfected cell extract; Lane 2, PXR-M2 transfected cell extract; Lane 3, PXR-M3 transfected cell extract; Lane 4, PXR-M4 transfected cell extract; Lane 5, PXR-M6 transfected cell extract and Lane 6, Untransfected cell extract (Un). ***B.*** *COS-1 cells were transiently transfected and expressed with different GFP-tagged PXR isoforms like PXR-M2, PXR-M3, PXR-M4, PXR-M6 and wild type PXR for immunofluorescence study. Cell images were recorded using a fluorescence microscope equipped with water–immersion objectives. Live cell images illustrate the sub-cellular localization of potential isoforms of PXR. The left panel shows the distribution pattern of receptor without drug treatment. The right panel shows the distribution pattern of receptor rifampicin (10μM) treatment. The GFP fluorescence visualizes the distribution pattern of receptor. Hoechst fluorescence was done for visualizing the corresponding nuclei and the merged images for visualizing the corresponding nuclei with different isoforms of PXR.*

Overall, this part of the study revealed differential sub-cellular distribution pattern of translational isoforms of PXR. It is possible that differential localization or expression of PXR isoforms in different patho-physiological states may exhibit altered functional responses.

Abrogation of transcriptional activity of the putative translational isoforms of PXR

To understand the functional role of the potential translational isoforms of PXR, we attempted to explore whether these could be transcriptionally activated by PXR ligand or not. For this purpose, promoter-reporter based luciferase assays were performed in HepG2 cells. Cells were transiently co-transfected with XREM-Luc reporter gene and wild type PXR expression plasmids or independently with translational isoforms like PXR-M2, PXR-M3, PXR-M4 and PXR-M6. Following transfection, cells were incubated with either rifampicin or vehicle (DMSO). As a result, rifampicin significantly enhanced wild type PXR transcriptional activity while all its translational isoforms showed abrogated transcriptional activity(**Figure 30**).

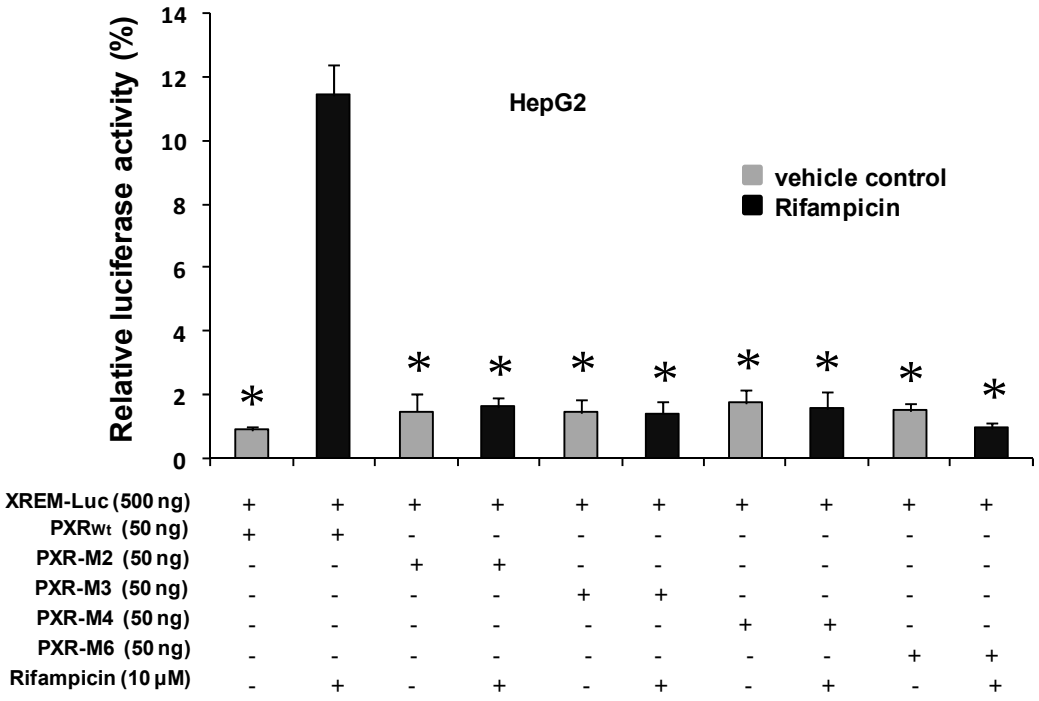


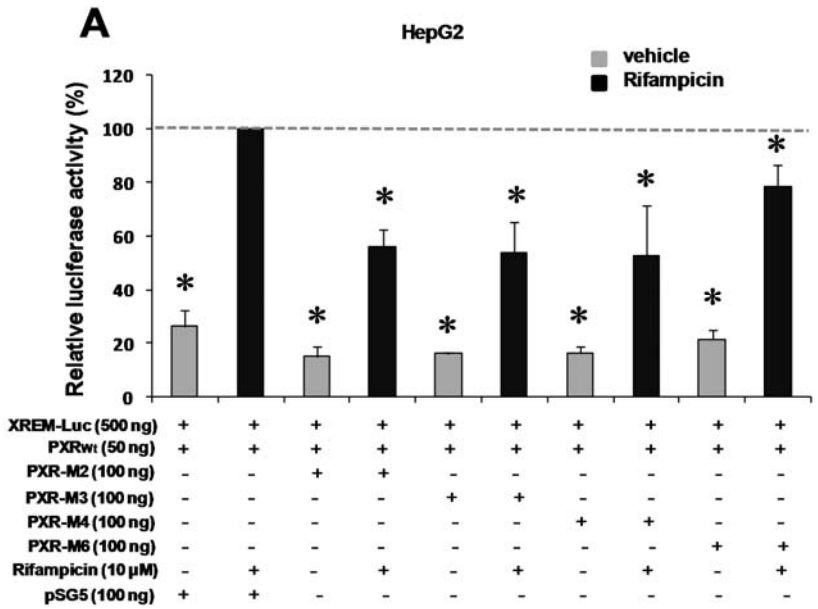
Figure 30: Loss of transactivation potential of PXR translational isoforms.*HepG2 cells were transfected with constructs XREM-Luc, wild type PXR expression plasmid and different PXR translational isoforms (PXR-M2, PXR-M3, PXR-M4 and PXR-M6 plasmids) according to the scheme shown in figure. After transfection, the cells were treated with rifampicin for 36 h and then harvested for luciferase assay. Relative luciferase activity was calculated in comparison to rifampicin-induced wild type PXR luciferase activity, which was taken as 100%. From the graph it is appeared thatdifferent translational isoforms of PXR have abrogated transactivation potential. The value represents the means ± SD of three independent experiments. Asterisks (*) signify luciferase values that differed significantly from rifampicin treated PXR transfected cells (P<0.05 in Student's T-test).*

The results imply that the loss of transcriptional function of these translational isoforms may be due to the loss of 'Activation Function 1 (AF1)'domain in all constructs. In general, from physiological view no isoform(s) remain in isolation, rather they always co-express in combination with its other forms including the wild type protein.So, we further examined the combinatorial effect of these constructs in presence of wild type PXR protein to examine if their presence influences the wild type PXR functions.

Transcriptional activity of wild type PXR is impeded by its minor translational isoforms

To investigate the possibility of influence of PXR isoforms on transcriptional activity of wild type PXR, co-transfection experiments were performed in HepG2 cells. We observed that these isoforms down-regulate the wild type induced PXR transcriptional activity and thereby affect the basic function of the wild type receptor (**Figure 31A**).Taking this into account, it can be speculated that the level and combination of different PXR isoforms being expressed in different cell/tissue types may differ and play combinatorial roles in executing and regulating cellular function of wild type PXR via isoform interaction.

To examine the effect of different translational isoforms on wild type PXR expression, western blot analysis of PXR and its potential isoforms was done with transient co-transfection in COS-1 cells (**Figure 31B**). This experiment confirmed that in presence of different isoforms, the expression of wild type PXR protein remains unaffected. Therefore, it may be inferred that the reduced transcriptional activity of wild type PXR may be attributed to different isoforms which may be competitively binding to PXR co-factors, DNA response elements or its hetero-dimerizing partner RXR.



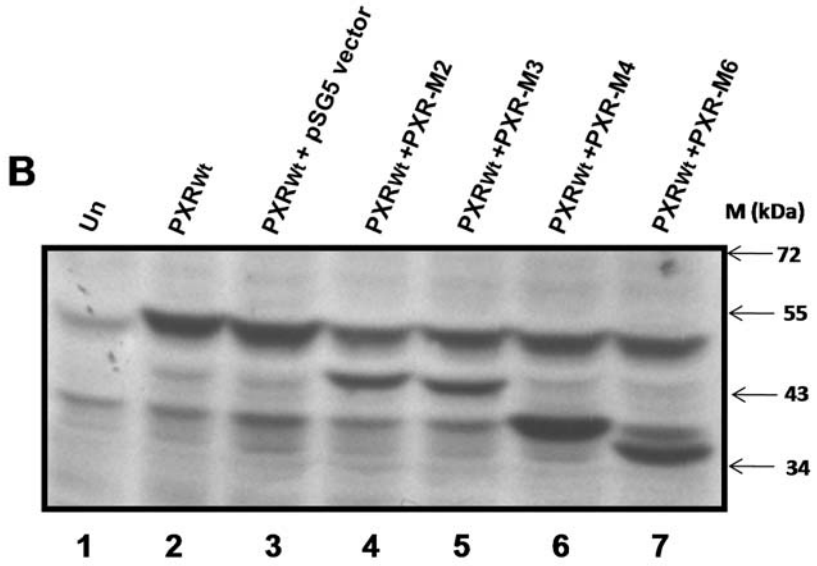


Figure 31: Transcriptional activity of wild type PXR is impeded by co-expression of its minor translational isoforms.*A.* *Different translational isoforms of PXR repress wild type PXR-mediated transactivation. In HepG2 cells different constructs XREM-Luc, wild type PXR expression plasmid along with either of the PXR translational isoforms PXR-M2, PXR-M3, PXR-M4 and PXR-M6 according to the scheme shown in the figure. Carrier plasmid was used to keep the total amount of transfected plasmid constant in each well. After plasmid transfection, the cells were treated with rifampicin for 36 h and then harvested for luciferase assay. Relative % activity was calculated in comparison to rifampicin-induced wild type PXR luciferase activity, which is taken as 100. The value represents the means ± SD of three independent experiments. Asterisks (*) signify luciferase values that differed significantly from rifampicin treated PXR transfected cells (P<0.05 in Student's T-test).* ***B.*** *COS-1 cells were co-transfected with PXR and its translational isoforms as indicated in figure.Cell extracts were prepared after 48 h of transfection and electrophoresed through 10% SDS-PAGE, blotted on to PVDF membrane and probed with rabbit anti-hPXR polyclonal antibodies at a 1:2,000 dilution. Lane 1, Untransfected cells extract; Lane 2, Cells transfected with wild type PXR; Lane 3, Cells transfected with wild type PXR with pSG5 vector as carrier plasmid; Lane 4, Cells transfected with wild type PXR and PXR-M2 isoform; Lane 5, Cells transfected with wild type PXR and PXR-M3 isoform; Lane 6, Cells transfected with wild type PXR and PXR-M4 isoform; Lane 7, Cells transfected with wild type PXR and PXR-M6 isoform. A specific reactivity with a major protein band having molecular weight of 50 kDa was detected along with different potential PXR isoforms. It is evident that co-transfection with PXR isoforms had no significant effect on the expression of wild type PXR.*

Differential co-factor recruitment by translational isoforms

In the present study, we demonstrated that the transcriptional activity of wild type PXRis reduced in presence of differenttranslational isoforms, due tocompetition for co-regulatorrecruitment like SRC-1 as assessed by mammalian two-hybrid assay. The **Figure 32A** shows pictorial representation of working strategy for mammalian two-hybrid assay.It has been recently reported that PXR interacts with SRC-1 and co-repressor SMRTin a ligand-independent manner (Navaratnarajah et al, 2012).In accordance with this finding, we also did not observe the effect of rifampicin on PXR interaction towards SRC-1. However, unlike previous reports for other nuclear receptors which showed the ligand-dependent interaction with co-factors (Kishimoto et al, 2006), we observed that addition of a ligand does not have much effect (less than two- fold) for the recruitment of SRC-1 with full-length PXR.Interestingly, when both NTD and DBD were deleted, theligand-dependent interaction ofPXR-M4 and PXR-M6 isoforms with SRC-1 increased by 2.5-fold while PXR-M2 and PXR-M3 isoforms did not show any interaction with SRC-1 in both rifampicin treated or untreated conditions (**Figure 32B**). Although PXR-M2 and PXR-M3 isoforms have intact LBD and partial DBD domain eventhey do notinteractwith SRC-1. This observation suggested that DBD of PXR may exhibit negative impact on its interaction with SRC-1. However, in case of wild type PXR the presence of full-length DBD didnot totally compromise its affinity towards SRC-1 in contrast to PXR-M2 and PXR-M3 isoforms. It possibly suggests the involvement of amino/carboxyl-terminalinteractionof PXR in SRC-1 recruitment.In case of PXR-M2 and PXR-M3 isoforms the presence of partial DBD along with LBD totally abrogatesits interaction with SRC-1.

Taken together, these data suggest that PXR-M4 and PXR-M6 isoforms recruit SRC-1 preferentially over wild type PXR that results in depletion of 'SRC-1 pool' which in turn results into a crisis for this co-activator recruitment by the wild type PXR activation. This also implies that the differential expression of PXR isoforms in cancerous cells may compete similarly for co-factor recruitment resulting into a distinct cellular out-put.

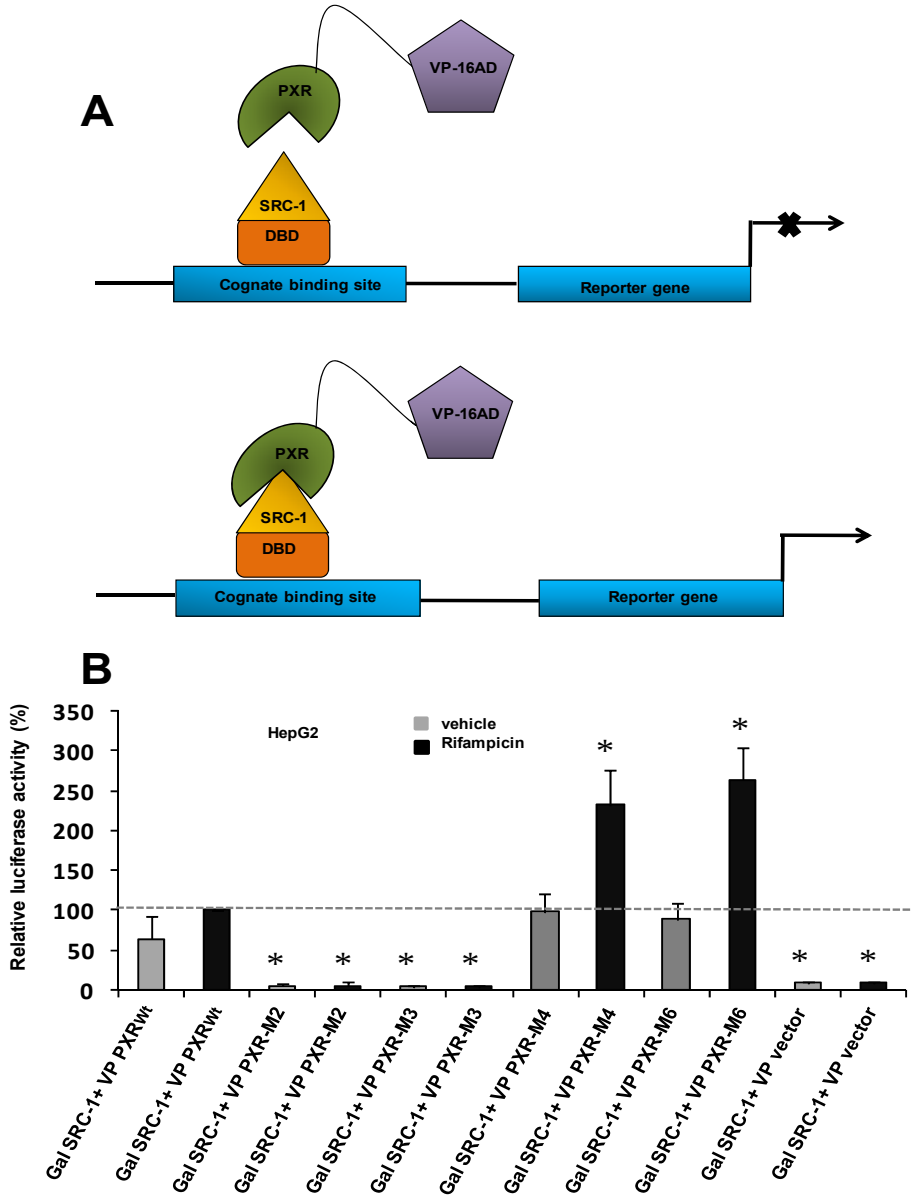


Figure 32: Differential recruitment of SRC-1 by different translational isoforms of PXR.*A.* *Schematic representation of the working strategy of mammalian two-hybrid system from which data are retrieved and shown in Figure B.* ***B.****Mammalian two-hybrid assay was executed in HepG2 cells. Full-length wild type PXR and different PXR isoforms PXR-M2, PXR-M3, PXR-M4 and PXR-M6 fused to VP16 (VP16-PXR*^{WT}*, PXR-M2, PXR-M3, PXR-M4 and PXR-M6) and co-activator SRC-1 fused to Gal4-DNA binding domain (Gal4-SRC-1) were co-transfected in HepG2 cells along with Gal4 promoter-reporter construct FR-Luc. After transfection, the cells were treated with rifampicin for 36 h and harvested for luciferase assay. Relative % activity was calculated in comparison to Gal4-SRC-1 and VP16-PXR*^{WT}*with rifampicin treatment, which was taken as 100. All values represent mean ± SD of three separate experiments. Asterisks (*) signify luciferase values that differed significantly from rifampicin treated VP-PXR*^{WT} *transfected cells (P<0.05 in Student's T-test).*

Furthermore, we found that the ability of SRC-1 to interact with PXR-M4 and PXR-M6 isoforms ismore profound as compared to wild type PXR *in vivo*. Removal of the NTD domain and partial DBD significantly diminished interaction of SRC-1 with PXR-M2 and PXR-M3 isoforms while removal of both the NTD and DBD domains restores maximal interaction with SRC-1.

Differential DNA binding activity of PXR translational isoforms

In addition to predominantly nuclear residency during interphase, PXR was observed to be associated with condensed chromosomes during all stages of mitosis that may have physiological implication in its function as a transcription factor (Saradhi et al, 2005b). The PXR/RXRα hetero-dimer pair is capable of binding a direct repeat with a 3nt spacer (DR3), DR4 and everted repeat with 6nt spacer (ER6) response elements in the promoters of a variety of xenobiotic genes and number of nucleotide spacers determines the specificity of binding for heterodimer partners. This is known from previous study from our laboratory that, PXR binds to condensed chromatin during all the stages of mitosis (Saradhi et al, 2005b). So, it was be interesting to assess the mitotic chromatin binding profile of all translational isoforms of PXR. We observed that PXR-M2 and PXR-M3 bound with lesser extent to mitotic chromatin but PXR-M4 and PXR-M6 remain unbound to mitotic chromatin when compared to chromatin association of wild type PXR (**Figure 33**).

In brief, the observation implies that PXR-M2 and PXR-M3 may competitively bind to response element of PXR that in turn reduce the transcriptional activity of wild type PXR.

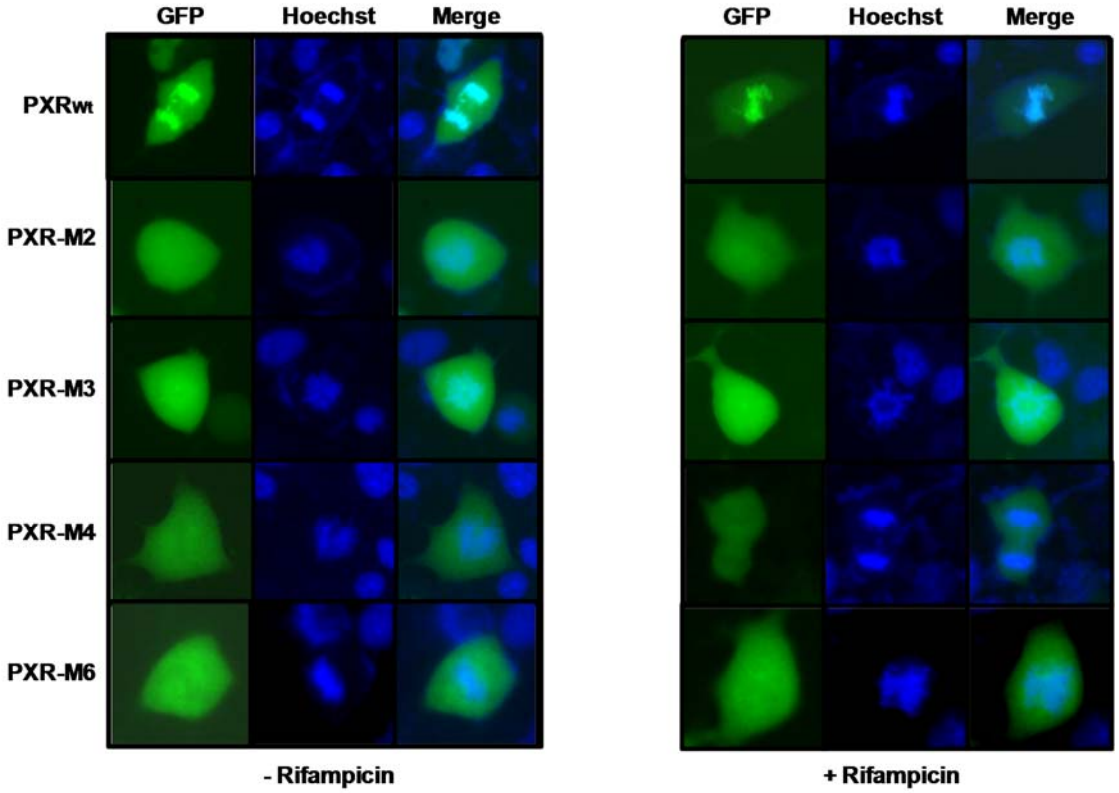


Figure 33: Differential binding of translational isoforms of PXR to mitotic chromatin. *COS-1 cells were transiently transfected with GFP-tagged wild type PXRWT, PXR-M2, PXR-M3, PXR-M4 and PXR-M6 plasmids. After 20 h of expression, mitotic stages of different cells were monitored and recorded by fluorescence microscopy.PXRWT, PXR-M2 and PXR-M3 associate with the condensed chromatin during mitosis while PXR-M4 and PXR-M6 do not bind to mitotic chromatin in presence or absence of rifampicin. In all the cases, cells were incubated with Hoechst to visualize the corresponding nuclei or mitotic chromatin.*

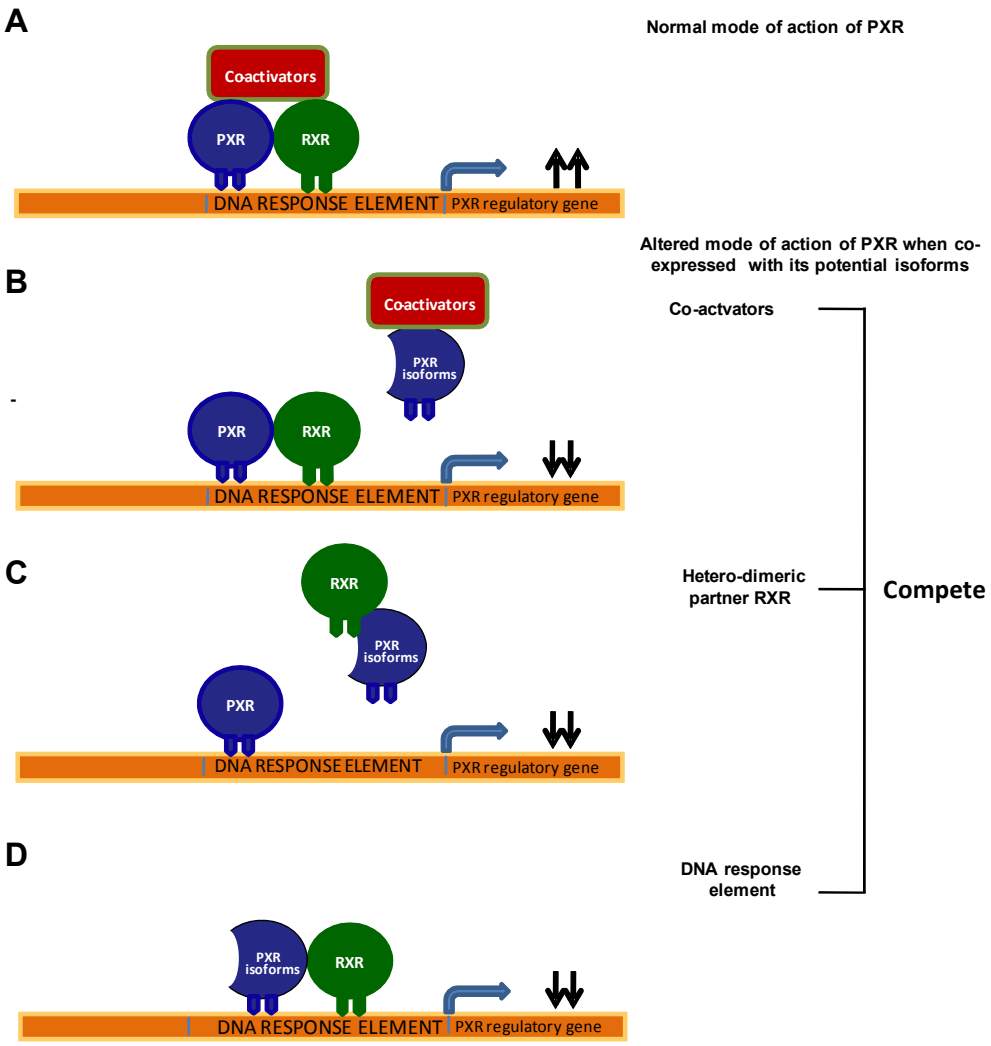


Figure 34: Depiction of inhibitory action of translational isoforms on wild type PXR. *When activated wild typePXR regulates the cellular process by binding to the promoters of its responsive genes thereby modulating their functions (**A**). Translational isoforms of PXR contributes to ligand-mediated repression of wild type PXR activity. Competition with PXR co-activators (**B**), hetero-dimeric partner RXR (**C**) and DNA response element binding (**D**),may contributes to repression of wild type PXR activity. Based on the present study we suggest a novel mode of repression of PXR activity by generation of itstranslational isoforms.*

DISCUSSION

Primary function of PXR is in xenobiotic metabolism and clearance from the body. However, mechanisms by which PXR exertsadditional effects like cancer progression, cell-cycle regulation, bloodbrain barrier functionand

several more are not well understood (Zhou et al, 2009; Qiao et al, 2013; Koutsounas et al, 2013). It has been reported that chemotherapy in cancerous diseases causes up-regulation of various drug transporters resulting into chemoresistence in an individual. In an endeavor to reverse the failure of such therapies, it is important to understand how PXR works.

PXR functions with respect to its potential isoforms are not well explored till date. We examined the existence of different isoforms of PXR and their cellular importance. While the splice isoforms of PXR are reported, in this study we examined the existence of additional translational isoforms of PXR. The PXR protein sequence and amino-terminal methionines alignment with different species appears to be conserved across the species.Therefore, we speculated that different in-frame methionine may contribute to differential expression of PXR protein in broader range of cells and tissues. Also, this differential expression of isoforms may contribute to differential action and may be important for the homeostasis of endobiotics and several other functionsattributed to PXR. In the present study we confirmed the existence of additional forms of PXR generated by alternative translational initiation mechanism. To arrive at this conclusion, initially we applied *in vivo* techniques. In our preliminary study when COS-1 cells were transiently transfected with PXR, several bands appeared along with the well-characterized 50kDa band of PXR protein. Endogenous co-expression of these bands was also apparent in different cancerous cell lines and in a liver cell line stably transfected with PXR. From the observation we speculated theseto beeither a consequence of proteasomal degradation or additional forms of PXR. To rule out the possibility of these being proteasomal degradation products, we treated the transiently expressed PXR with proteasomal inhibitors MG132 and LLnL.We observed that these bands did not disappear by treatment with proteasomal inhibitors. So, it suggested thatsome additionalpotential forms of PXRmay co-exist along with 50 kDa main form of PXR.

Protein synthesis is believed to be initiated with the amino acid methionine which lies in optimal Kozak context. However, downstream

methionines also get alternatively translated by internal ribosome entry site-mediated translation, leaky scanning or ribosomal shunting mechanisms. So, the presence of these isoforms wasconfirmed by site-directed mutagenesis at different downstream methioninesand analyzing the protein expression profile of these mutated constructs by western blot analysis. Furthermore,promoter-reporter based assays were performed to determine the functional role of these mutated constructs. The results from these experiments suggested that methionine-1 is most important determinant of retention of PXR transcriptional activity. Another outcome from these experimentssuggests that the retention of methionine-1 even in m2 and m23 mutatedreceptor resulted in attenuation of PXR transcriptional activity. So, from this perspective methionine-2 and -3 are also important determinant of PXR activity. Based on the data retrieved from different cancerous cell lines,we predictedthat a differential expression of PXR isoforms may execute differential cellular response under varied physiological situations. It is tempting to speculate that the differential expression of PXR isoforms may require cis/trans regulatory factors which determinetheirexpression levels in different metabolic disorders and cancers.Further,to characterize the functionality of these isoforms we made expression constructs comprising of different in-frame methionine derived from wild typePXR. Expected molecular weight of these potential isoforms of PXR when alternatively translated from different in-frame methionine was calculated asPXR-M1 =49.77, PXR-M2=43.72, PXR-M3=42.23, PXR-M4= 37.72 and PXR-M6=36.96. This observation was strengthened in our western blot analyses by using different polyclonal and monoclonal antibodies raised against the full-length PXR.Translational isoforms of PXR lack theamino-terminal domain which includes activation function domain 1 (AF-1).In addition to this PXR-M4 and PXR-M6 isoforms are also devoid of DNA binding domain and therefore were observed to be transcriptionally non-functional when compared to wild type PXR.We also examined the sub-cellular localization of these isoforms by live cell imaging and observed that PXR-M2 and PXR-M3 localized in the nucleus with atypical punctuate pattern whereas PXR-M4

and PXR-M6 isoforms exhibits both cytoplasmic and nuclear localization without any punctuate pattern.

Interestingly, a few reports suggest NRs (like GRβ, v-erbA) have naturally occurring dominant negative isoforms that impedes the activity of the wild type receptor when the two are co-expressed in the same cell (Damm et al, 1989; Yudt et al, 2003).It has been recently reported thatprogression of mouse skin carcinogenesis is associated with increased ERα levels and is repressed by a dominant negative form of ERα (Logotheti et al, 2012).Therefore, functional significance of PXR isoforms was demonstrated by the co-expression of these isoforms with the wild type PXR protein. Interestingly, the co-expression of PXRisoforms reduced the transcriptional activity of wild type PXR protein. So,from the observations it was obvious that these isoforms exhibita dominant-negative effect on the wild type PXR function. However, the most pertinent query at this point was to reveal how PXR isoforms repress PXR-mediated gene transcription? Depending upon the tissue or cell type, these isoforms may repress transcription i) directly, ii) indirectly via cofactor recruitment or displacement, or iii) via conformational DNA changes.Elucidation of the exact mechanism applying to these isoforms requires further detailed studies.

In an attempt to explore the molecular mechanisms involved in PXR isoforms-mediated repression of wild type PXR transcriptional activity, we enquired whether PXR transcriptional repression reflects a direct interaction between co-regulators and PXR isoforms. Our observations suggested that PXR isoforms PXR-M4 and PXR-M6 competitive recruit or sequester co-activator SRC-1 suggesting a novel mechanism of action of PXR isoform formodulating PXR transcriptional activity. The PXR isoform-mediated transcriptional repression may thus help in auto-regulation of PXR functionality in different cancerous cell lines. Although this work primarily aimed to focus on detection and identification of PXR isoforms some of our observations exhibit a novel mode of regulatory action of PXR isoforms-mediated functions, when co-expressed concomitantly with wild type PXR. Subsequent experiments designed to resolve such inhibitory effect of PXR

isoforms on wild type PXR activity may have tissue specific function involving trans- and cis-acting factors. The inhibitory effect of these isoforms may have been due to the competitive binding to PXR-responsive elements. It is reasonable to interpret, that these isoforms may have promoter binding affinity similar to wild type which will then impart reduced PXR-mediated gene transcription. A previous study from our laboratory demonstrated that PXR associates with condensed chromatin during all mitotic stages (Saradhi et al, 2005b; Kumar et al, 2012).We studied the mitotic binding of all these translational isoforms by live cell imaging. We observed mitotic binding property of PXR-M2 and PXR-M3 isoforms whereas PXR-M4 and PXR-M6 isoforms do not show any binding with mitotic chromatin. It was evident that the differential binding of different PXR isoforms to mitotic chromatin may result in distinct cellular functions.

Accumulating evidences from *in vivo* studies like site-directed mutagenesis and endogenous expression profile in different cancerous cell lines strongly suggestedthe presence of novel translational isoforms of PXR.Taken together, our results demonstrate thatPXR isoforms do not contribute to transcription function directly rather repress wild type PXR activity.It is logical to hypothesize that this function may be exerted via competition for differential co-factor recruitment, competition for promoter binding or competition for hetero-dimeric partner RXR. However, unraveling the detailed mechanism of action of these isoforms awaits further work. In this perspective, the present study suggests a hitherto an unidentified mode of action of PXR isoforms through interaction with wild type PXR, co-activator SRC-1 and differential chromatin binding as described in a hypothetical model presented in **Figure 34**. In brief, the highlight of the present study demonstrates theexistence of novel translational isoforms of PXR whichhave been identifiedto function as dominant negative forms for wild type PXR transcriptional activity.

CHAPTER III

Post-translational modifications and functional analysis of PXR

INTRODUCTION

Post-translational modifications (PTMs) and their inter-relationships play an important role in altering protein behavior and functional output of a protein. In addition to different exogenous and endogenous factors, PXR activity is also being reported to be regulated by different PTMs that further expand the receptor activity. Recent reports suggests, PXR undergo different PTMs like phosphorylation, SUMOylation, acetylation and ubiquitination that in turn modulate PXR activity and thereby execute differential and distinct biological roles (Pondugula et al, 2009a; 2009b; Lichti-Kaiser et al, 2009a; 2009b; Hu et al, 2010; Masuyama et al, 2000; Staudinger et al, 2011; Biswas et al, 2011).

PTM of a protein by the small ubiquitin-like modifier (SUMO) is increasingly recognized as an important regulatory mechanism. SUMOylation is a dynamic and reversible process regulating gene expression by altering transcription factor stability, protein–protein interactions to favor recruitment of co-regulators and sub-cellular localization of target proteins (Hay, 2005; Heun, 2007). SUMOylation involves series of enzymes which is analogous to ubiquitination consisting of an E1-activating enzyme, an E2-conjugating enzyme (UBCh9), three different groups of E3-ligases (PIAS) to conjugate and several SUMO proteases (SENPs) to deconjugate SUMO from target proteins (Hay, 2007; Zhao, 2007). Whereas ubiquitination primarily mediates protein degradation, SUMOylation is emerging as a significant regulatory mechanism which affects diverse cellular processes such as transcriptional activity, protein-protein interaction, chromatin structure, DNA binding activity, signal transduction, apoptosis, autophagy, cell-cycle control, sub-cellular and intranuclear localization of its target protein (Melchior, 2000; Muller et al, 2001; Seeler & Dejean, 2003). Higher eukaryotes have four SUMO isoforms, SUMO-1, SUMO-2, SUMO-3 and SUMO-4, which are encoded by separate genes (Melchior, 2000; Holmstrom et al, 2003, Guo et al, 2004). Of these, SUMO-1, SUMO-2 and SUMO-3 are ubiquitously expressed, whereas SUMO-4 is primarily expressed in the kidney, lymph node and spleen (Guo et al, 2004). The mature forms of

SUMO-2 and SUMO-3 have 90% sequence homology but only 20% sequence homology with SUMO-1. SUMO-1 covalently modifies a number of nuclear receptor (NR) proteins including GR (Tian et al, 2002; Le et al, 2002; Tirard et al, 2007), AR (Poukka et al, 2000; Rytinki et al, 20011), PR (Abdel et al, 2002; Chauchereau et al, 2003; Abdel-Hafiz and Horwitz, 2012), ER (Sentis et al, 2005; Picard et al, 2012), MR (Pascual–Le Tallec et al, 2005) etc. In this context, it is clear that multiple PTM events may affect NRs that modulate protein-protein interactions, sub-cellular localization and stability for the fine-tuning of gene transcription events. Therefore, a thorough investigation of these processes will be essential for an in-depth understanding of the cellular implications of these modifications.

A recent study has shown that PXR gets SUMOylated to repress the inflammatory response (Hu et al, 2010). *In vitro* study suggested that PXR undergoes SUMOylation by all the SUMO isoforms but an *in vivo* study confirms that PXR undergo SUMOylation only by SUMO-3 isoform not by SUMO-1 and SUMO-2 isoform (Hu et al, 2010). PXR SUMOylation has been under growing field of investigation and further work is warranted to give insight into this PTM. Recognition of large number of SUMO substrates raises questions about how SUMOylation alters the functional diversity of protein, how substrate-dependent activities of SUMO are determined and how SUMOylation gets regulated. A consensus SUMO-acceptor site, consisting of the sequence ΨKXE, where Ψ is a large hydrophobic amino acid and K (lysine) is the site of SUMO conjugation, has been identified, although nonconsensus SUMO-acceptor sites are also reported in some cases (Rodriguez et al, 2001). Recently, bioinformatics analyses identified a subset of SUMO consensus sites called the PDSM (Phosphorylation Dependent SUMOylation Motif), which associates a classical SUMO consensus site with an adjacent proline-directed phosphorylation site (ΨKXEXXSP) and NDSM (Negative charge amino acid Dependent SUMOylation Motif) (Hietakangas et al, 2006; Yang et al, 2003; Yang et al, 2006). The significance of PDSM is a proline-directed phosphorylation site that cannot be SUMOylated unless phosphorylated (Hietakangas et al, 2006)

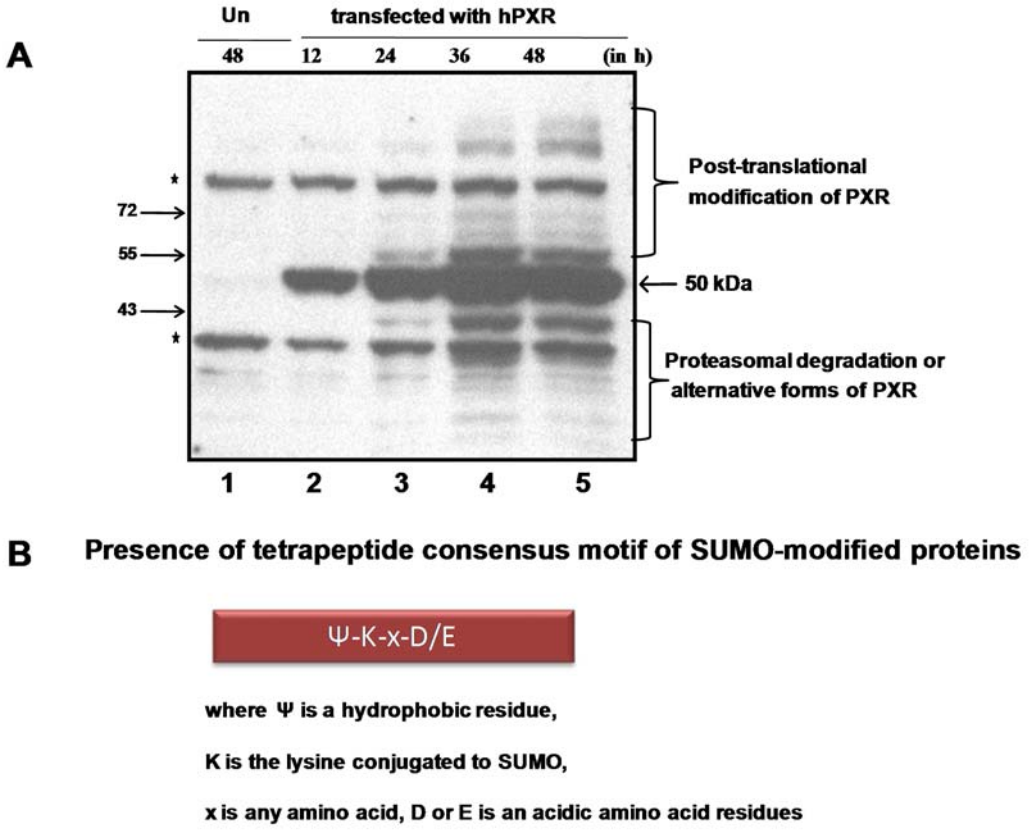
whereas acidic amino acids present in NDSM are essential for SUMO conjugation and bind to a positively charged patch on E2- conjugation enzyme UBCh9 (Yang et al, 2006). So, next question arises whether presence of these motifs regulate the PXR SUMOylation which in turn affect its function. Furthermore, by using established bioinformatic tool 'SUMOplot' (http://www.abgent.com/doc/sumoplot) four SUMOylation sites in human PXR are predicted to be present, with one high probability SUMOylation motif in hinge region and three low probability SUMOylation motifs in ligand binding domain. In the present study, we show for the first time that PXR is modified under *in vivo* conditions by the covalent attachment of SUMO-1 in ligand-independent manner, a PTM with regulatory functions for an increasing number of proteins. In the present study, following *in silico* and *in vivo* analyses, we attempted to explore the importance of putative SUMOylation sites on PXR mediated transcriptional output. Overall our data appear to suggest that transcriptional activation by SUMO-1 conjugation may play an important role in controlling the ability of PXR to interact with distinct transcriptional co-factors and finally regulate transcriptional output. These findings identify PXR SUMOylation, a novel mechanism for modulating PXR function.

RESULTS

Characterization of post-translational modification of human PXR protein

To study the PTM of PXR, we first transiently transfected the COS-1 cells with human PXR plasmid and the generation of the expressed protein was detected by western blotting. The results with transfected cells showed multiple bands along with the well-characterized 50 kDa band of PXR protein (**Figure 35A**). The presence of additional upper bands suggested the existence of different PTMs while lower bands appearing below 50 kDa could be considered as additional forms of PXR or proteasomal degradation products. So, this experiment notifies the possible existence of modified forms of PXR. Since much information is focused on PXR phosphorylation,

ubiquitination and acetylation but so far no definitive *in vivo* evidence for SUMOylation has been reported. **Figure 35B** show the presence of tetrapeptide consensus motif of SUMO-1 modified proteins. To study the SUMOylation PTM on PXR a bioinformatic tool 'SUMOplot™ Prediction' predicted four SUMOylation sites in human PXR which are restricted to hinge region, $_{107}$MKKE$_{110}$ (motif with high probability) and to ligand binding region $_{128}$KKSE$_{131}$, $_{159}$MKTF$_{161}$ and $_{169}$FKNF$_{172}$ (motifs with low probability) (**Figure 35C**). Taken together, it suggested the presence of SUMO interacting motifs on PXR protein.



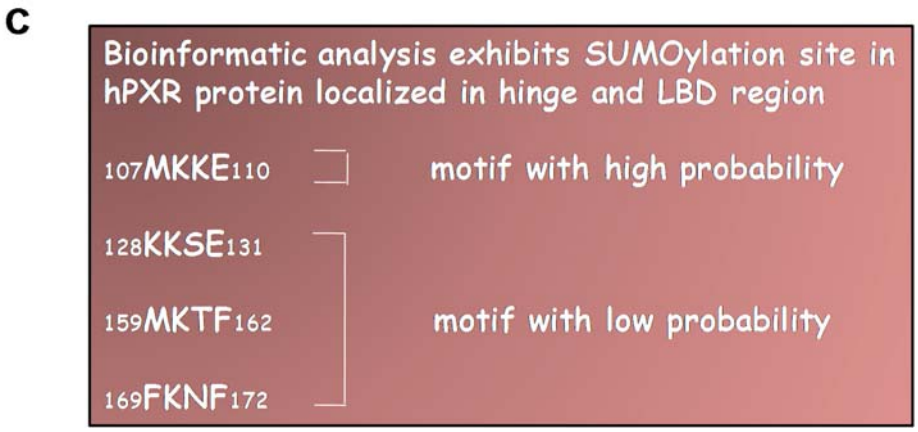


Figure 35: Western blot analysis shows post-translational modification of PXR. ***A.*** *COS-1 cells transfected with PXR plasmid and cell extracts were prepared after 12, 24, 36 and 48 h of post-transfection. The cell extracts were electrophoresed through 10% SDS-PAGE, blotted onto PVDF membrane and probed with rabbit anti-hPXR polyclonal antibodies at a 1:2,000 dilution. Lane 1, Untransfected cells extract (Un); Lane 2, 3, 4 and 5, cell extracts prepared at 12 h, 24 h, 36 h and 48 h post-transfection respectively. A specific reactivity with a major protein band having molecular weight of 50 kDa was detected. Upper bands reveal the presence of potential post-translationaly modified forms of PXR. Lower bands reveal the presence of potential isoforms or proteasomal degradation product of PXR protein. Asterisks (⋆) signify the non-specific band pattern.* ***B.*** *Pictorial representation of tetrapeptide consensus motif of SUMO-modified proteins.* ***C.*** *In silico analysis revealed that potential SUMO interacting motifs are present in the hinge and LBD region of PXR.*

Generation of polyclonal antibody against SUMO-1

Investigation into the relevance of PXR SUMOylation and its detailed functional aspects, necessitated development of a highly specific, sensitive and well-characterized antibody for SUMO-1. Here, we report a successful expression of full-length SUMO-1 protein, development and characterization of polyclonal antiserum against SUMO-1. The protein purification was done as described in 'Materials and Methods' section. Though a homogeneous preparation of SUMO-1 protein was clearly evident we preferred to inject the protein after excising the KCl stained band from polyacrylamide gel. Electrophoresed SUMO-1 protein could be exclusively excised from the gel and homogenized for immunization. For primary injection and corresponding booster injections the purity and recovery of gel-excised SUMO-1 protein was always reconfirmed. Therefore, homogenized preparation containing a single protein for SUMO-1 could be confidently

used for immunizing the *Swiss albino* mice. After collecting the pre-immune serum, the antiserum was collected after the first injection and three subsequent boosters injection. We confirmed the specificity and sensitivity of the developed polyclonal antibody against SUMO-1 in our western blot studies. The COS-1 cells were transiently transfected with GFP-SUMO-1 plasmid and the expressed protein was detected by western blotting at a dilution of 1:2,000. The results with transfected cells expressing SUMO-1 protein showed a predominant band of 40 kDa corresponding to GFP-SUMO-1 (**Figure 36**). Therefore, in our western blot analysis, this antibody successfully detected SUMO-1 in cultured cells.

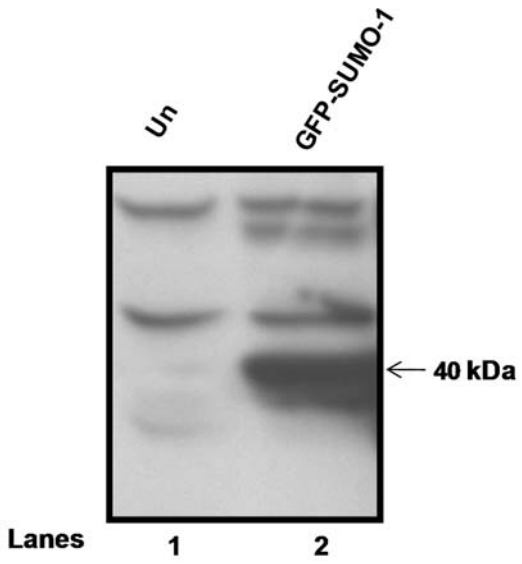


Figure 36: Analysis of polyclonal antibody raised against full-length human SUMO-1 by *Western blotting*. *Extract from untransfected and SUMO-1 expressing COS-1 cells were electrophoresed through 10% SDS-PAGE, blotted on to PVDF membrane and probed with mouse anti-SUMO-1 antibody at 1:2,000 dilutions. Lane 1: Untransfected COS-1 cells extract (Un); Lane 2: COS-1 cell extract from GFP-SUMO-1 transfected cells.*

Transcriptional activity of PXR is modulated by SUMO-1

Highly promiscuous nuclear receptor PXR can be activated by a diverse group of xenobiotic and endobiotic compounds in addition to this there are several endogenous factors that affect PXR functions. In order to shed light on the mechanisms responsible for regulation of PXR transcription function, we attempted to explore the effect of SUMOylation on

functional activity of PXR. To address the effects of SUMO-1 modification on PXR functions, we first examined the influence of SUMO-1 modification on PXR transcriptional activity. To investigate the possible existence of functional cross-talk between PXR and SUMO pathway, transient transfection assays were performed in a human liver cell line HepG2. For this purpose, HepG2 cells were co-transfected with XREM-Luc promoter-reporter and PXR expression plasmid and increasing dose of SUMO-1 expression plasmid. Interestingly, when SUMO-1 was exogenously co-transfected, rifampicin mediated PXR transcriptional activity was moderately increased. Increasing amount of SUMO-1 expression led to progressive increment of promoter activity of rifampicin activated PXR. These results demonstrate that the SUMOylation substantially induced rifampicin mediated PXR trans-activity in a dose dependent manner. The effect of SUMO-1 expression on the wild-type PXR function in HepG2 cells is shown in **Figure 37**. Our results showed that SUMO-1 modification significantly activates PXR-mediated transcriptional activity in dose-dependent manner.

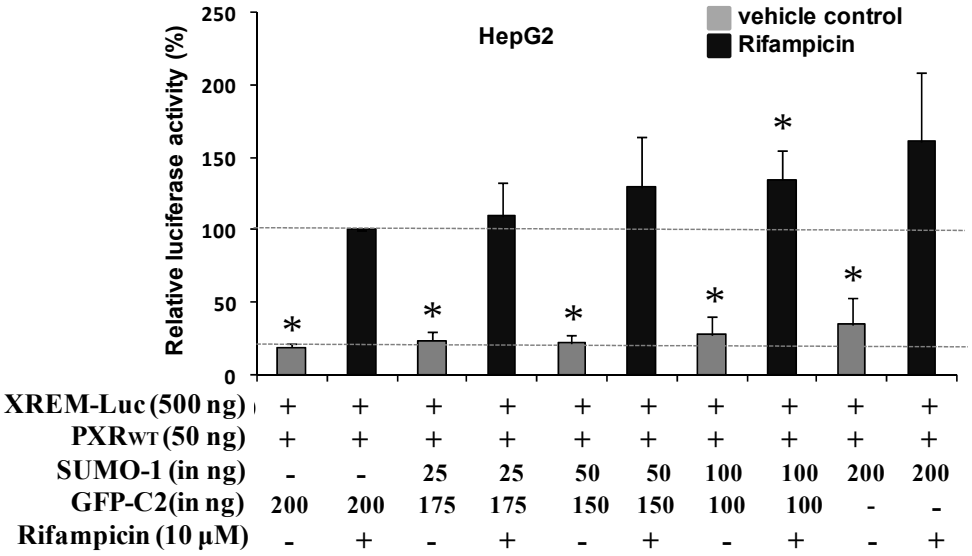


Figure 37: **Enhancement of transcriptional activity of PXR with increasing co-expression of SUMO-1**. *To determine PXR transcriptional activity XREM-Luc, wild type PXR expression plasmids and increasing concentration of GFP-SUMO-1 plasmids were co-transfected in HepG2 cells according to scheme shown in figure. Carrier plasmid (GFP vector) was used*

to maintain the total amount of plasmid constant in each well. After plasmid transfection, the cells were treated with rifampicin for 36 h and subsequently harvested for luciferase assay. Relative fold activity was calculated in comparison to rifampicin-induced luciferase activity which was taken as 100%. The values represent the means ± SD of three independent experiments. Asterisks (*) signify luciferase values that differed significantly from PXR transfected cells (P<0.05 in Student's T-test).

Investigation of interaction of PXR and SUMO-1

Understanding the protein–protein interactions is important for the assessment of various intracellular signaling pathways, modeling of protein complex structures and for gaining insights into various biochemical processes. Regulation of function of several by SUMOylation suggests that there may also be direct interaction between SUMO-1 and PXR. From the reports it is conceivable that SUMOylation may also recruit co-factor to PXR leading to transcription regulation of PXR. Protein–protein interactions occur when two or more proteins bind together, often to carry out their biological function. Therefore, for this purpose various methods have been utilized that could lead to identification of PXR interacting proteins involved in SUMOylation. So, in this study approaches like live cell imaging, two hybrid assay and co-immunuprecipitation were utilized to explore the possible interaction between PXR and SUMO-1.

Co-localization of PXR and SUMO-1 by live cell imaging

Modulation of PXR activity by SUMO-1 prompted us to examine if the two receptors co-localize/interact in the cells. To study the sub-cellular co-localization of PXR upon interaction with SUMO-1, we first cloned SUMO-1 in pDsRed-express C1 vector and designated as RFP-SUMO-1. The sub-cellular localization of RFP-SUMO-1 was shown as nuclear. We observed GFP-PXR and RFP-SUMO-1 co-localize as distinct nuclear foci to produce yellow fluorescence (**Figure 38**). This observation suggests the co-localization of two proteins and possible existence of interaction between them.

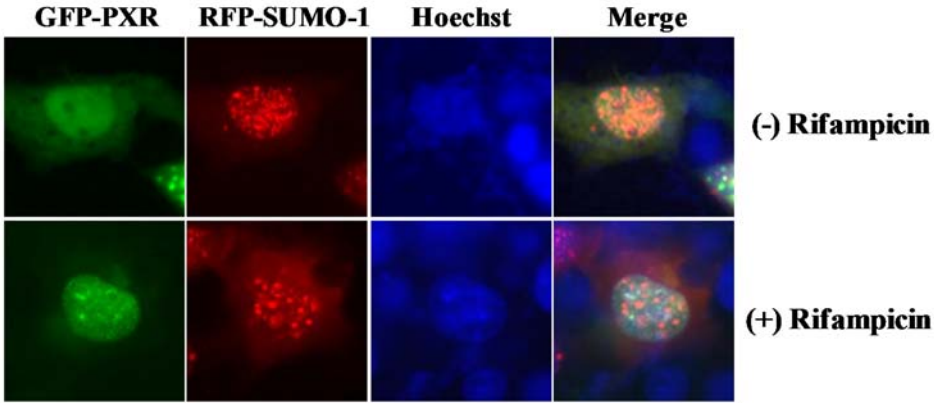


Figure 38: **Co-localization of GFP-PXR with RFP-SUMO1.** *COS-1 cells were transiently co-transfected with GFP-PXR and RFP-SUMO-1. Following transfection and receptor expression period, cells were incubated with Hoechst (1 µg/ml). The GFP fluorescence shows the predominantly nuclear distribution pattern of GFP-PXR and RFP fluorescence shows the predominantly nuclear distribution pattern of SUMO-1. Hoechst staining was performed for visualizing the corresponding nuclei and merge images for visualizing the nuclei with PXR and SUMO-1.*

SUMOylation of PXR by SUMO-1: an *in vivo* analysis

To determine whether PXR undergoes SUMOylation, we analyzed COS-1 cells were transiently transfected with constructs encoding PXR and GFP-tagged SUMO-1. Transfected cells were lysed in modified RIPA buffer in the presence of N-ethylmaleimide (NEM), an inhibitor of SUMO-1 hydrolase. Cellular extract were first pulled by PXR antibody and subsequently western blot analysis was performed with SUMO-1 antibody. Anti-SUMO-1 antibody detected a major band of approximately 90 kDa corresponding to covalently modified forms of SUMO-1 (**Figure 39**). None of these bands were detected either in COS-1 cells transfected with PXR construct alone or in untransfected COS-1 cells.

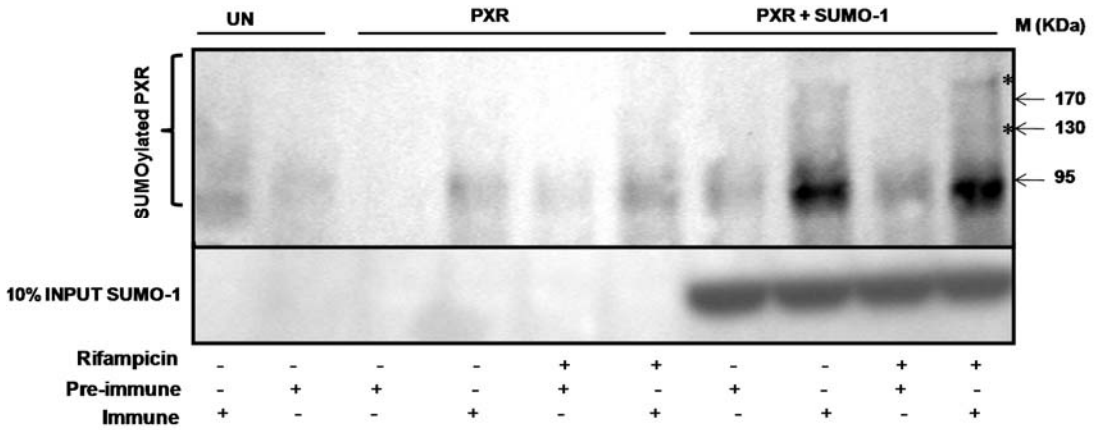


Figure 39: **SUMO-1 coimmunoprecipitates with PXR.** *COS-1 cells were co-transfected with PXR alone or along with SUMO-1 plasmids as shown in above figure. Untransfected COS-1 (UN) and cells were transfected with PXR were taken as negative control. Interaction between PXR and SUMO-1 was detected by co-immunoprecipitation. Cells were treated with or without rifampicin for 36 h and harvested for immunoprecipitation with rabbit anti-PXR immune sera and pre-immune sera as indicated in figure. PXR was immunoprecipitated using a PXR polyclonal antibody followed by immunoblotting with SUMO-1 antibody. Immunoprecipitated complex at 90 kDa was detected by mouse anti-SUMO-1 antibody at 1:2,000 dilution by western blotting corresponding to PXR-GFP-SUMO-1 complex. In addition to this multiple-SUMOylated (*) PXR species were identified.*

Taken together, our results indicated that PXR acts as a substrate for SUMO-1 modification *in vivo,* suggesting the possible role of SUMOylation in modulation of PXR function.

UBCh9-E2 conjugation system directly interacts with PXR

To investigate whether E2-conjugation enzyme UBCh9 and PXR directly interact with each other and form UBCh9–PXR complex, we carried out mammalian two-hybrid experiment. **Figure 40A** represents the working strategy of mammalian two-hybrid experiment. Mammalian two-hybrid experiment was performed using VP16 transactivation domain fusion protein with UBCh9 (VP16-UBCh9) together with a Gal4-PXR (Gal4 DNA binding domain fused with PXR) and a Gal4-luciferase reporter gene (FR-Luc) as a read out for interaction. The Gal4-PXR fusion protein where transfected along with FR-Luc promoter-reporter construct, the transcriptional activity of this was taken as 100% in the presence of

rifampicin. When both fusion proteins were co-expressed, interaction of Gal4-PXR with VP16-UBCh9 was apparent from the two-hybrid signal (**Figure 40B**).

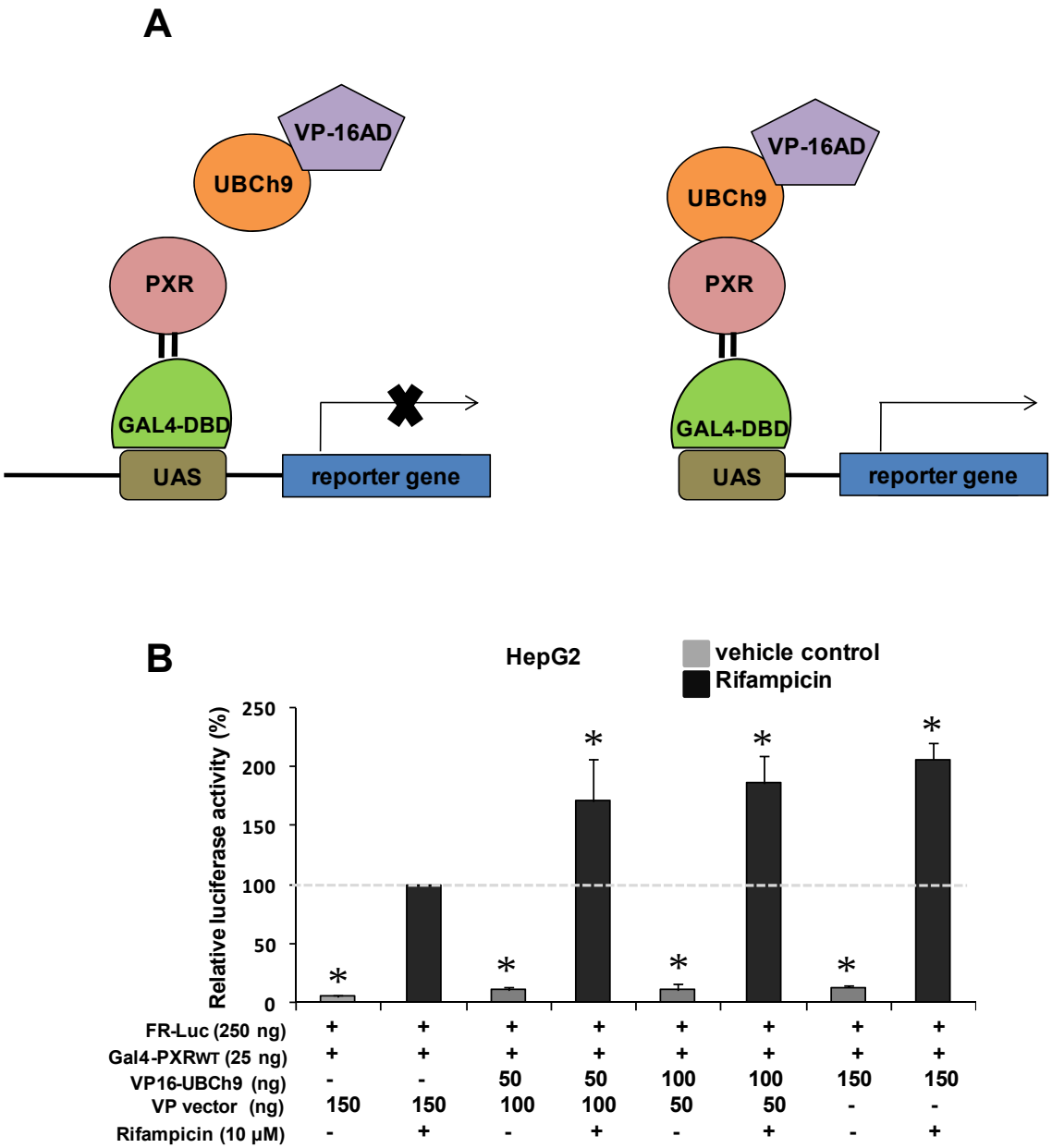


Figure 40: **UBCh9 physically interacts with PXR.** ***A.*** *Schematic presentation of the working strategy of mammalian two-hybrid assay.* ***B.*** *This assay was executed in HepG2 cells. E2-conjugation enzyme UBCh9 fused to VP16 (VP-16 UBCh9) and full-length PXR fused to GAL4-DNA binding domain (GAL4-PXRWT) were co-transfected in HepG2 cells along with Gal4 promoter-reporter construct FR-Luc. After transfection, the cells were treated with rifampicin for 36 h as shown in the scheme and then harvested for luciferase*

assay. Relative luciferase activity is plotted in comparison to Gal4-PXR alone transfected in presence of rifampicin which was taken as 100%. Asterisks (*) signify luciferase values that differed significantly from Gal4-PXR and VP-16 transfected cells (P<0.05 in Student's T-test).

By mammalian two-hybrid assay, it was evident that E2-conjugation system human UBCh9 directly bound to PXR and enhance substrate specificity for SUMO-1 interaction, which further strengthened the fact that SUMO-1 directly bind to PXR. So, this experiment identified the E2 enzyme UBCh9 serve as an interacting partner of PXR, providing a possible molecular explanation for SUMO-dependent modulation of cellular target proteins.

Determination of SUMO interacting motifs in PXR

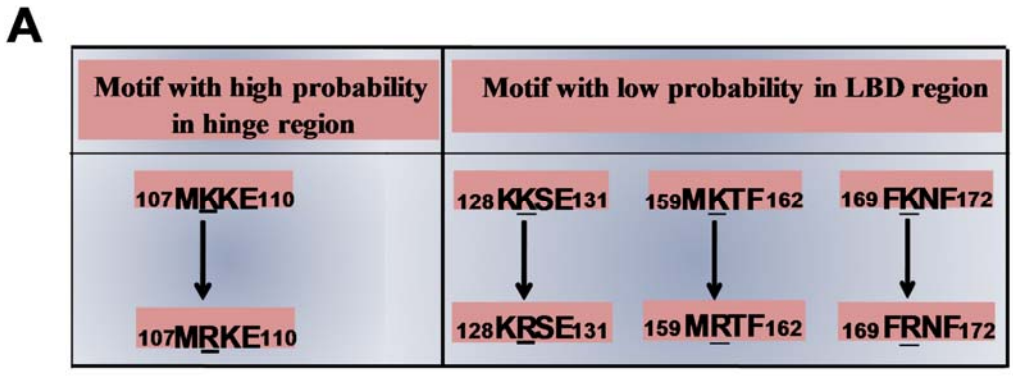
SUMOylation is known to occur on the lysine residue with a consensus sequence of ΨKXE/D, with Ψ representing a hydrophobic residue and X represents any amino acid. By SUMOplot prediction it has been known PXR hinge and ligand binding domain region contains four putative lysine residues for SUMOylation. Another SUMOylation prediction tool SUMOsp 2.0 addresses 26 sites among them lys-108 lies in consensus sequence and rest of them in non-consensus sequences. To determine which residue of PXR acts as the SUMO attachment site, we focused on lys-108, lys-129, lys-160 and lys-170 since these residues are common in both prediction tools. Among these, lys-108 motif lies in hinge region with high probability while the other three resemble the low probability motif (**Figure 35C**). To investigate the involvement of these lysine residues as SUMO-1 acceptor, each of the four lysine residues was individually mutated to arginine (**Figure 41A**). Single lysine mutation were designated as $PXR_{K108R,}$ $PXR_{K129R,}$ PXR_{K160R} and PXR_{K170R}; triple lysine mutation designated as $PXR_{3KR(108-)}$ (with all three sites mutated except 108 site), $PXR_{3KR(129-)}$ (with all three sites mutated except 129 site), $PXR_{3KR(160-)}$ (with all three sites mutated except 160 site), $PXR_{3KR(170-)}$ (with all three sites mutated except 170 site); and ultimately a mutant construct where all the four lysines were mutated, designated as PXR_{4KR}. At first we examined the transcriptional activity of

these PXR mutants and compared to the wild type PXR to determine if the introduction of these mutations affected transcriptional function of the receptor. We observed that PXR_{K108R}, PXR_{K129R}, PXR_{K160R} mutation, all PXR_{3KR}, and PXR_{4KR} mutations having lowered transcriptional activity in compare to wild type PXR (**Figure 41B**). Further, we examined the protein expression profile of these mutants and observed no change in protein expression profile of these mutants as compared to wild type PXR (**Figure 41C**).

This implied that lower transcriptional activity was not due to altered or lowered expression of the PXR protein. So, these experiments show that disruption of SUMO interacting motif results in lower transactivation potential of PXR.

SUMOylation does not affect intra-cellular stability of PXR protein

To analyze the effect of SUMOylation on PXR protein stability, we co-transfected plasmid-DNA expressing SUMO-1, wild type PXR and either of the different PXR SUMO mutants as shown in **Figure 42**. To investigate any possible alteration PXR expression by presence of SUMO-1, we performed western blot analysis with the cell extracts. No significant effect on PXR protein expression level was observed in the presence of SUMO-1 implying that enhancement in PXR-mediated transcriptional activity is neither due to the increase PXR expression nor due to the potential artificial effects linked to exogenous transfection of SUMO-1.



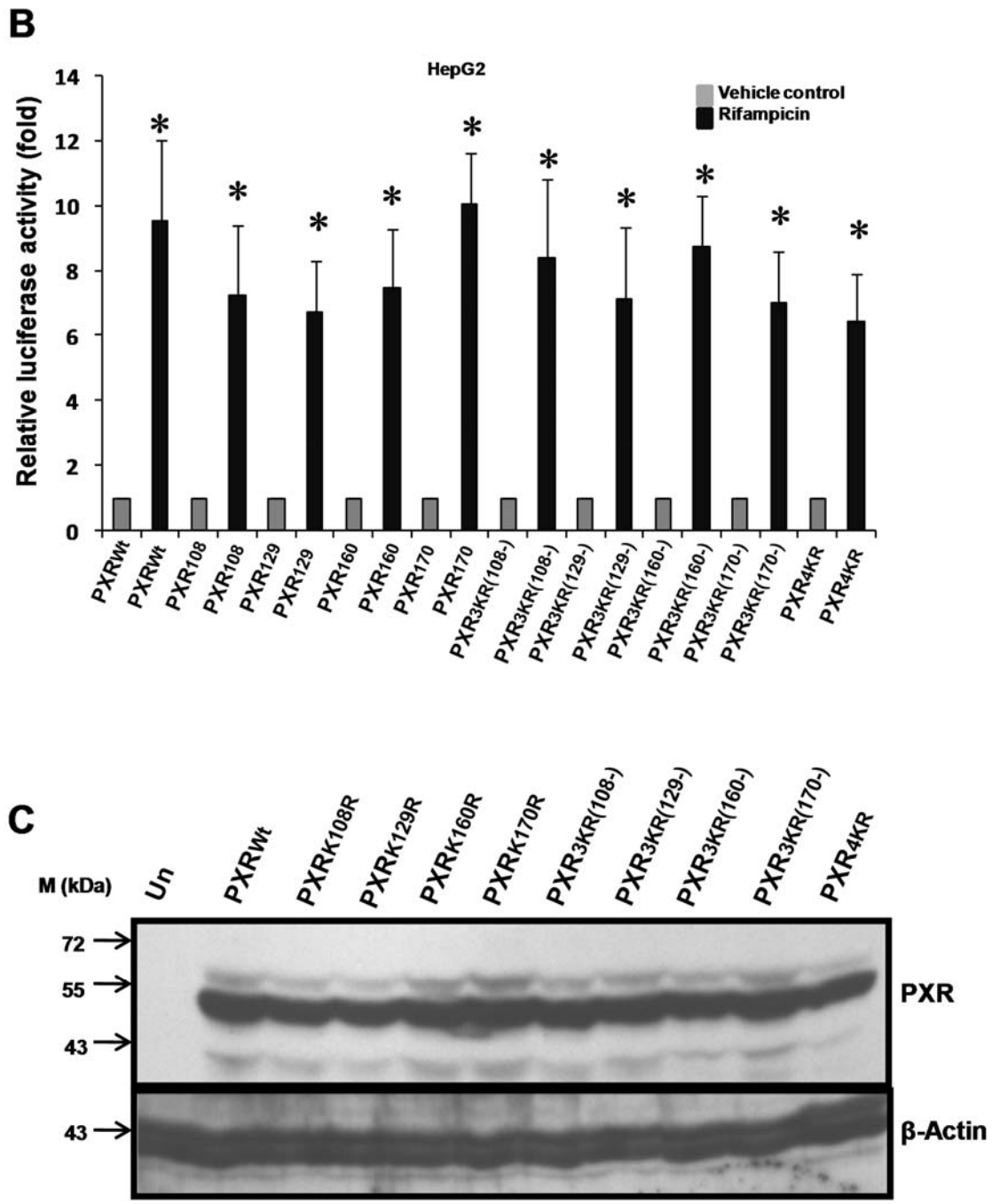


Figure 41: **Determination of transcriptional activity and protein expression profile of SUMO mutants of PXR.** ***A.*** *The pictorial representation of site-directed mutagenesis of different SUMO mutants of PXR.* ***B.*** *For determination of transcriptional activity of each construct, wild type PXR expression plasmid and one of the SUMO mutants of PXR were transfected individually along with XREM-Luc in HepG2 cells according to the*

scheme shown in figure. After plasmid transfection, the cells were treated with rifampicin for 36 h and then harvested for luciferase assay. Relative fold luciferase activity was calculated for each construct from rifampicin treatment to no treatment, which was taken as 1. The value represents the means ± SD of three independent experiments. Asterisks (*) signify luciferase values that differed significantly from vehicle treated wild type PXR and SUMO mutants of PXR independently transfected in cells (P<0.05 in Student's T-test). ***C.*** *Western blot analysis of SUMO mutants of PXR transfected in COS-1 cells. Cell extracts were prepared in RIPA buffer after 48 h of transfection and electrophoresed through 10% SDS-PAGE, blotted onto PVDF membrane and probed with PXR monoclonal antibody as described in 'Materials & Methods' and rabbit anti–β-Actin polyclonal antibody at a 1:2,000 dilution. No significant change of PXR protein level was observed when using β-Actin as loading control (lower panel).*

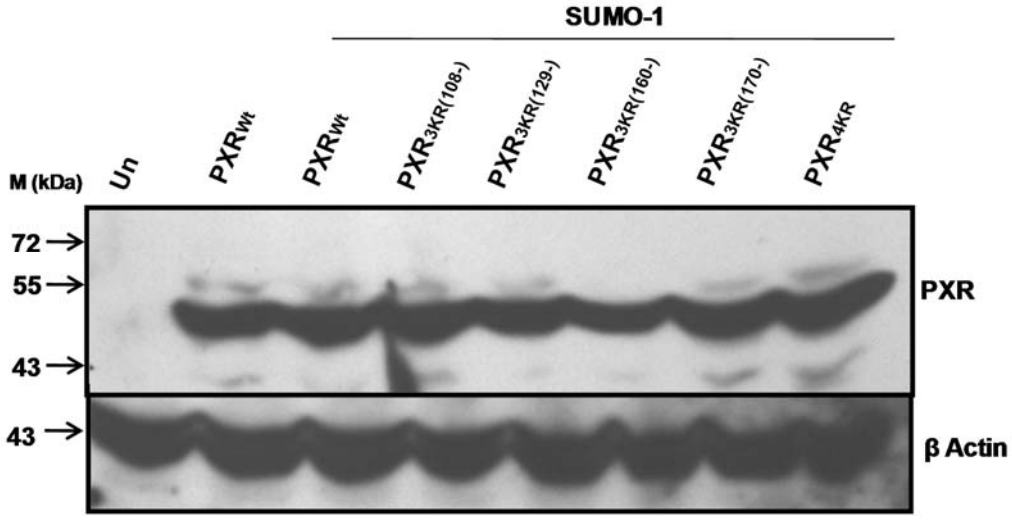


Figure 42: **PXR protein stability is not affected by SUMOylation**. *COS-1 cells were co-transfected with PXR and* different SUMO interacting motif mutants *of PXR along with SUMO-1 as shown in figure. Cell extracts were prepared in RIPA buffer after 48 h of transfection and electrophoresed through 10% SDS-PAGE, blotted onto PVDF membrane and probed with PXR-L13 monoclonal antibody as described in 'Materials & Methods'. There is no significant change of PXR protein level was observed.*

Sub-cellular localization of SUMO mutants of PXR

SUMOylation of a protein/receptor may have altered sub-cellular localization (Chauchereau et al, 2003; Hamard et al, 2007; Cho et al, 2009; Besnault-Mascard et al, 2005). To test whether SUMOylation influence the sub-cellular localization of PXR, COS-1 cells were transfected with GFP tagged wild-type PXR or its mutant PXR_{K108R}, PXR_{K129R}, PXR_{K160R}, PXR_{K170R}

and PXR4KR and subjected to live cell imaging. When transiently expressed in COS-1 cells, none of the mutants showed any substantial effect on receptor sub-cellular localization. Like the wild type PXR, the mutant receptors also localized in the nuclear compartment revealing no significant differences, in presence or absence of rifampicin (**Figure 43A & B**).

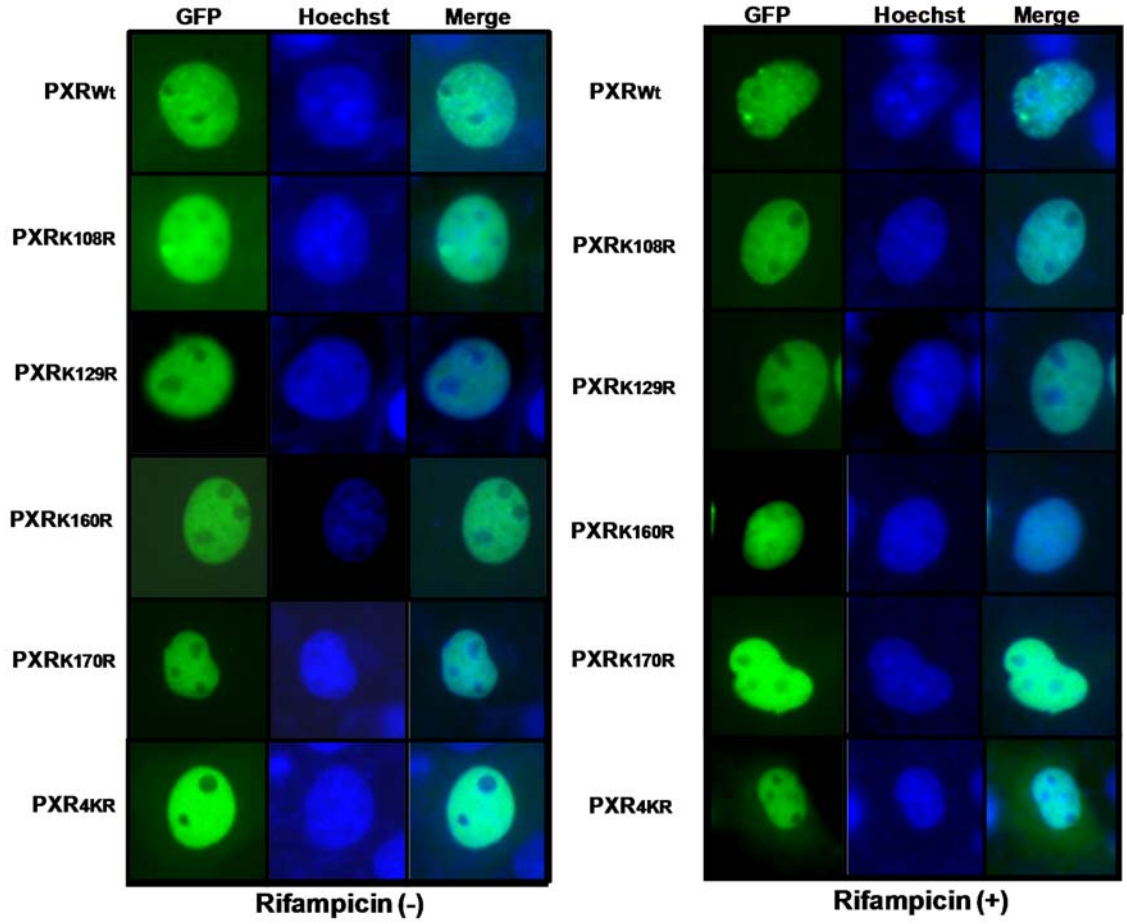


Figure 43: **Sub-cellular localization profile of GFP-tagged SUMO mutants of PXR.** *COS-1 cells were transiently transfected with GFP-tagged wild type and SUMO mutants of PXR for immunofluorescence study. Live cell images show the sub-cellular localization of SUMO mutants of PXR with nuclear compartment. Images were recorded using a fluorescence microscope equipped with water–immersion objectives. The left panel **(A)** shows the distribution pattern of receptor in absence of drug treatment. The right panel **(B)** shows the distribution pattern of receptor after rifampicin (10 µM) treatment. The GFP fluorescence visualizes the predomi```*

nantly nuclear distribution pattern of wild type PXR and SUMO mutants of PXR receptor. Hoechst

fluorescence was done for visualizing the corresponding nuclei and the merged images for visualizing the corresponding nuclei with different SUMO mutants of PXR.

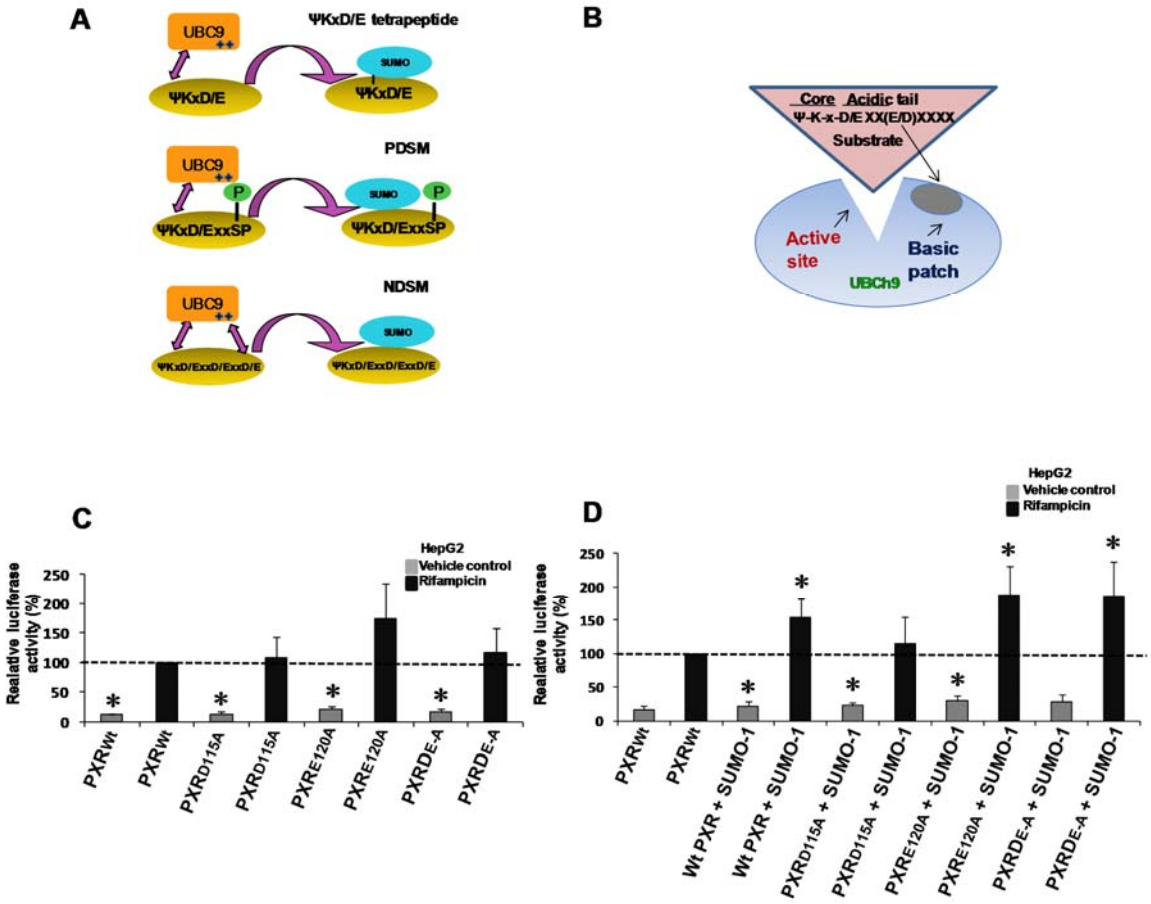
Presence of Negative charge amino acid Dependent SUMOylation Motif (NDSM) in PXR

Recently, an acidic tail located downstream from a hydrophobic patch has been shown to be an important determinant of non-covalent interactions between SUMO and other proteins (Yang et al, 2003). Bioinformatic tool SUMOFI (cbg.garvan.unsw.edu.au/sumofi/form.do) may be used to identify a subset of SUMO consensus sites called the PDSM (Phosphorylation Dependent SUMOylation Motif), which associates a classical SUMO consensus site with an adjacent proline-directed phosphorylation site (ΨKXEXXSP) and NDSM (Negative charge amino acid Dependent SUMOylation *M*otif) (Hietakangas et al, 2006; Yang et al, 2003; Yang et al, 2006) (**Figure 44A**). The recognition of a negatively charged patch within the NDSM of substrate proteins is required for efficient interaction with basic patch of UBCh9 (**Figure 44B**).

Through analysis with SUMOFI programme, we observed presence of a NDSM in PXR at 107-120 amino acid residues, $_{107}$MKKEmims**D**eave**E**$_{120}$. We, therefore, made a detailed analysis to examine if this acidic tail plays a role in PXR SUMOylation. Specific alanine substitution of acidic patches proximal to the SUMO core consensus sites, ΨKxE, of PXR has been utilized for analysis of NDSM in PXR. We made three different mutants to establish the existence of NDSM in PXR, we designated these mutants as PXR$_{D115A}$ (aspartic acid at 115 position gets mutated to alanine), PXR$_{E120A}$ (glutamic acid at 120 position gets mutated to alanine) and PXR$_{DE-A}$ (aspartic acid at 115 position and glutamic acid at 120 position gets mutated to alanine).

For functional analysis we transfected these mutants individually to HepG2 cells alongwith XREM-Luc and observed that transcriptional activity of PXR$_{D115A}$ and PXR$_{DE-A}$ were nearly similar to that of wild type PXR. However, transcriptional activity of PXR$_{E120A}$ was observed to be higher than wild type PXR (**Figure 44C**). This, increase in transcriptional activity

suggested the possibility of co-activator recruitment by PXR$_{E120A}$. Furthermore, co-transfection of NDSM mutant PXR$_{D115A}$ in HepG2 cells alongwith XREM-Luc and SUMO-1, reduced the transcriptional activity but the transcriptional activity of PXR$_{E120A}$ and PXE$_{DE-A}$ was found to be enhanced in comparison to wild type PXR (**Figure 44D**). These result support the speculation that aspartic acid residue at 115 position must be contributing to NDSM in PXR. Additionally, we also examined the sub-cellular localization of these mutants in COS-1 cells. It was observed that the PXR protein was efficiently expressed and there was no significant change in sub-cellular localization of these mutants (**Figure 44E**). These observations potentially provide new perspectives for a better understanding of the functions of NDSM in PXR.



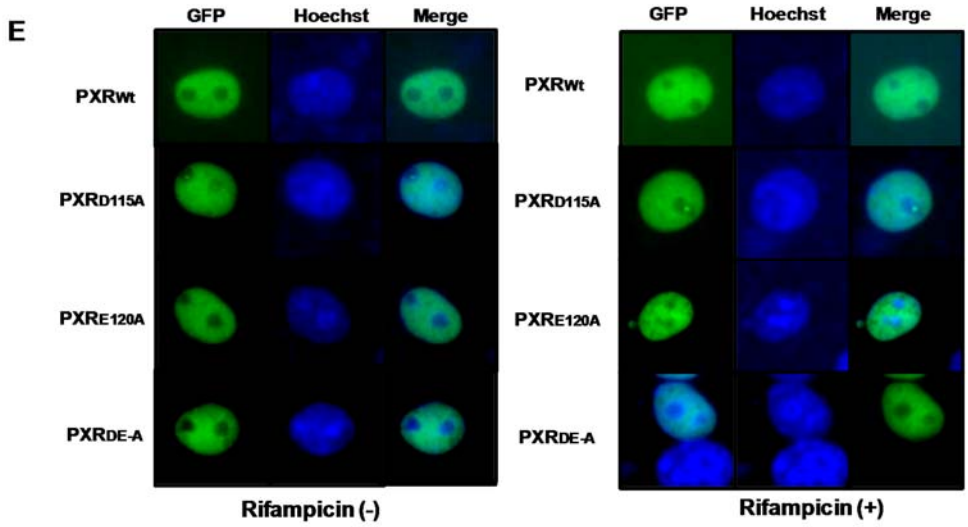


Figure 44: The characterization of Negative charge Dependent SUMOylation Motif (NDSM) mutants of PXR by promoter reporter-based assays and sub-cellular localization. ***A****. Pictorial representation of SUMO consensus sites called the PDSM and NDSM which associates a classical SUMO consensus site.* ***B.*** *Pictorial representation of interaction of NDSM with the basic patch of UBCh9. This figure represents a basic patch that resides within UBCh9 and interacts with the acidic tail present adjacent to SUMO consensus motif* ***C****. Comparison of transcriptional activity of each of the NDSM mutants of PXR with wild type PXR.* ***D****. Comparison of transcriptional activity of each of the NDSM mutants of PXR with wild type PXR when co-transfected with SUMO-1. For determination of transcriptional activity constructs XREM-Luc, wild type PXR expression plasmid, NDSM mutants of PXR and SUMO-1 plasmids were co-transfected in HepG2 cells according to scheme shown in figure C & D. Carrier plasmid (GFP vector) was used, to keep the total amount of plasmid constant in required well of figure C. After plasmid transfection, the cells were treated with rifampicin for 36 h and then harvested for luciferase assay. Relative fold activity was calculated in comparison to rifampicin-induced luciferase activity of wild type PXR, which was taken as 100%. The value represents the means ± SD of three independent experiments. Asterisks (*) signify luciferase values that differed significantly from PXR transfected cells (P<0.05 in Student's T-test).* ***E.*** *Sub-cellular localization profile of GFP-tagged SUMO mutants of PXR. COS-1 cells transiently transfected with GFP tagged SUMO mutants of PXR for immune fluorescence study. Live cell images illustrates the sub-cellular localization of SUMO mutants of PXR. The left panel shows the distribution pattern of receptor in absence of drug treatment. The right panel shows the distribution pattern of receptor with rifampicin (10 µM) treatment. The GFP fluorescence indicates the predominantly nuclear distribution pattern of PXR. Hoechst fluorescence was done for visualizing the corresponding nuclei and the merged images for visualizing the corresponding nuclei with different SUMO mutant of PXR.*

Regulation of PXR functions by drugs modulating different signaling cascades

a) Effect on transcription function of PXR

There are several previous reports available in literature suggesting that PXR may undergo several PTMs resulting into distinct cellular out-put via modulation of different signaling pathways (Masuyama et al, 2000; Pondugula et al, 2009; Lichti-Kaiser et al, 2009; Hu et al, 2010; Biswas et al, 2011; Staudinger et al, 2011). To explore this fact, further we used different drugs that modulate various signaling pathways like TSA (Trichostatn A, inhibitor of class I and II mammalian histone deacetylase), CPTH2 (Histone Acetyltransferase Inhibitor IV), PMA (Phorbol 12-Myristate 13-Acetate, an activator of Protein Kinase C) and OKA (Okadaic Acid, a potent inhibitor of PP1 and PP2A phosphatases). We treated HepXREM cells (HepG2 cells stably integrated with PXR and promoter-reporter CYP3A4-Luc) as mentioned in 'Materials and Methods'. We observed that both PMA and OKA do not significantly alter human PXR transcriptional activity in presence of rifampicin (**Figure 45A**). Contrary to this observation, a previous study by Ding & Staudinger, 2005 suggested repression of transcriptional activity of mouse PXR via activation of the PKC signaling pathway in mouse hepatocytes treated with PMA and OKA. These discrepancies in altering PKC signaling pathway between our findings and earlier report could be due to the differences between human PXR and mouse PXR. In terms of sequence homology, sub-cellular localization and ligand preferences the PXR from these two species has been reported to differ from each other.

Other two drugs, TSA and CPTH2 are known to regulate modulatory events of chromatin configuration by acetylation and deacetylation respectively. They significantly alter the transcriptional activity of PXR (**Figure 45B**). We observed that treatment HepXREM cells with TSA increased transcriptional activity while CPTH2 treatment decreased transcriptional activity of PXR in presence and absence of rifampicin. A previous study showed that hypoacetylation of PXR protein results into an increase in transactivation potential of PXR (Biswas et al, 2011). It is well

known that TSA is responsible for hyperacetylation of histone or non-histone proteins while CPTH2 reverses this phenomenon. Therefore, our results suggest that enhancement of PXR activity may not be due hyperacetylation rather of PXR protein due to chromatin modification by TSA. Similarly, repression of PXR activity by CPTH2 may be due to deacetylation of chromatin. The results shown in **Figure 45B** substantiate our claims.

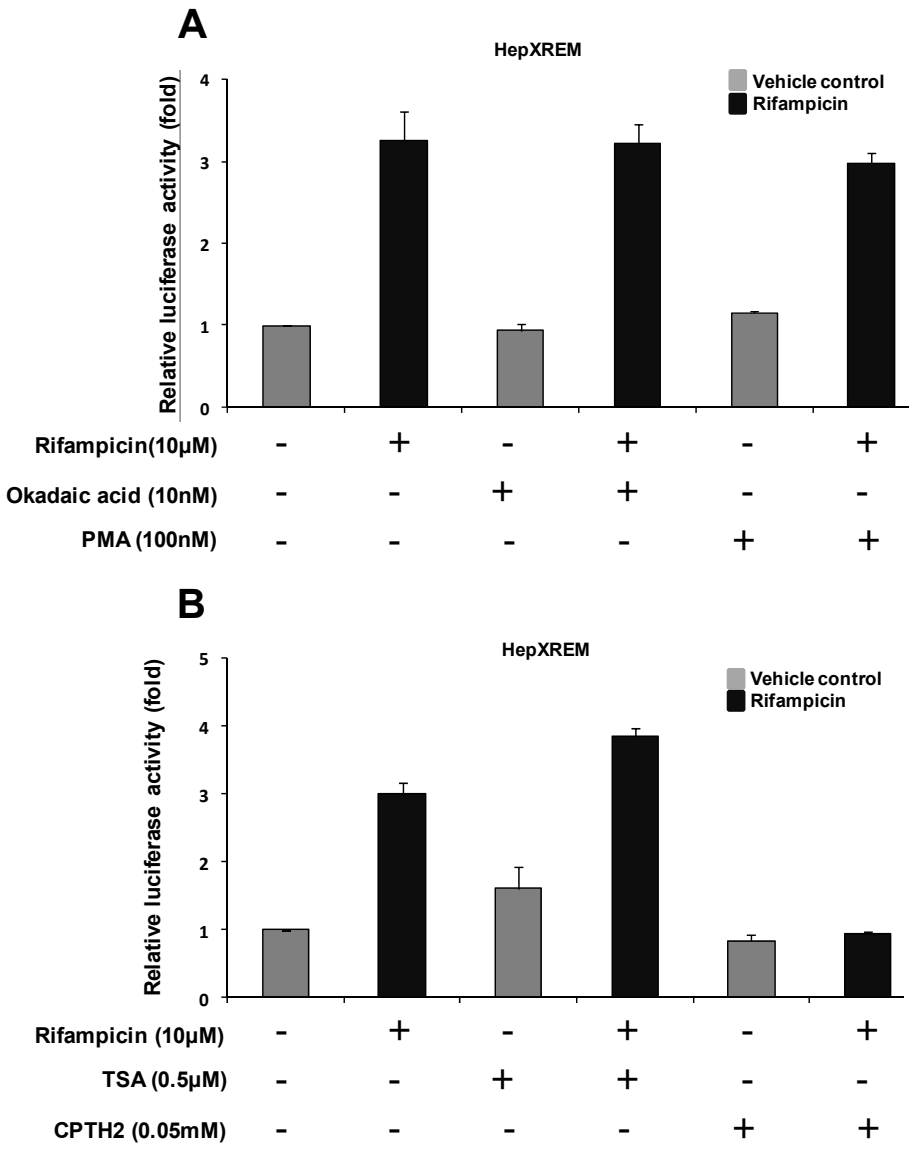


Figure 45: Effect of TSA, CPTH2, OKA and PMA on PXR transcriptional activity. *The HepXREM* (HepG2 cells stably integrated with PXR and promoter-reporter CYP3A4-Luc) *cells were seeded in 24-well plate one day before the drug treatment. The following day, cells were treated with either*

*vehicle (DMSO) or different drugs as indicated in figure. Transcriptional activity of PXR subsequent to 24 h treatment with different drugs alone or in combination with rifampicin shown in figure **A** & **B**. Results are shown in fold induction of luciferase activity determined relative to the activity in absence of drug which was taken as 1. The values represent the means ± SD of three separate experiments. Asterisks (*) signify luciferase values that differed significantly from HepXREM untreated cells (P<0.05 in Student's T-test).*

b) Alteration of DNA binding activity of PXR by drugs modulating cell signaling cascades: an analysis by ChIP assay

Being a transcription factor, PXR binds to the specific responsive elements in the promoter regions of its regulated gene. Therefore, we performed ChIP assays to examine the possibility of differential DNA binding of PXR on ER6 region of CYP3A4 promoter in presence of different drug treatments (as already mentioned above) (**Figure 46**). For this assessment HepXREM cells were treated with different drugs for 24 h and analysed by ChIP assay. As shown in **Figure 46**, no change was observed in PXR binding to ER6 before or after rifampicin (RIF) (10 μM) treatment in HepXREM cells. This is in agreement with an earlier observation (Tiangang & Chiang, 2006). However, in case of either TSA (0.5 μM), CPTH2 (0.05 mM), OKA (10 nM) and PMA (100 nM) treatments alone, a reduced PXR DNA binding activity was observed as compared to rifampicin treatment. Among all the drugs CPTH2 exhibited maximum reduction in DNA binding activity followed by PMA, OKA and TSA.

The study confirmed that in presence of TSA alone, increase in DNA binding activity could be attributed to the accessibility of different co-activator due to open configuration of chromatin. This resulted in an increase in transcriptional activity of PXR. On the contrary, CPTH2 inhibits DNA binding activity via initiation of closed configuration of chromatin that results into reduced transcriptional activity of PXR. When used in combination with rifampicin, CPTH2 exhibited a reduction in PXR DNA binding activity that correlated with the reduction of transcriptional activity of PXR.

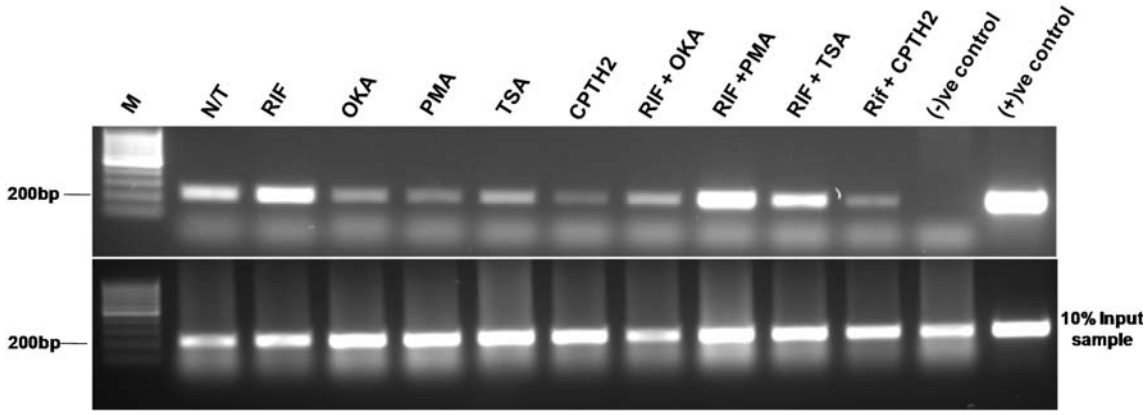


Figure 46: Different drug treatment to HepXREM cell line show differential binding of PXR to proximal ER6 region. *Cell lysates prepared from HepXREM cells treated with different drugs were examined by ChIP assay as indicated in 'Materials and Methods' using anti-PXR antibody. The sonicated DNA fragments present in immunoprecipitates were amplified in PCR using ER-6 region primer pairs. Results of PCR amplification from control cell lysate (input) and the immunoprecipitate are shown. N/T=no treatment.*

No change in PXR DNA binding activity was detected subsequent to co-treatment of cells with rifampicin and either of the drugs i.e. OKA, PMA and TSA. No significant change in transcriptional activity with rifampicin+OKA and rifampicin+PMA treatments were observed when compared to rifampicin treatment alone. Interestingly, an increase in PXR transcriptional activity with rifampicin+TSA treatment was observed but no enhancement in DNA binding activity was apparent. Thus, depending on the modulatory properties, these drug treatments appeared to mediate differential PXR binding onto the ER6 promoter that ultimately resulted in modulation of PXR functions.

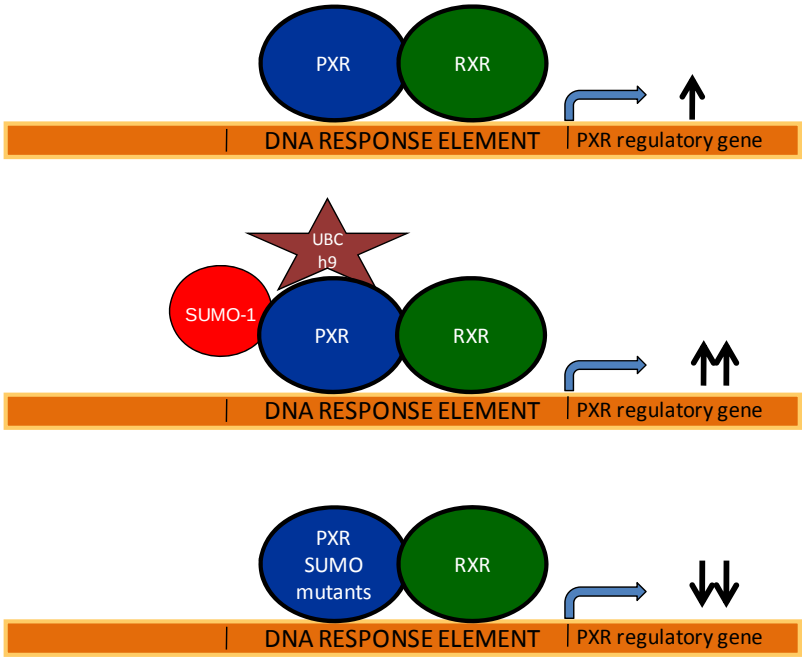


Figure 47: Hypothetical model depicting the regulatory mechanisms operating on xenobiotic response element: interaction between SUMO-1 and PXR modulates transcription function. Activated *PXR regulate cellular processes by binding to the promoter of its responsive genes. PXR SUMOylation contributes to further enhancement of ligand-mediated activation of wild type PXR. Whereas PXR SUMO mutants binding contributes to transcription repression compare to wild type PXR activity as SUMO interacting motifs gets mutated in these mutants. The model suggests an alternative mode of regulation of PXR activity attributed to receptor PXR SUMOylation.*

DISCUSSION

Substantial evidences suggest that in addition to the classical mode of ligand-dependent receptor regulation, nuclear receptor activity can also be regulated by post-translational modification (PTM). PTMs like phosphorylation, SUMOylation, acetylation, ubiquitination etc. are reported to contribute to modulation of steroid/nuclear receptor functions including AR, ER, PR, GR, MR and also including PXR albeit to lesser extent. In the current study, we have examined the effect of SUMOylation on PXR transcription function (**Figure 47**). In the initial part of our study we observed ligand-independent direct interaction between SUMO-1 and PXR by co-immunoprecipitation and co-localization studies using GFP-tagged PXR and RFP-tagged SUMO-1. In an earlier study, SUMO-1 has been

reported to interact with ERα strictly in a ligand-dependent manner (Sentis et al, 2005). In our preliminary studies, utilizing classical approach of promoter-reporter assays, we tested the effect SUMOylation on PXR transcriptional activity. We observed that with increasing concentration of SUMO-1 expression, there is a progressive increase in PXR transcriptional activity on XREM-Luc both in presence or absence of rifampicin. These observations imply that SUMOylation enhances transcription function of PXR irrespective of its association with its ligand. Overall, the observations suggest the existence of a novel interaction between PXR and SUMO-1 that results into an enhanced PXR transcriptional activity.

In context to PXR regulation, we have identified a SUMO Interacting Motif (SIM) located in the DBD and LBD region of PXR. Most importantly, we have shown that SUMO binding is involved in enhancement of transcriptional activity of this nuclear receptor. The functionality of this motif was assessed by mutational analysis that resulted in substantial reduction of the transactivation potential of PXR. Interestingly, in HepG2 cells different PXR SUMO mutants like PXR^{K108R}, PXR^{K129R} and PXR^{K160R} exhibited 2-fold reduction in transcriptional activity compare to wild type PXR, suggesting the possibility of these sites being involved in SUMOylation of PXR. Studies conducted with disruption of SIM resulting in reduction of transcription function of PXR suggest the importance of SUMOylation involved in regulation of this receptor. Mechanistically, we would assume that the SIM functions through the interaction with a SUMOylated protein, exerting an activating effect on PXR.

Next, we evaluated the regulatory influence of Negative charge Dependent SUMOylation Motif (NDSM) (lysine at 115 position) on PXR transcriptional activity by promoter-reporter assay. One of the most intriguing finding was that NDSM mutant, PXR^{D115A} and $PXR^{DE\text{-}A}$, did not show any effect on PXR transcriptional activity but PXR^{E120A} mutant which substantially enhanced the PXR transcriptional activity. Additional work is needed to explain the enhanced PXR transcriptional activity in case of PXR^{E120A} mutant which may be presumably attributed to an increase in co-

activator or decrease of co-repressor recruitment. Furthermore we did co-transfection of SUMO-1 plasmid with wild type PXR and NDSM mutants or alone wild type PXR as control. We observed that SUMOylation increases PXR transcriptional activity but PXR_{D115A} mutant shows no alteration in transcriptional activity and same as alone wild type PXR control. While another NDSM mutants PXR_{E120A} and $PXR_{DE\text{-}A}$ shows increase in transcriptional activity which may be due to co-activator recruitment and further has to be investigated. In addition to recruitment of protein co-factors, the transcriptional activity of nuclear receptors may also be affected by (1) protein degradation/stability; (2) alterations in affinity for DNA response elements; (3) alterations in protein sub-cellular localization; and (4) alterations in gene expression profile. We also examined the sub-cellular localization of NDSM mutanst and we have shown that there is no alteration in sub-cellular localization of PXR NDSM mutants. So this confirms that alteration in transcriptional activity not attributed by change in sub-cellular localization of these mutants.

Furthermore, we have examined the regulation of PXR functions by using different drugs (like PMA, OKA, TSA and CPTH2) modulating cell-signaling cascades. We have examined PXR-mediated transcriptional and DNA-binding activities in absence or presence of these drugs along with or without rifampicin treatment. Previously, it has been reported that PCN (a ligand for mouse PXR), PMA (protein kinase C agonist) and OKA (protein phosphatase 1 & 2A inhibitor) treatments significantly represses 'mouse PXR' transcriptional activity (X Ding et al, 2005). On the contrary present study shows that in presence of rifampicin there is no significant effect of PMA and OKA on 'human PXR' transcriptional acivity. This may be due to species-specific differences between mouse and human PXR protein. On the other hand there is significant effect of TSA (HDAC inhibitor) and CPTH2 (HAT inhibitor) on PXR transcriptional activity. TSA induces PXR transcriptional activity while CPTH2 abolishes PXR transcriptional activity in ligand-independent manner. Furthermore, we examined DNA binding activity of PXR *in vivo* by ChIP assay in presence of rifampicin and these

drugs either alone or in co-treatment with rifampicin. We observed that treatments of PMA, OKA, TSA alone exhibited reduction in DNA binding activity when compared with presence or absence of rifampicin treatment while there is no change in DNA binding activity when co-treated with rifampicin. Interestingly, CPTH2 reduces the DNA binding activity of PXR in ligand-independent manner. In conclusion, our findings show that the involvement of phosphorylation and acetylation signaling cascades in modulation of PXR functions.

In brief, this section of study documents post-translational modification of PXR by SUMO-1 and existence of NDSM in modulation of PXR transcription function. Ligand-independent co-localization of PXR and SUMO-1 in the nucleus is reported. Existence of a SUMOylation motif in PXR by site directed mutagenesis is revealed. Involvement of phosphorylation and acetylation signaling cascades in modulation of PXR functions are reported. Taken together, the present study suggests a synergistic cross-talk between SUMO with PXR and provides a novel insight into functional relationship between different PTMs and PXR function.

Summary & Conclusions

Nuclear Receptors (NRs) constitute a super-family of ligand-inducible transcription factors. Multiple regulatory factors associated with these transcription factors assure that signal transduction results into an accurate regulation of the targeted gene networks. Apart from NRs binding to the specific hormone response elements in their target genes, additional mechanisms are also accountable for cell and promoter specific transcription activation. Such mechanisms involve a precise interplay with various factors binding to complex target gene promoters, cell-specific modulators of transcriptional activity of the receptor, as well as homo- and hetero-dimerization and interference with other signaling pathways. Moreover, NR isoforms may initiate different gene programs attributed to their differential target gene specificities. Regulation of these processes is found to occur at the level of receptor expression or differential expression of receptor isoforms, ligand availability and more recently, through Post-Translational Modifications (PTMs) of the receptor and interaction of a variety of co-activators/co-repressors with the receptor protein. The ongoing recognition of more than a single form for many of the receptors has directed the interest of investigators toward defining the promising roles for these 'extras'. So, the present study was undertaken to examine the possible existence of different isoforms of PXR and how it may provide an additional level of regulation. Although, the role of PXR has been rather clearly established as a xenobiotic sensor that coordinately regulates xenobiotic clearance in the liver and intestine via induction of genes involved in drug and xenobiotic metabolism but its subsequent expression and functions in other tissues has remained unclear.

In the past few years, research has revealed new and mostly unsuspected roles for PXR in modulating both as positive and negative regulators in inflammation, cytokine signaling pathways, bone homeostasis, vitamin D metabolism, lipid homeostasis, energy homeostasis, cholesterol biosynthesis, apoptosis and cancer. The differential expression of PXR and its isoforms in cancer and metabolic disorders points to important role of PXR but the molecular events underlying this dysregulation are still poorly understood. So far, only differential expression of PXR in different patho-

physiological situations have been studied by several research groups but the details of regulatory mechanisms controlling the differential expression of its isoforms remains to be elucidated. The overall aim of the study described in this thesis was to detect and investigate physiological relevance of the translational isoforms and post-translational modifications of PXR. The first section of the study deals with the generation of monoclonal antibodies for detection of novel PXR isoforms and to investigate their physiological significance on the function of wild type PXR. The second section deals with post-translational modification of PXR by SUMO-1.

The major findings from this first part of the study are outlined below:

- From the literature it is apparent that PXR expression was well reported in different cancerous states like endometrial cancer (Masuyama et al, 2007), breast cancer (Dotzlaw et al, 1999), prostate cancer (Chen et al, 2007) and epithelial ovarian carcinoma (Gupta et al, 2008) but due to apparent lack of specific polyclonal as well as domain specific monoclonal antibodies against PXR, the expression of PXR and its potential isoforms in different pathological states could not been concretely explored. So, the first part of the study was based on the detection of potential isoforms of PXR, and to investigate this possibility we needed one or more specific monoclonal antibodies which could specifically recognize various isoforms of PXR. So, to address this issue we generated polyclonal and monoclonal antibodies which were raised against full-length PXR protein with ability to detect various potential isoforms of PXR. Western blot analysis with a polyclonal antibody generated against full-length PXR, reveals reactivity against all the major domains of PXR. Though, monoclonal antibodies against DBD and LBD of PXR have been successfully raised we were not successful in retrieving the monoclonal antibody against amino-terminal part (NTD) of PXR. Specificity and efficacy of these monoclonal antibodies were determined by utilizing different immunological tools like isotype mapping, western blot analysis, domain mapping, cross-reactivity, flow cytometry, immunocytochemistry, immunohistochemistry, chromatin immunoprecipitation and co-immunoprecipitation analyses. However, we

could not assess the efficacy of these monoclonal antibodies in EMSA experiments.

- In our preliminary studies in western blot analysis, when the human PXR-1 (a splice isoform) was transiently expressed in COS-1 cells, multiple protein bands were detected along with the well-characterized 50 kDa band. The observations suggested that these other bands may be either proteolytic fragments or possible PXR isoforms derived from a single mRNA (Kliewer, 1998; Saradhi, 2005a).

  There are several lines of evidences suggesting that a significant number of NR isoforms, including GR, chicken VDR, ERα and MR may be generated by the process of alternative translation initiation (Lu et al, 1997; Barraille et al, 1999; Pascual Le-Tallec et al, 2004; Lu & Cidlowski, 2005). The sequence analysis of PXR gene revealed that it contains several in-frame AUG codons which act as putative multiple initiation sites in PXR gene as was convincingly reported in the case of GR (Lu & Cidlowski, 2005). It was conceivable that some of alternative translation initiation at amino-terminal AUG codons of PXR could also produce polypeptides differing by their appropriate molecular masses to generate the same pattern of PXR isoforms which correspond with the pattern of bands observed in our western blot studies (Saradhi et al, 2005a). True to our speculations, our initial studies suggested that multiple isoforms of PXR may result from the use of alternative translation initiation at different in-frame AUG codons.

- Therefore, in the present study attempt was made to explore the molecular nature of these potential PXR isoforms by using site-directed mutagenesis approach. For this, we sequentially mutated different in-frame methionines into alanine and designated the products as m1, m2, m3, m4 and m6 and also did combinatorial site-directed mutagenesis as m23 and m123 in the cDNA coding sequences of PXR. We observed that the respective methionine mutations in different constructs resulted into disappearance of corresponding protein bands in western blot analysis. So, specific mutation analysis confirmed the role of putative amino-

terminal AUG codons in generation of PXR isoforms by alternative translation initiation**.**

- For characterization of possible isoforms of PXR, we cloned different translational isoforms harboring different in-frame methionines as PXR-M2, PXR-M3, PXR-M4 and PXR-M6. Further, we examined the protein expression profile of different isoforms by transient transfection in COS-1 cells and western blot analysis. In contrast to wild type PXR, expression of these deletion constructs showed respective expression of truncated protein bands that matched with respective additional bands of wild type PXR present in western blot profile. Results confirmed that these deletion constructs may be putative translational isoforms derived from different in-frame methionine of PXR protein.
- We examined the transcriptional behavior of these translational isoforms of PXR by transient expression in HepG2 cell-line along with promoter-reporter construct XREM-Luc. We observed that, none of these translational isoforms have any transcriptional activity when compared to the wild type PXR.
- Several NR isoforms may exhibit differential sub-cellular localization to modulate their transcription functions (Lu & Cidlowski, 2005; Pasqualini et al, 2001). So, to investigate the sub-cellular localization profile, different translational isoforms of PXR were cloned into GFP vector. Results revealed nuclear localization of PXR-M2 and PXR-M3 with a typical punctuate pattern in both ligand (rifampicin) treated or untreated conditions. However, in contrast to ligand-independent nuclear localization of wild type PXR (Saradhi et al, 2005b), PXR-M4 and PXR-M6 isoforms exhibited uniform nuclear and cytoplasmic localization.
- When compared to the mitotic chromatin binding property of wild type PXR some differences were observable with its isoforms (Saradhi et al, 2005b). PXR-M2 and PXR-M3 bound to mitotic chromatin to lesser extent whereas PXR-M4 and PXR-M6 were totally incapable of associating with the mitotic chromatin. These observations could be correlated to the fact that PXR-M2 and PXR-M3 have one zinc finger motif remaining while PXR-

M4 and PXR-M6 are devoid both of the zinc finger motifs which are conserved structure in NRs.

- To investigate the possibility of any influence of PXR isoforms on transcriptional activity of wild type PXR, co-transfection in HepG2 cells was performed with wild type PXR and either of its potential isoforms. We observed that in presence of different isoforms the transcriptional activity of wild type PXR gets down-regulated. This indicated that the co-existence of these isoforms inhibits the transcription function of the wild type receptor. Taking this into account, it can be speculated that the level and combination of different PXR isoforms, being expressed in different cell/tissue types, may differ and play combinatorial roles in normal physiological and disease states.
- In any cellular milieu, co-expression of multiple isoforms may influence the expression of the wild type form. To examine whether co-expression of the either translational isoforms of PXR affects the expression of wild type form, western blot analysis was performed with transient co-transfection of PXR and its potential isoforms in COS-1 cells. Experiments confirmed that co-expression of PXR isoforms, do not enhance or inhibit the expression of wild type PXR.
- The altered transcriptional behavior of wild type PXR in presence of either of its different translational isoforms may be due to alteration in co-regulator interaction profile. To explore this possibility mammalian two-hybrid assay was performed with Gal-SRC-1 and VP-PXR$_{WT}$, VP-PXR-M2, VP-PXR-M3, VP-PXR-M4 and VP-PXR-M6 constructs. We observed that when compared to the VP-PXR$_{WT}$ the translational isoforms VP-PXR-M4 and VP-PXR-M6 exhibit increased interaction with co-activator Gal-SRC-1 whereas VP-PXR-M2 and VP-PXR-M3 do not show any interaction to this key co-activator. So, this competitive recruitment of co-activator SRC-1 by PXR-M4 and PXR-M6 isoforms may affect transcriptional activity of wild-type receptor.

In conclusion, the work presented in this part of the study presents some of the novel insights into PXR isoforms. We have identified the existence of several translational isoforms which may differentially modulate PXR

functions. The modulation of PXR transcriptional activity by its different translational isoforms may be attributed to different mechanisms like i) differential co-factor interaction profile, ii) competitive binding of PXR hetero-dimeric partner RXRα and iii) competitive binding to PXR response element.

In the second part of this study based on post-translational modification of PXR, we have documented a novel interaction between PXR and SUMO-1. PXR is unique among all nuclear receptors as it is highly promiscuous in nature and is activated by a diverse array of endogenous hormones, dietary steroids, pharmaceutical agents and xenobiotic compounds (Saradhi et al, 2005; Zhou et al, 2009). In this study, a direct interaction between PXR and SUMO-1 demonstrates a novel mechanism of regulation of PXR transcriptional activity in liver cell line HepG2.

The major highlights from the second part of this study are outlined below:

- Our preliminary cell-based studies revealed that when the human PXR cDNA was transiently expressed in COS-1 cells, higher molecular weight protein bands were also detected along with its well-characterized band of 50 kDa. Though a few, there are reports suggesting that PXR undergoes several post-translational modifications like phosphorylation, ubiquitination, acetylation etc. However, no significant work has been done on SUMO-1 modification which is reported to influence the function several other NRs. Initially, utilizing bioinformatic tool 'SUMOplot™ prediction' four SUMOylation sites in human PXR were predicted. Three of the SUMO-1 sites were located in the LBD region while the fourth was located in the hinge region of the receptor. When compared, motif $_{107}$MKKE$_{110}$ had the highest probability for SUMOylation while the remaining three motifs ($_{128}$KKSE$_{131}$, $_{158}$MKTF$_{161}$ and $_{169}$FKNF$_{172}$) had lower probability for SUMOylation. To confirm our speculations derived from *in silico* analysis we conducted a series of *in vivo* experiments.
- To address the potential effects of SUMO-1 modification on PXR functions, we first examined the impact of SUMO-1 modification on PXR transcriptional activity. To investigate this, promoter-reporter based

luciferase assay was performed in HepG2 cells which were transiently co-transfected with XREM-Luc reporter gene and PXR expression plasmid and increasing doses of SUMO-1 expression plasmid. Rifampicin treatment led to significant increase in wild type PXR-mediated transactivation of the XREM-luciferase reporter activity. Interestingly, when SUMO-1 was exogenously co-transfected, rifampicin mediated PXR transcriptional activity was significantly increased by 30%. Increasing amount of SUMO-1 expression led to progressive increment of promoter activity imparted by the rifampicin activated PXR. Our results imply that SUMOylation activates PXR transcriptional activity, suggesting the possible role of SUMOylation in modulation of PXR function.

- The increase in PXR transcriptional activity does not necessarily imply that PXR directly interact with the SUMO-1. Several reports suggest that the trans-acting function of SUMO executed by modifying receptors' co-activator or non-covalent interaction with participating proteins may result into distinct physiological out-put. Therefore, by co-immunoprecipitation assay we further confirmed that enhancement in PXR activity is due to direct SUMOylation of PXR protein. COS-1 cells were transfected with constructs encoding PXR and GFP-SUMO-1 and whole cell extracts were immunoprecipitated with PXR polyclonal antibody. Further, western blot analysis with SUMO-1 antibody, detected a major band of approximately 90 kDa that corresponds to the covalently modified forms of PXR and GFP-SUMO-1. Results obtained from co-immunoprecipitation assay confirmed a direct interaction between PXR and SUMO-1 for modulation of PXR functions.
- Furthermore, modulation of PXR transcriptional activity by SUMO-1 prompted us to examine if the two molecules interact/co-localize in the cells. The intracellular dynamics studies using GFP-tagged PXR and RFP-tagged SUMO-1GG (active form of SUMO-1) revealed that the two proteins co-localizes in the nucleus in ligand-independent state to produce a yellow fluorescence. These co-localization studies of PXR and SUMO-1 partly explain the mechanisms involved in SUMO-1-mediated modulation of PXR-dependent functions.

- To explore the possibility of direct interaction between E2-conjugation enzyme UBCh9 with PXR for SUMOylation event, mammalian two-hybrid assay was performed. For execution of experiment, VP16-UBCh9 (VP16 transactivation domain fusion protein with UBCh9) together with a Gal4-DBD-PXR and a Gal4-luciferase reporter gene (FR-Luc) were taken as a read out for molecular interactions. Mammalian two-hybrid assay with GAL4-PXR and VP16-UBCh9 confirmed a direct interaction between the two proteins. This study further highlighted the ability of UBCh9 to interact with PXR and provide substrate specificity towards SUMOylation.
- SUMOylation is known to occur on the lysine residue with a consensus sequence of ΨKXE, with 'Ψ' representing a hydrophobic residue and 'X' representing any amino acid. To determine which residue of PXR serving as the SUMO attachement site, we focused on lys-108, lys-129, lys-160 and lys-170 because they reside in a motif that fits the ΨKXE consensus SUMOylation site. Among these, one motif lies in hinge region with high probability while other three lie in low probability motif. Each of the four lysines was mutated individually to arginine. We also performed combinatorial site-directed mutagenesis to examine the effect of these mutations on transcription function of PXR. We first examined the transcriptional activity of all these mutants and observed that $PXR_{K108R,}$ $PXR_{K129R,}$ PXR_{K160R} mutation, PXR_{3KR} and PXR_{4KR} mutations have lowered transcriptional activity when compared to the wild type PXR. So, this observation suggested that lys-108, lys-129, lys-160 may be the potential sites for SUMO-1 conjugation. We also examined the sub-cellular localization of all the mutants and revealed nuclear localization of all mutants similar to the wild type PXR. This implied that reduced transcription function is not due to altered sub-cellular localization of PXR.
- To elucidate whether the attachment of SUMO-1 to PXR affects PXR protein stability, we performed western blot analysis in COS-1 cells co-expressing SUMO-1 and either of the constructs encoding wild type PXR or different PXR SUMO mutants. No significant effect on PXR protein expression level was observed in the presence of SUMO-1 implying that

increment in wild type PXR-mediated activity is neither due to the enhancement of PXR expression nor due to the potential artificial effects linked to exogenous transfection of SUMO-1.

- Recently, bioinformatics analyses identified a subset of SUMO consensus sites called the PDSM (Phosphorylation Dependent SUMOylation Motif), which associates a classical SUMO consensus site with an adjacent proline-directed phosphorylation site (ΨKXEXXSP) and NDSM (Negative charge amino acid Dependent SUMOylation Motif). Through bioinformatic analysis we found that NDSM is present in human PXR at 107-120 amino acid residues, $_{107}$MKKEmims**D**eave**E**$_{120}$. Specific alanine substitution of acidic patches like PXR$_{D115A}$, PXR$_{E120A}$ and PXR$_{DE-A}$ proximal to the SUMO core consensus sites of PXR, was utilized for analysis of NDSM. We examined the luciferase activity on XREM-Luc promoter with PXR$_{D115A}$, PXR$_{E120A}$ and PXR$_{DE-A}$ constructs in absence or presence of SUMO-1. We observed that aspartic acid at 115 position of PXR may contribute to NDSM, since from our study it has been observed that SUMOylation enhances the transactivation potential of PXR, and mutation at asp-115 position leads to no significant change of PXR transcriptional activity even in presence of SUMO-1.
- Further, we examined the transcriptional activity of PXR using different drugs which are responsible for modulation of distinct signaling pathways. In this perspective, we treated HepXREM cells (HepG2 cells stably integrated with PXR and promoter-reporter CYP3A4-Luc) with different drugs like TSA (Trichostatn A, inhibitor of class I and II mammalian histone deacetylase), CPTH2 (Histone Acetyltransferase Inhibitor IV), PMA (Phorbol 12-Myristate 13-Acetate, an activator of Protein Kinase C) and OKA (Okadaic Acid, a potent inhibitor of PP1 and PP2A phosphatases) in presence or absence of rifampicin. Subsequent to treatments, we examined the transcription activity of PXR by promoter-reporter based assay. Upon PMA or OKA treatment, we did not observe any significant change in PXR transcriptional activity either in the presence or absence of rifampicin. However, on the contrary, previous experiment reported by Ding & Staudinger, 2005b showed reduced mouse PXR transcriptional

activity upon PMA or OKA treatment in presence of rifampicin. This difference in PXR transcriptional activity could be attributed towards species specific difference is regulating specific signaling cascades in distinct manner. Treatment with TSA increased the PXR transcriptional activity both in presence or absence of rifampicin. While under same conditions CPTH2 treatment decreased the PXR transcriptional activity. A previous study has shown that hypoacetylation of PXR leads to increase in transactivation potential of PXR (Biswas et al, 2011). It is well known that TSA is responsible for hyperacetylation of histone or non-histone proteins while CPTH2 reverses this phenomenon. Therefore, results from our study suggest that enhancement of PXR activity may not be due hyperacetylation rather due to chromatin modification by TSA. Similarly, repression of PXR activity by CPTH2 may be due to deacetylation of chromatin.

- We next examined the effect of these drugs on DNA binding activity of PXR by ChIP assay. In case of individual drug treatments with TSA, CPTH2, OKA and PMA, less PXR DNA binding activity was observed when compared to presence or absence of rifampicin treatment. Among all drug treatments TSA treatment resulted in enhanced DNA binding activity while CPTH2 treatment showed reduced DNA binding activity in HepXREM cells. The enhanced DNA binding activity of PXR by TSA may be explained by the fact that TSA treatment makes chromatin more accessible to PXR and different co-activators resulting in enhanced transcription function of PXR. Furthermore, CPTH2 treatment probably makes chromatin less accessible to PXR and different co-activators resulting in reduced transcriptional activity of PXR. No change in DNA binding activity of PXR was detected on ER6 promoter even after rifampicin co-treatment with OKA, PMA and TSA treatments. Further investigation involving the role of different cell signaling pathways in PXR-mediated differential functions and crosstalk between different signaling pathways will be necessary to fully understand the functional implication of these signaling events.

In brief, last part of the study demonstrated that SUMO-1 modification of PXR results into an enhancement of PXR transcriptional activity. Another finding suggested the presence of NDSM in PXR which may contribute to fine regulation of PXR SUMOylation event. In this study, it has also been found that SUMOylation of PXR does not alter sub-cellular localization and stability of PXR protein. We also observed that signaling pathways involved in phosphorylation or acetylation of PXR alter its transcriptional function and DNA binding activity. Investigation based on PXR SUMOylation may provide a basic insight into some novel mechanisms involved in PXR regulation and function.

Future Perspective

The present study documents the preparation and characterization of PXR monoclonal antibody which acts as a tool to detect different isoforms of PXR and their functional significance. Collectively, our study indicates the existence of multi-faceted nature of PXR isoforms and their cellular relevance. This study promises to explore, alteration in PXR isoforms expression and its interaction with distinct co-regulators likely has numerous consequences in PXR-dependent pathological conditions. The findings hold substantial importance in view of the fact that PXR plays key roles in xenobiotic clearance cascades of exogenous and endogenous metabolites and different pathological conditions. From clinical point of view, regulation of levels of these isoforms may prove to be an important prognostic marker for certain diseased states. Although the differential expression patterns can be detected either by polyclonal or monoclonal antibodies, the functional significance of these isoforms in different patho-physiological condition still remains unanswered. Additionally, what regulates the differential expression of these isoforms under different cancerous conditions also warrants further study which may provide novel targets for pharmaceutical intervention.

While it is clear that PXR undergoes various post-translational modifications including acetylation, ubiquitination, SUMOylation, it remains unclear as to which particular lysine residues are important for these

competitive modifications. A thorough study of these post-translational modifications is likely to provide more insight into PXR activation and repression. Future studies need to be focused on defining the molecular basis for these post-translational modifications to assess the absolute effects of these modifications on PXR functions. Ligand binding is the primary mode of PXR activation, but several other pathways also interface with PXR function like alteration of PXR protein modifications and subsequent modulation of interacting proteins. Further examination of the effects of post-translational modifications on gene expression and chromatin interaction is another important avenue of research persual. Ultimately, improved understanding of molecular signaling events of PXR action are expected to provide a better understanding of PXR functions in terms of its different isoforms and SUMOylation in different patho-physiological states.

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Publications

Patent

1. Rakesh K. Tyagi, **Priyanka**, Niti Puri. Generation and characterization of monoclonal antibodies against specific domains of human Pregnane and Xenobiotic Receptor (PXR) as a useful immunological tool in biological research and immunodiagnostics (under preparation).

Papers/manuscripts will be communicated subsequent to patent filing.

Conference Presentations/Abstracts

1. **Priyanka**: Transcription function of a nuclear receptor PXR is modulated by sumoylation *in* 81st Annual Meeting of the Society of Biological Chemists (India) at IICB, Kolkata, 8th-11th November, 2012.
2. **Priyanka,** Deepak Kotiya and Rakesh K. Tyagi: Novel insights into PXR isoforms and their relevance in cellular functions *at* the Annual Symposium of the Society for Reproductive Biology and Comparative Endocrinology (India) at Mohanlal Sukhadia University, Udaipur, 30 January to 1 February, 2012.
3. **Priyanka** and Rakesh K. Tyagi: Novel insights into PXR isoforms and their relevance in cellular functions *at* the annual research festival Biosparks 2012 conducted by School of Life Sciences, Jawaharlal Nehru University. 14th & 15th March 2012.