A comprehensive approach towards validation of small molecules on nuclear receptor PXR platform

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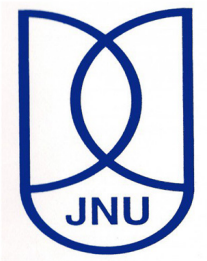

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CERTIFICATE

The research work embodied in this thesis entitled "**A comprehensive approach towards validation of small molecules on nuclear receptor PXR platform**" 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.

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Dedicated to

MY PARENTS

&

FAMILY

CONTENTS

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Shashi

ABBREVIATIONS

ABCB1	ATP Binding Cassette Subfamily B Member 1
ACTR	Activator of Thyroid Receptor
ADME	Absorption, Distribution, Metabolism and Elimination
AF1	Activation Function 1
AF2	Activation Function 2
AMP	Adenosine Mono Phosphate
AMPK	AMP-activated Protein Kinase
APS	Ammonium Persulphate
AR	Androgen Receptor
ATCC	American Type Cell Culture
ATP	Adenosine Tri Phosphate
bp	Base Pair
BCRP	Breast Cancer Resistance Protein
8-Br-cAMP	8-Bromo-Cyclic AMP
BSA	Bovine Serum Albumin
BXR	Benzoate X Receptor
˚C	Degree Celsius
cAMP	Cyclic AMP
CAR	Constitutive Androstane Receptor
CBP	CREB-Binding Protein
CDKs	Cyclin Dependent Kinases
CDS	Coding Sequence
CIP	p300/CBP Cointegrator-Associated Protein
CITCO	6-(4-Chlorophenyl) Imidazo [2,1-b][1,3]Thiazole-5-Carbaldehyde O-(3,4 dichlorobenzyl)oxime
COUP	Chicken Ovalbumin Upstream Promoter
CPT1A	Carnitine Palmitoyl Transferase 1A
CREB	Cyclic AMP Response Element Binding Protein
CYP	Cytochrome P450
DAX-1	DSS-AHC critical region of the X chromosome, gene 1
DBD	DNA-Binding Domain
DDIs	Drug-Drug Interactions
DEPC	Diethyl Pyrocarbonate
DMD	Drug Metabolism and Disposition
DMEs	Drug Metabolising Enzymes
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
dNTP	Deoxyribonucleotide Tri Phosphate
DPP-4	Dipeptidylpeptidase 4
DR	Direct Repeat
DTT	Dithiothreitol
EDTA	Ethylene Diamine Tetra Acetic Acid
ER	Everted Repeat
ERK	Extracellular Signal-Related Kinase
ERR	Estrogen-Related Receptor
ERs	Estrogen Receptors

FBS	Fetal Bovine Serum
FOXO1	Forkhead Transcription Factor 1
FXR	Farnesoid X Receptor
g	Gram
GLP-1	Glucagon-Like Peptide-1
GLUT	Glucose Transporter
G6Pase	Glucose 6-Phosphatase
GPCR	G-protein Coupled Receptor
GR	Glucocorticoid Receptor
GRIP1	Glucocorticoid Receptor Interacting Protein 1
GSH	Glutathione
GSK	Glycogen Synthase Kinase
GSTs	Glutathione S-Transferases
hr	Hour
HAT	Histone Acetyl Transferase
HDAC	Histone Deacetylase
HMGCS2	3-Hydroxy-3-Methy Glutarate Coenzyme Synthase-2
HNF-4	Hepatocyte Nuclear Factor-4
HRE	Hormone Responsive Element
HRP	Horse Radish Peroxidase
IBD	Inflammatory Bowel Disease
IDF	International Diabetes Federation
IR	Inverted Repeat
JNK	c-Jun-N-terminal Kinase
kDa	Kilodalton
LBD	Ligand-Binding Domain
LCA	Litocholic Acid
Luc	Luciferase
LXR	Liver X Receptor
M	Molar
MAPKs	Mitogen-Activated Protein Kinases
MDR1	Multi-Drug Resistance 1
mg	Milligram
min	Minute
ml	Millilitre
mM	Millimolar
µg	Microgram
µl	Microlitre
µM	Micromolar
MR	Mineralocorticoid Receptor
mRNA	Messenger RNA
MRP	Multidrug Resistance Associated Protein
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazoliumbromide
MW	Molecular Weight
NCo	Nuclear Receptor Coactivator
N-CoR	Nuclear Receptor Corepressor
NF-κB	Nuclear Factor-κB
ng	Nanogram
NHRs	Nuclear Hormone Receptors

NLS	Nuclear Localization Signal
NP-40	Nonyl Phenoxypolyethoxylethanol
NR	Nuclear Receptor
NTD	N-Terminal Domain
NURR-77	Nuclear Receptor Related Protein-77
OATP	Organic Anion Transporter Polypeptide
ONPG	O-Nitophenyl-Beta-Galactopyronoside
PAGE	Polyacrylamide Gel Electrophoresis
PBP	Peroxisome Proliferator Activated Receptor (PPAR)-Binding Protein
PBREM	Phenobarbital-Responsive Enhancer Module
PBS	Phosphate Buffered Saline
PCN	Pregnenolone-16α-Carbonitrile
PCR	Polymerase Chain Reaction
PDB	Protein Data Bank
PEPCK1	Phosphoenol Pyruvate Carboxykinase
PGC-1	Peroxisome Proliferator Activated Receptor Gamma Coactivator 1
P-gp	P-Glycoprotein
pH	Power of Hydrogen
PKA	Protein Kinase A
PKC	Protein Kinase C
PMSF	Phenyl Methyl Sulphonyl Fluoride
PPAR	Peroxisome Proliferator-activated Receptor
PP2C	Protein Phosphatase 2C
PPRE	Peroxisome-proliferator-Activator Response Element
PR	Progesterone Receptor
PVDF	Poly-Vinyl-di-Fluoride
PXR	Pregnane& Xenobiotic Receptor
RAR	Retinoic Acid Receptor
RE	Response Element
RFP	Red Fluorescent Protein
ROR	Retinoic Acid Receptor-Related Orphan Receptor
RT	Room Temperature
RXR	Retinoid X Receptor
s	Second
SAPKs	Stress-Activated Protein Kinases
SCD-1	Stearoyl-CoA Desaturase-1
SDS	Sodium Dodecyl Sulphate
SE	Standard Error
SF-1	Steroidogenic Factor-1
SGK	Serum/Glucocorticoid Regulated Kinase 2
SGLT-2	Sodium-Glucose Co-transporter-2
SHP	Small Heterodimer Partner
SJW	St John's Wort
SLC	Solute Carrier
SMRT	Silencing Mediator for Retinoid and Thyroid-Hormone Receptors
SRC-1/2/3	Steroid Receptor Coactivator-1/2/3
SREBP-1c	Sterol Regulatory Element-Binding Protein-1c

SUs	Sulfonylureas
SULTs	Sulfotransferases
SWI/SNF	SWItch/Sucrose Non Fermentable
TAE	Tris Acetate EDTA
T2DM	Type II Diabetes Mellitus
TEMED	N, N, N', N'-Tetra Methyl Ethylene Diamine
TFs	Transcription Factors
TIF2	Transcriptional Intermediary Factor 2
TR	Thyroid Receptor
TRAM	Thyroid Receptor Activator Molecule
Tris	Tris-(hydroxyl methyl)-Amino Methane
TZDs	Thiazolidinediones
U	Units
UGT1A1	UDP-Glucuronosyltransferase 1A1
VDR	Vitamin D Receptor
XREM	Xenobiotic Responsive Enhancer Module

INTRODUCTION

Nuclear Receptors (NRs) are ligand-modulated transcription factors playing important roles in various physiological processes of cellular proliferation, differentiation, reproduction, development and metabolism (Theotokis et al., 2013). Pregnane and Xenobiotic Receptor (PXR; NR1I2) is one of the 48 members of NR superfamily. It is a 'master-regulator' of 'drug metabolism and disposition machinery' comprised of phase I, phase II drug metabolizing enzymes (DMEs) and phase III membrane transporters which eliminates chemicals from cells. In this manner, PXR acts as a 'xenosensor' playing a pivotal role as a constituent of the defense mechanism of our body. It is reported to respond against myriads of exogenous (therapeutic drugs, dietary supplements, endocrine disruptors etc.) or endogenous compounds (steroids, lithocholic acid etc.) (Goodwin et al., 2002). This characteristic of ligand promiscuity differentiates PXR from the other members of NR superfamily. PXR is predominantly expressed in liver and intestine.PXR heterodimerizes with RXR (Retenoid X Receptor) upon ligand bindingand interacts with the response element of its target genepromoters (including components of DMD machinery) to regulate their expressions. The components of 'drug metabolism and disposition machinery' which it activates are i) Phase I enzymes (CYP3A4, CYP3A7, CYP3A11, CYP3A23, CYP2B6, CYP2B9, CYP2C8, CYP2C9, CYP2C19, CYP2C55 and CYP1A), which are involved in oxidation, reduction, hydrolysis and hydration of lipophilic xenobiotics to make them water soluble (Koki et al., 2007); ii) Phase II enzymes are transferases (UDP-glucuronosyltransferases UGTs, sulfotransferases SULTs and glutathione S-transferases GSTs) which add some polar groups to further increase the polarization and solubility of xenobiotics and make them convenient for biliary and urinary excretions (Sonoda et al., 2002; Wang et al., 2012); and iii) Phase III transporter [multidrug resistant proteins (MDR1, MDR2), multidrug resistance associated protein 2 (MRP2) and the organic anion transporter polypeptide 2 (OATP2) etc.] that finally excrete the noxious chemicals out of the cell (Staudinger et al., 2001). Small molecule modulators have profound effect on the 'yin and yang' mode of PXR activation. On one hand PXR activation by small molecule modulators enable this receptor to play an instrumental role in 'detoxification and elimination' of toxic xenobiotics/endobiotics, while, on the other hand, PXR activation also imposes a serious concern for drug-drug interactions (DDIs). Such DDIs could decrease the efficacy (by fast elimination of parent drug) or increase the toxicity (causing parent drug to generate reactive and toxic metabolites or leading to drug accumulation at

toxic level) of co-administered drugs by altering the metabolism of small molecules that it senses in the cellular milieu. Therefore, it is desirable that during drug development process, small drug molecules are screened on PXR-platform prior to their clinical trial to avoid late stage failure due to activation of 'drug metabolism and disposition' machinery.

PXR shares a structurally conserved, similar domain structures like other NRs including, highly conserved and centrally located DNA binding domain (DBD). DBD contains two highly conserved zinc fingers and P-box. This P-box is involved in receptor dimerization and providing response element binding sequence specificity in the corresponding target genes of NRs. At the N-terminus of DBD is a highly variable N-terminal domain (NTD) which harbors constitutively active AF-1 (activation function domain) region. Towards the C-terminus of DBD is, moderately conserved ligand binding domain (LBD). LBD contains ligand binding sites and transcriptional activation function domain AF-2, which acts in ligand-dependent fashion. AF-1 and AF-2 are present at the extreme of N-terminal and C-terminal regions respectively to provide platform for binding of co-regulators (co-activators and co-repressors). LBD connects with DBD through a less conserved hinge region (D domain) which contains nuclear localization signal (NLS) (Huang et al., 2010). PXR is devoid of AF-1 region (Pondugula et al., 2009). The DBD of PXR is 95% conserved across all mammals but ligand binding domain shows more sequence variations. Ligand binding domain of PXR is relatively less conserved among mice, rat, rabbit and human (LeCluyse, 2001). Therefore, ligand preference and pharmacological activation of PXR also differs across these species (Jones et al., 2000). Interestingly, rifampicin acts as a ligand of human PXR but not for mouse PXR. Similarly, PCN (5-pregnen-3β-ol-20-one-16α-carbonitrile) is known as a potent ligand of mouse PXR and poor ligand of human PXR (Wang et al., 2012). Therefore, inferences drawn from animal studies may not always hold true for human subjects. PXR is unique among NRs because of its broad ligand specificity and low affinity which is attributed to its distinctive LBD structure (Ngan et al., 2009).

The cytochrome P450 (CYPs), are heme containing proteins with monooxygenase activity (Jonsson-Schmunk et al., 2018). CYP3A (CYP3A4, CYP3A5 and CYP3A7) forms are abundantly expressed in humans and account for

metabolizing various clinical drugs. In 1986, CYP3A4, the first member from human CYP3A family was identified (Jonsson-Schmunk et al., 2018). CYP3A4 is most abundant in humanand is responsible for the metabolism of more than 50% of endogenous and exogenous compounds (Goodwin et al., 1999). CYP3A4 is transcriptionally regulated by various NRs like PXR, CAR, GR and HNF-4α by binding with CYP3A4 promoter regions. Among these transcription factors, PXR has been reported as the master regulator of CYP3A4 (Jonsson-Schmunk et al., 2018).

PXR is reported to regulate about 40 genes of the 'drug metabolizing and clearance machinery' (Aouabdi et al., 2006). PXR is also reported to cross-talk with other NRs or signaling pathways (Zhou et al., 2006; Pascussi et al., 2008). Thus, its dysregulation is suggested to be involved in various pathological conditions of cancer or other metabolic disorders (Qiao et al., 2013), inflammatory bowel disease, inflammation (Zhou et at., 2006) and also in the regulation of energy homeostasis, bone homeostasis, bile acid homeostasis, vitamin D metabolism (Pascussi et al., 2000) and lipid metabolism.

One of the major metabolic disorders causing global concern is diabetes mellitus (Kaiser et al., 2014). This metabolic disease is characterized by hyperglysemia, glycosuria, coronary artery disease and congestive heart failure. There are two types of diabetes mellitus one is type I (T1DM) that is insulin-dependent and genetic. This is characterized by deficiency of insulin because of autoimmune destructions of β-cells. Type II is insulin-independent and is characterized by insulin resistance. Type II diabetes (T2DM) is diagnosed in ~95% of the overall cases of diabetes (Agrawal et al., 2013).

There are various reports proposing the ambiguous roles of PXR in diabetes (Hukkanen et al., 2014). Some reports suggest the anti-hyperglycemic role of PXR due to suppressing gluconeogenic genes (Konno et al., 2008). There are different TFs (FOXO1, HNF-4 and CREB) known to regulate the expression levels of rate-limiting enzymes PEPCK and G6Pase by binding with the promoter of these gluconeogenesis genes. PXR is known to interact with these TFs and hinders their binding with promoters of gluconeogenesis genes.When mice were given the treatment of PXR agonist PCN (pregnenolone 16α-carbonitrile), PEPCK, G6Pase, carnitine palmitoyl transferase1A and 3-hydroxy-3-methylglutaryl-CoA synthase 2 expression levels

were found to be down-regulated (Rysa et al., 2013; Gotoh et al., 2015). Similar effects were observed when PXR (VP-PXR) was expressed in transgenic mice (Hakkola et al., 2016). Conversely, in other reports, PXR expression in transgenic mice was suggested to promote the accumulation of triglycerides in liver, leading to diabetes.This deposition was independent of the involvement of lipogenic transcription factor SREBP-1c. Expressions of free fatty acid transporter CD36 and other lipogenic gens like SCD-1 (steroyl-CoA desaturase-1) and long chain free fatty acid elongase were increased in PXR transgenic mice (Zhou et al., 2006). In healthy human volunteers, rifampicin was shown to increase the glucose level during oral glucose tolerance test (Rysa et al., 2013). In such a case, drug-activated PXR was shown to induce gluconeogenic genes. Also, serum and glucocorticoid-regulated kinase 2 (SGK2) and G6Pase were found to be induced in HepG2 cells expressing PXR ectopically (Gotoh et al., 2015). Hitherto, the exact role of PXR in diabetes is still obscure. Since the activation of PXR is species-specific thus conflicting results may be observed in mouse model and in human or human derived cell lines.

Various types of anti-diabetic drugs are used to treat type II diabetes, some are well-known, newly approved and some are withdrawn due to their adverse side effects causing liver damage, heart failure or renal failure (Kaiser and Oetjen, 2014). Troglitazone is one of the drugs from withdrawn category, which has been stopped due to its hepatotoxicity. Later, it was found that troglitazone oxidatively metabolized into a cytotoxic quinone product and CYP3A4 played a major role behind this oxidation (He et al., 2001). PXR is believed to be an essential regulator of CYP3A4. Rosiglitazone and pioglitazone are other oral anti-diabetic formulations which are also members of thiazolidinedione like troglitazone and have been withdrawn from the several countries owing to their association with cardiotoxicity and bladder cancer respectively (Nissen and Wolski, 2007; 2010; Zhu et al., 2012). It has also been reported that some anti-diabetic drugs regulate PXR expression (Krausova et al., 2011). Thus, it will be interesting to unravel whether PXR has any role in working of anti-diabetic drugs or in the withdrawal of these anti-diabetic drugs.

Prescription of combination therapy is a common practice during the treatment of many metabolic disorders and infectious diseases. In such combination therapies one drug may modulate the expression of genes of ‘DMD’, influencing the

metabolism of another co-administered drug. This leads to decreased bioavailability or increased toxicity of the latter. Many of the drugs like terfenadine, suprofen, rofecoxib, mibefradil, cisapride etc. have been withdrawn for showing toxicity (Sun et al., 2010). Evaluation of drug-drug interactions (DDIs) has now become a major safety concern during drug discovery and development processes (Sinz, 2013; Wang et al., 2014). TZDs are also recommended in combination therapy. Co-administration of other oral anti-diabetic drugs is followed in the cases where there is no effect of monotherapy (Tornio et al., 2012). Because of such a combination therapy, patients are at high risk of having severe side-effects of DDIs. Attenuation in the effect of SUs has already been known when it was prescribed for co-medication (Tornio et al., 2012). Being a principal regulator of 'DMD' machinery, PXR is suggested as a therapeutic target in drug screening process (Goodwin et al., 2001; Cecchin et al., 2016).Thus, regulation of CYP3A4, MDR1, MRP2 and OATP2 is critical because of their role in drug-drug interaction by which one drug exacerbate the metabolism of second drugs if used in combination (Moore et al., 2000). So, PXR-mediated altered metabolism of anti-diabetic drugs would culminate into clinical and market failure. It is therefore, reasonable to assess the pharmacokinetic properties of a drug for PXR activity at initial cellular level before going to human clinical trial.

There are various approaches for screening a library of drugs. Some of them are cell-free ligand binding assays (fluorescence polarization), cell-based two-hybrid assay, cell-based transactivation assays etc. Ligand binding assays are rapid but being cell-free, they do not reflect the exact image of what is happening in cellular environment. Though, two hybrid assays are cell-based and able to predict the therapeutic behavior of drugs inside the cells, but they are not much reliable as it is carried out with only a portion of target protein of interest so could not represent same structure and functionality of target protein of interest. Now-a-days, cell-based trans-activation assays are used more often for high-throughput screening of drugs, which surmount the drawbacks of assays mentioned above (Pinne and Raucy, 2014).It is apparent that in cell-based assays, there is no experimental variations in stable transfection and is also cost effective than transient transfection based assays (Kim et al., 2010). The primary goals of the drug discovery process are to develop such agents that show only targeted action without any adverse effects. From all these studies, it is apparent that, to select the best drug candidate PXR assay must be included in high-

throughput screening of small molecules. There are various levels at which PXR screening could be performed. It could be at PXR protein level/PXR-responsive promoter level (e.g. CYP3A4 promoter, components of detoxification machinery), and at the PXR-promoter level. Several therapeutic drugs used in the treatment of metabolic disorders (including novel, established and redundant drugs) have not been concretely examined on PXR-platform levels owing to the absence and standardization of multi-level screening protocols. We judiciously selected some novel (dapagliflozin), established (metformin, glimepiride, repaglinide, tolbutamide, chlorpropamide, gliclazide) and redundant (rosiglitazone, pioglitazone, troglitazone) anti-diabetic drugs and screened them at dual level of PXR platform by using stable liver cell lines as our tools. Investigation and identification of a multi-tier, cell culture-based drug screening approach on the nuclear receptor PXR platform may explain why some drugs are clinically successful while others fail or exhibit drug-drug interactions. Keeping this background in view the following '*Aims and Objectives*' were framed for the present study:

1) To develop a multi-tier high-throughput screening for endobiotics and xenobiotics (including clinical drugs) with the involvement of nuclear receptor PXR and other associated components of detoxification machinery.

2) To evaluate some of the selected novel, established and redundant therapeutic drugs used in treatment of common metabolic disorders.

3) To formulate a judicious proposal for a working model having the competence to predict the efficacy for clinical success, drug-drug interactions of a molecule etc.

The aim of this study was to investigate the modulation of PXR both at its transcriptional level and post-translational level by utilizing the stable cell lines generated in our laboratory. Also, the PXR-dependent regulations of the components of 'DMD' machinery and cross-talk between PXR and certain signaling pathways have been examined. To study the effectof small molecules on PXR-promoter level, we generated Hepx-497/+43 stable cells in HepG2 (liver cell line). This cell line was stably transfected with PXR-promoter region -497/+43 cloned in frame with Luc gene, coding for luciferase enzyme. Next, to screen drugs for their modulatory effect on PXR transcriptional activity, another HepG2 derived cell line, HepXREM was

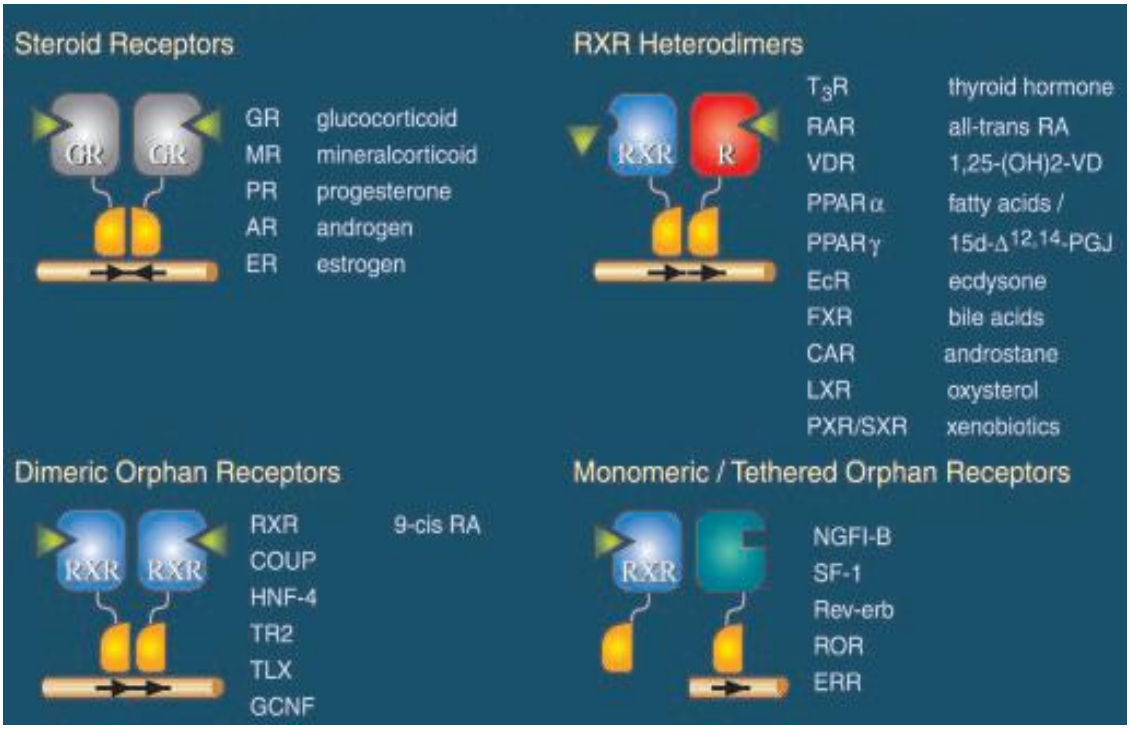
generated. In this cell line, PXR protein was expressed along with CYP3A4-promoter-reporter construct (XREM-Luc). In the above contexts, studying PXR modulation by therapeutic drugs appeared to be important to understand the pharmacokinetic profile of drugs. Pre-assessment of the anti-diabetic drugs for modulatory effects on PXR and induction of the components of 'drug metabolism and disposition' machinery can resolve the safety concerns, treatment failures and drug withdrawals due to the harmful drug-drug interactions.

REVIEW OF LITERATURE

An overview of Nuclear Receptor Superfamily

Human Genome codes for 48 members of Nuclear Receptor superfamily which are ligand-modulated transcription factors (Jin and Li, 2010). Ligand-activated Nuclear Receptors are implicated to play an important role in most of the fundamental aspect of physiological processes like development, homeostasis, metabolism and reproduction (Theotokis et al., 2013). Regulation of these biological processes isattributed by either genomic/non-genomic as well as ligand-dependent/ligand-independent activities of NRs. NRs are evolved in early metazoans long before the bifurcation of phylogenetic tree into vertebrates and invertebrates (Germain et al, 2006). In mid 1980s, the first NR gene was cloned by Evans laboratory named GR (Glucocorticoid receptor) followed by ER (Estrogen receptor) cloning by Chambon group (Laudet et al, 1992). Since then, many NRs have been cloned and reported to show high degree of sequence and functional similarities with each members. NR superfamily includes two subfamilies named, nuclear hormone Receptors (NHRs) and orphan Nuclear Receptors. Within 48 members of NR superfamily, 24 members are liganded-receptors and rests are orphans (**Table I)** (Gronemeyer et al, 2004). Nuclear Hormone Receptors are activated by their ligands to perform the activation or repression of target genes. NHRs are also known as classic receptors activated by steroidal ligands like steroids, vitamin D3, thyroid hormone, retinoic acids (Huang et al, 2010) and corresponding NRs for these endocrine ligands are GR, ER, PR, AR and MR Nuclear Receptors (Jin and Li, 2010). Another subfamily of NR superfamily has orphan as well as adopted orphan nuclear receptors and they show sequence similarities with the existing NHRs. They are named orphan because of their unidentified ligands atleast at the time of their discovery, thus possibly known to be regulated by some another means of post-translational modifications (Huang et al, 2010). Over the past few years, some endogenous ligands for few of the orphan NRs have been reported. These intracellular ligands are cholesterol derivatives like pregnane, bile acids, and byproducts of lipid metabolism like fatty acids, prostaglandins, leukotrines and benzoate derivatives. Therefore, now these orphan NRs are called as adopted orphan Nuclear Receptors due to their currently reported ligands. Members of this adopted orphan NR subfamily are PXR, CAR, LXR, PPARs, FXR and RXR. While, for rest of NRs their ligands are still unidenfified, thus remain orphan Nuclear Receptors like COUP, HNF-4, SF-1, ROR, ERR etc.

Manglesdorf has classified NR superfamily into four classes, in which Class I) consists of NRs responding to their hormonal ligands and bind as homodimers to the half-site inverted repeat RE present in their target genes promoter. Class II) includes those members which heterodimerize with RXR and bind with the inverted repeat RE half-site of target genes. Class III) and IV) consists of putative orphan NRs with unreported ligands and bind as homodimer to the direct repeat RE half-site and as monomer to the single half site RE present in the promoter of their target genes, respectively (Olefsky, 2001). Schematic representation of NR superfamily classification into four classes with ligands of each member is shown in **Figure 1**.



(*Adapted from JBC, 2001)*

Figure 1: Classification of NR superfamily. *NR superfamily has been sub-divided in four classes based on the types of ligands (known or unidentified), types of dimerization (homo or heterodimerization), types of response element (RE) repeat (direct or inverted) present on target gene promoters etc. Class I) members are steroids hormone receptors having affinity for hormonal ligands, class II) members heterodimerize with common partener RXR, class III) include orphan receptors functioning as homodimer and class IV) members are also orphan but bind as monomer to the single repeat RE of target genes promoter. In case of class I and class II NRs, the ligands of each member have also been shown except orphan receptors (classes III and IV), because their ligands are still known to be identified.*

Table-I: Human Nuclear Receptors

Name	Abbreviation	Nomenclature	Ligand
Thyroid hormone receptor	TRα TRβ	NR1A1 NR1A2	Thyroid hormone Thyroid hormone
Retinoic acid receptor	RARα RARβ RARγ	NR1B1 NR1B2 NR1B3	Retinoic acid Retinoic acid Retinoic acid
Peroxisome proliferator-activated receptor	PPARα PPARβ PPARγ	NR1C1 NR1C2 NR1C3	Fatty acids, leukotriene B4, fibrates Fatty acids Fatty acids, prostaglandin J2,
Reverse erbA	Rev-erbα Rev-erbβ	NR1D1 NR1D1	Orphan Orphan
RAR-related orphan receptor	RORα RORβ RORγ	NR1F1 NR1F2 NR1F3	Cholesterol, cholesteryl sulphate Retinoic acid Retinoic acid
Liver X receptor	LXRα LXRβ	NR1H3 NR1H2	Oxysterols, T0901317, GW3965 Oxysterols, T0901317, GW3965
Farnesoid X receptor	FXRα FXRβ*	NR1H4 NR1H5	Bile acids, Fexaramine Lanosterol
Vitamin D receptor	VDR	NR1I1	1,25-dihydroxy vitamin D_3, litocholic acid
Pregnane X receptor	PXR	NR1I2	Xenobiotics, PCN
Constitutive androstane receptor	CAR	NR1I3	Xenobiotics, phenobarbital
Human nuclear factor 4	HNF4α HNF4γ	NR2A1 NR2A2	Orphan Orphan
Retnoid X receptor	RXRα RXRβ RXRγ	NR2B1 NR2B2 NR2B3	Retinoic acid Retinoic acid Retinoic acid
Testis receptor	TR2 TR4	NR2C1 NR2C2	Orphan Orphan
Tailless	TLL	NR2E2	Orphan
Photoreceptor-specific nuclear receptor	PNR	NR2E3	Orphan
Chicken ovalbumin upstream promoter-transcription factor	COUP-TFI COUP-TFII	NR2F1 NR2F2	Orphan Orphan
ErbA2-related gene-2	EAR2	NR2F6	Orphan
Oestrogen receptor	ERα ERβ	NR3A1 NR3A2	Oestradiol-17β, tamoxifen, raloxifene Oestradiol-17β, various synthetic compounds
Oestrogen receptor-related receptor	ERRα ERRβ ERRγ	NR3B1 NR3B2 NR3B3	Orphan DES, 4-OH tamoxifen DES, 4-OH tamoxifen
Glucocorticoid receptor	GR	NR3C1	Cortisol, dexamethasone, RU486
Mineralocorticoid receptor	MR	NR3C2	Aldosterone, spirolactone
Progesterone receptor	PR	NR3C3	Progesterone, medroxyprogesterone acetate, RU486
Androgen receptor	AR	NR3C4	Testosterone, flutamide
NGF-induced factor B	NGFIB	NR4A1	Orphan
Nur related factor 1	NURR1	NR4A2	Orphan
Neuron-derived orphan receptor 1	NOR1	NR4A3	Orphan
Steroidogenic factor 1	SF1	NR5A1	Orphan
Liver receptor homologous protein 1	LRH1	NR5A2	Orphan
Germ cell nuclear factor	GCNF	NR6A1	Orphan
DSS-AHC critical region on the chromosome, gene 1	DAX1	NR0B1	Orphan
Short heterodimeric partner	SHP	NR0B2	Orphan

(Adapted from Nature reviews, drug discovery, 2004)

Structural organization of NR superfamily

Crystal structure has revealed the critical insight into the domain organization of nuclear receptors (Jin and Li, 2010). Flexibility of domains of NRs has made crystallization of NRs challenging (Jin and Li, 2010). All members of NR superfamily share a common structural organizationas well as sequence similarity. They contain six distinct domains with highly variable A/B domain present at the extreme of N-terminus of NRs. Within this A/B domain, there is a constitutively active AF-1 region present which is required for ligand-independent activation of NRs along with some other activation domains also. Numbers of amino acids within the A/B region vary from less than 50 to more than 500 among different NRs. Therefore, length of A/B region also varies, as in case of CAR A/B region is short in length without AF-1 function (Baes et al, 1994). Till date, no structure for AF-1 is available. Towards the C-terminal of A/B domain is a centrally located and highly conserved DNA binding domain (DBD) C is present. Based on the 3D structure available for some of the NRs, it is found that there are two highly conserved zinc finger motifs present in DBD, which are responsible for binding with response element (RE) present in the regulatory regions of their target genes. Despite having sequence conservation among all NRs, in order to provide sequence specificity in terms of binding with different target genes there is a motif present, named P-box which is responsible for receptor dimerization also. It is the DBD which allow NRs to bind to their targets differently (Jin and Li, 2010). Nuclear receptor DAX-1 and SHP are those NRs which lack DBD but regulate target genes by acting as co-repressors as they compete with co-activators to bind with NRs (Jin and Li, 2010). Positioned along the C-terminus of DBD is, a moderately conserved and largest domain E, which is required for ligand binding, thus named named ligand binding domain (LBD). LBD is a complex domain, because it serves as a dimerization interface and also harbors the AF-2 region required for ligand-dependent activation of NRs. DBD and LDB show highest sequence similarity among NRs (Jin and Li, 2010). Ligand binding triggers the conformational changes in AF-2 region,rendering it to bind with co-activators (coregulators), which help in making the promoters of gene, accessible to bind basal TFs and RNA polymerase (Zassadowski et al., 2012). LBD is made up of 11-13 α-helices and ligand (after binding with NRs) contacts H3, H5, H6, H7 and H10 helices while helices 3, 4 and 12 are organized in a manner to form hydrophobic groove where coregulators usually

bind (Jin and Li, 2010). In order to provide flexibility, NRs encompass a less conserved D domain (Hinge region), connecting DBD (C domain) to LBD (E domain). This flexible hinge region possess NLS (Nuclear localization sequence) required for NRs localization towards nucleus which overlaps with DBD. At the extreme C-terminus, F domain is present whose function is hitherto unknown (Rechavi et al, 2003; Bhasin et al, 2004). Domain organization of NRs is shown in **Figure 2**.

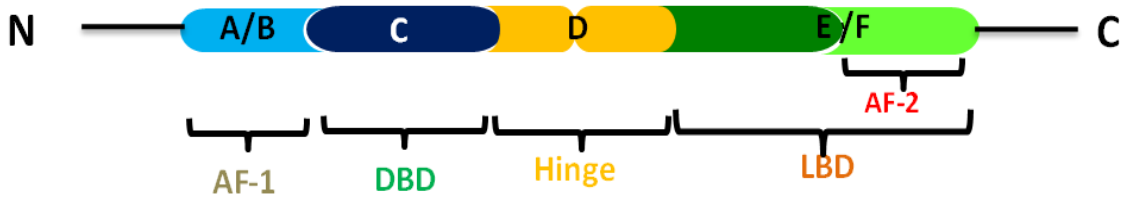


Figure 2: Schematic representation of domain structure of Nuclear Receptors. *At the extreme N-terminal domain A/B, an AF-1 region is present which acts as an interacting site for other transcription factors. At the centre, DNA binding domain (C) is present, through which NR bind with target genes regulatory elements. At the extreme C-terminus, ligand binding domain E/F is present, which acts as a binding site for ligands. Also, it harbors AF-2 region to interact with co-activators. A hinge region (D domain) encompasses NLS and forms a link between C and E/F domain.*

Mode of action of NRs

Nuclear Receptors are ligand-activated and DNA binding transcription factors regulating the broad spectrum of biological processes of development, differentiation, apoptosis and metabolism (McKenna, 2016). Beside the regulation of these physiological phenomenons, they are also involved in patho-physiological conditions of cancer, inflammation and metabolic diseases like diabetes. Being ligand-inducible transcription factors they get activated by binding of ligands. To execute their transcriptional functions (activation or repression), NRs require different co-regulators. Around 300 different types of co-regulators have been reported (Jin and Li, 2010). Most of the NRs in the absence of ligands remain in the complex form in association with co-repressors, to repress their target genes. Ligand binding introduces conformational changes in the NRs, leading to the dissociation of co-repressors, followed by recruitment of co-activators. Multi-component complex of co-repressors possess histone de-acetylase activity. Histone de-acetylation makes chromation more compact thus, promotes transcriptional repression of genes. Interactions of co-

activators with NRs help in chromatin remodeling and de-compaction to generate transcriptionally permissive environment at the RE (response element) present within the regulatory sites of target genes. Binding to the RE is mediated by P-box of the DNA binding domain of the NRs. These REs are organized as two hexameric half-sites with different orientations and named as direct repeat (DR), inverted repeat (IR) and everted repeat (ER), separated by variable numbers of nucleotides spacer. The consensus half-site sequence is AGGTCAnx which orient themselves i) in DR as AGGTCAnx-AGGTCA; ii) in ER as TGACCTnx-AGGTCA iii) AGGTCAnx-TGACCT in IR (Zassadowski et al, 2012). Co-activators are divided in two categories. One is the members of SWI/SNF group of proteins while second one belongs to HAT (Histone acetyl transferase). The most studied group of HAT category is p160 family. Within this p160 family, three members have been reported till date. I) include SRC-1/Nco-A1; II) include TIF2/GRIP1/Nco-A2 and III) include p/CIP/ACTR/RAC3/AIBE/TRAM1. SRC-1 further recruits cyclic AMP response element binding protein (CBP), p300 and p300/CBP, another acetyl transferase. Co-activators from HAT category add acetyl group to histones, which is responsible for chromatin de-compaction. Other co-activators, SWI/SNF chromatin remodeler unwinds DNA in ATP-dependent manner. Beside this, some co-activators execute their indirect action by facilitating communication between NRs and general transcription machinery by bridging them (Dilworth and Chambon, 2001). In order to de-condense the DNA from histones, large histone acetylase machinery has to work. General mechanism of action of NRs has been shown in **Figure 3**.

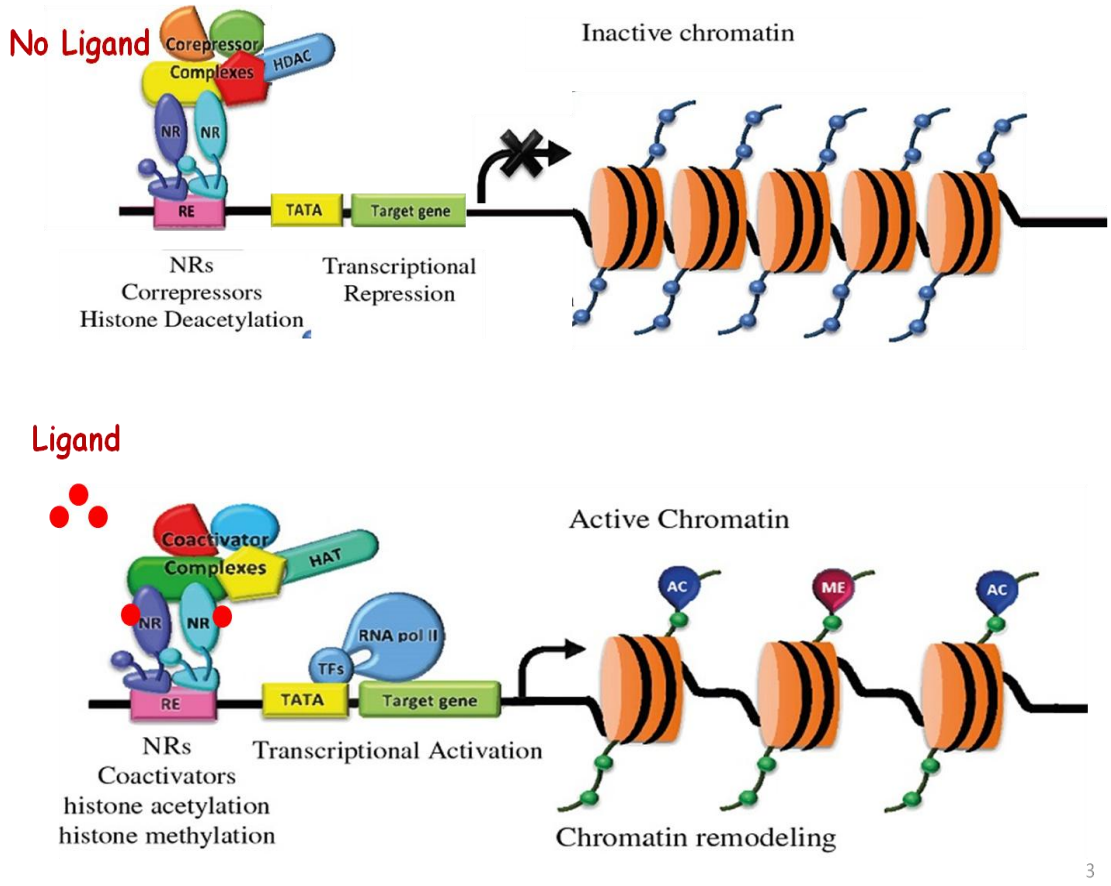


Figure 3: Pictorial representation showing mechanism of NRs action. *In absence of ligand, NRs remain bounded with co-repressor complexes of SMRT, NCoR along with HDACs, which de-acetylate histone, rendering chromatin in tightly bound inactive state. This results in transcriptional repression of target genes. In the presence of ligand, co-repressors become dissociated, followed by co-activators recruitment, acetylating the histone and making chromatin de-repression and promoting NR binding to specific DNA element (RE) present in the upstream promoter sequences of the specified NR target genes leading the expression of NR regulated genes.*

Pregnane & Xenobiotic Receptor (PXR)

Discovery and characterization of PXR has remained elusive until 1995, when Guzelian PS's laboratory started suspecting the presence of nuclear factors other than glucocorticoidsin transcriptionally up-regulating CYP3A1 gene. They suspected regulation of CYP3A1 gene at transcriptional level, as a mechanism behind its induction by dexomethasone and PCN synergistically. For this purpose, they had cloned 1.5kb promoter region of CYP3A1 gene and made the chimeric constructs of deletion mutants of this promoter fused with CAT (chloramphenicol acetyl transferase) gene. They had transfected primary hepatocytes of adult rat with these chimeric deletion constructs and delineate the presence of 33 bp minimal promoter

responsible for CYP3A1 gene induction. Suprisingly, this minimal promoter lacked the consensus GRE (glucocorticoid response element) sequence which was supposed to be bounded by steroid receptor GR, but instead showed pattern of response element recognized by non-steroidal nuclear receptor (direct repeat half-sites AGTTCA separated by three nucleotide spacer) (Kliewer et al., 1998). So, they concluded that GR indirectly induce CYP3A1 gene by binding with some other pre-assembled nuclear factors (Quattrochi et al., 1995). Later in 1996, same group tried to find the suitable animal models to understand the CYP3A4 heterogeneity and variability among humans by measuring the promoter activity of CYP3A family members across certain species, treated with different xenobiotics. They found that PCN (pregnenolone 16α-carbonitrile) has induced the CYP3A23 (a homologue of CYP3A4) gene in rat liver/hepatocytes but unable to induce CYP3A6 (a homologue of CYP3A23) gene in rabbit liver/hepatocytes, while rifampicin had shown similar induction of CYP3A in both the species. They performed sequence analysis of the 5' flanking region of CYP3A members in human, rat and rabbit and found a significant similarities among them. When they transfected the chimeric construct CYP3A6-CAT in rat, they observed the induction of CYP3A6 in same manner as of rat's own CYP isoform CYP3A23. So, they suspected about the differences in cellular environment, having different levels of trans-acting factors may be responsible for difference in the induction of CYP3A members across these species (Barwick et al., 1996). In 1998, PXR was identified by Steven A. Kliewer's laboratory by motif search from mouse EST database. They identified this new member of NR superfamily from mouse cDNA library which showed similarity with NRs reported at that time (Kliewer et al., 1998). Further, they tried to find the expression of PXR in different tissues of mouse embryo/adult by in situ hybridization and northern blot analysis and found the prominent expression of PXR in liver and intestine, while low expression was also detected in stomach and kidney (Kliewer et al., 1998). Parellely, Ron Evans cloned human orphan nuclear receptor from human genomic DNA library and named as SXR, because of getting activated by a variety of natural and synthetic steroids, which showed significant similarity with Xenopus Benzoate X Receptor (BXR) (Blumberg et al., 1998). Later on, SXR was found to have similarity of ~95% in DBD and ~73% in LBD to PXR (Blumberg et al., 1998). Therefore, on the basis of the findings from Guzelian's laboratory, about the presence of an un-identified cellular factor and atypical GRE in the CYP3A promoter, they suspected that, the response element may

be the PXR response element and cellular factor is nothing but PXR (Yan and Xie, 2016). Thereafter, CYP3A gene was found as a prototypical target gene of PXR (Yan and Xie, 2016). Later on, genes coding for CYP450s and other metabolizing enzymes were found to be activated by steroids in order to provide protection against them. Subsequently, it was found that, protection against xenobiotics was mediated by orphan nuclear receptors, but not by steroid receptors (Blumberg et al., 1998). CYPs are hemoproteins which get activated by xenobiotics and CYP3A4 is the main player among them, in catalysing biotransformations of endo/xenobiotics (McDonnell and Dang, 2013; Jonsson-Schmunk et al., 2018). Like other typical NRs, PXR also shares a common modular structure of its domain starting with conserved DNA binding domain (DBD) at N-terminal and ligand binding domain (LBD) at C-terminal region which binds with co-regulators. Unlike most of the NRs, PXR lacks AF-1 region. SRC-1, SRC-2, SRC-3 and PBP (peroxisome proliferator-activated receptor binding protein) have been reported to interact with ligand-activated PXR to de-compress the chromatin and recruit the transcriptional machinery to the target genes promoters (Smutny et al., 2013). Despite having similar domains organization, PXR exhibits some differences. Extra 45 amino acids are present between helix 1 and 3, making β-sheets five stranded, while other NRs possess two to three of the β-sheets. Helix 2 is now replaced with helix 1-3 insert, to make the floor of ligand binding pocket larger in volume than the other NRs. Crystal structure of PXR-LBD has revealed twice the volume of this domaincompared to other NRs and lined by 20 hydrophobic amino acids with four polar and four charged amino acids. This 3D-structure of PXR-LBD has provided insight into the molecular basis of its promiscuity to accommodate ligands of different shapes and sizes. The characteristic of PXR of having large binding pocket has allowedbinding with its prototypical ligand rifampicin, which is one of the largest ligands known for NRs (Jin and Li, 2010). Unlike to rifampicin, PXR also binds with small ligand SR12813. Crystal structure of SR12813, a cholestrol lowering drug in complex with PXR has shown that out of twenty, nineteen hydrophobic amino acids are involved in lining the pocket. SR12813 binds PXR in three different orientations with different sets of hydrogen bonding and vander waal's interaction. These molecular features enable PXR to bind with a wide range of xenobiotics, as the large spherical pocket allows ligands to bind with their multiple shapes or via multiple hydrogen-bonding interactions (Willson et al., 2002). PXR is abundantly expressed in liver and intestine, the organs where highest expression of

xenobiotic metabolism and elimination related enzymes and proteins occur. PXR transcriptionally regulates genes encoding; i) Phase I (CYP3A4, CYP3A7, CYP3A11, CYP3A23, CYP2B6, CYP2B9, CYP2C8, CYP2C9, CYP2C19, CYP2C55 and CYP1A enzymes) (Koki et al., 2007) involved in oxidation, reduction, hydrolysis and hydration of lipophilic xenobiotics, rendering them water soluble; ii) Phase II drug metabolizing enzymes (glutathione S-transferases GSTs, UDP-glucuronosyltransferases UGTs, sulfotransferases SULTs) (Sonoda et al., 2002) to further increase the xenobiotic polarization and solubility by adding exogenous moities; and iii) Phase III proteins including uptake and efflux transporters[organic anion transporter polypeptide 2 (OATP2), multidrug resistant proteins (MDR1, MDR2) and multidrug resistance associated protein 2 (MRP2)]. PXR–mediated xenobiotics elimination is one of the body's adaptive defense mechanism against daily confrontation to environmental chemicals or drugs. Ligand-activated PXR heterodimerizes with RXR and recruits co-activators after binding to a specific motif (DR3, DR4, DR5, ER6 and ER8) present in the 5' flanking region of PXR regulated target genes (**Figure 4**). These are the motifs containing two copies of AG(G/T)TCA, a consensus NR binding sites either present as direct repeats separated by 3, 4 or 5 spacer nucleotides and named, DR3, DR4 and DR5 or everted repeats separated by 6 or 8 nucleotides named as, ER6 and ER8 (Orans et al., 2005). Activation of CYP3A4 catalyzes the metabolism of ~60% of clinical drugs. Activation of CYPs in one hand provides an adaptive response by enhanced xenobiotic clearance; on the other hand, it mediates potentially life threatening drug-drug interactions, where one drug alters the metabolism of second drug given in combination therapy. Thus, unfolding the molecular mechanism for CYP3A4 activation, is indispensable for the development of safer small molecules. Since long, liver has been known as the front line organ for metabolism and elimination of prescription drugs, herbal drugs, dietary supplements, environmental pollutants and endobiotics. These diverse sets of chemicals also possess the ligand property of PXR. Such behavior of these ligands has paved the way for the discovery, identification and characterization of novel constituents of body's xenobiotic defense system. Beside xenobiotic protection, PXR is also implicated to regulate the expression of genes involved in bile acid homeostasis, carbohydrate and lipid metabolism.

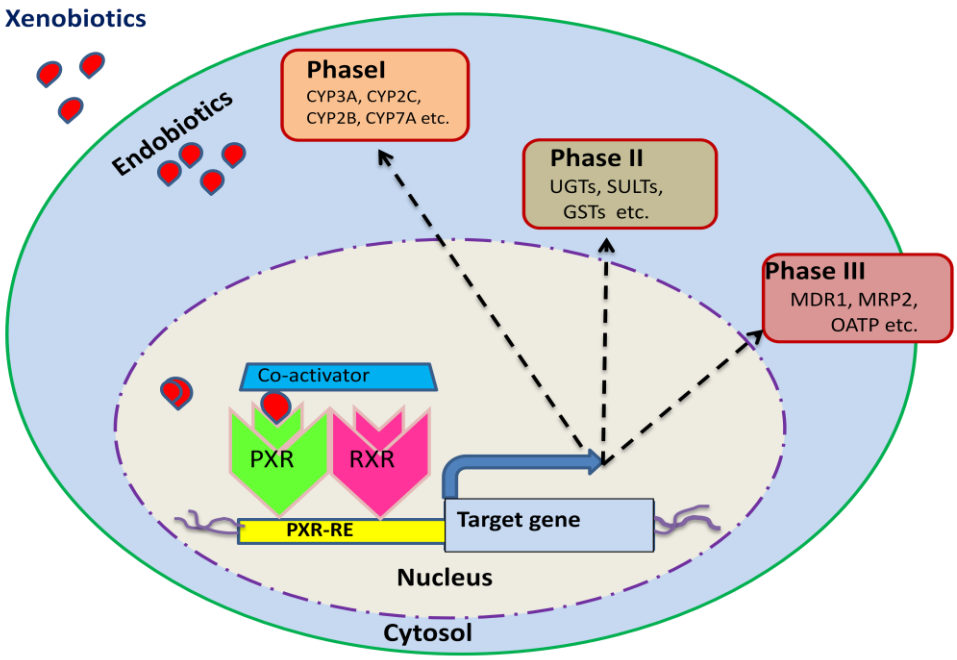


Figure 4: Schematic illustrations of activation of human PXR. *Human PXR resides inside the nucleus even in the absence of ligands but in association with co-repressors SMRT, NCoR etc. PXR gets activated followed by ligand (xenobiotics/endobiotics) binding and exerts its transcriptional function by heterodimerizing with RXR, binds with AGGTCA like direct repeats spaced by 3,4 or 5 bases (DR3, DR4, DR5) or everted repeats separated by 6 or 8 nucleotides (ER6 and ER8), present in the 5' flanking region of PXR (PXR-RE) target genes (phase I, phase II and phase III genes of drug metabolizing and disposition machinery), followed by recruitment of co-activators, resulting in their induction*

Functions of PXR

In our daily life, we are constantly exposed to myriads of potentially toxic lipophilic small molecules, called xenobiotics. They have potential to accumulate at toxic levels which has profound effect on health. To counter the harmful effects of these chemicals, body has evolved its defense system comprised of 'drug metabolism and disposition' machinery. The enzymes and transporter proteinsof this machinery are capable to catalyze the biotransformation reactions and eliminatethe harmful endobiotic/xenobiotic metabolites. PXR is 'master regulator' of the many of the genes of this machinery consisting of phase I and phase II drug metabolizing enzymes (DMEs) and phase III ABC family drug transporters. The cytochrome (P450) 3A4 (CYP3A4) and multidrug resistant 1 (MDR1), which encodes the P-glycoprotein (ABCB1) are the two most important target genes of PXR (Kliewer and Wilson, 2002; Rosenfeld et al., 2003). Alongside the recognition and binding with xenobiotics, PXR also acts as an 'endobiotic sensor'. It plays an important role in bile

acid and energy metabolism by regulating the metabolism of bile acids, fatty acids, lipids and glucose. PXR exerts its effects on energy metabolism either by directly regulating the genes or through cross-talk with other transcriptional regulators.

PXR, a 'master regulator' of drug metabolism and elimination machinery

PXR gets activated in response to structurally diverse range of xenobiotics and inducethe Phase I enzymes (cytochrome 450), Phase II conjugating enzymes (glutathione S-transferase, GST; sulfotransferases, SULT and UDP glucuronosyltransferases, UGT) catalyzing the biotransformation reactions and Phase III membrane transporters (MDR1 and OATP2) (Goodwin et al., 2001). In this way, PXR acts as a regulator of 'drug metabolism and disposition machinery'. The components of this machinery act in a coordinated manner to biotransform and facilitate the elimination of small toxic molecules from the cellular milieu. Down-regulation/inhibition of this machinery may leads to the accumulation of small molecules which may be harmful. Conversely, up-regulation/activation of the machinery may attenuate the bioavailabity or efficacy of small drug molecules. Therefore, modulation of the 'DMD' machinery via PXR also poses a serious health concerns concern of drug-drug interactions (Kliewer, 2005).

i) Regulation of phase I drug metabolizing enzymes

CYPs are the superfamily of heme-thiolate containing monooxygenases, catalysing the phase I biotransformation of small molecules (Venkatakrishnan et al., 2001). CYP enzymes are abundantly expressed in liver (Omura, 1999). Within the CYP family, major subfamily catalysing metabolism of xenobiotics in humans are; CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A isoforms CYP3A4, CYP3A5, CYP3A7 and CYP3A43 (Venkatakrishnan et al., 2001). CYP3A genes are present on 7q22.1 number chromosome (Gellner et al., 2001). Among the CYP450s, CYP3A4 is responsible for the metabolism of ~60% of the therapeutic agents and is highly expressed in liver and intestine like PXR (Venkatakrishnan et al., 2001; Singh et al., 2003). PXR has been shown to get activated by broad range of compounds and its activation leads to the transcriptional up-regulation of CYP genes including CYP3A4, CYP3A11, CYP3A23, CYP2B6, CYP2B9, CYP2C8, CYP2C9, CYP2C55, CYP2C19, CYP1A and CYP3A7 enzymes (Koki et al., 2007) involved in oxidation, reduction, hydrolysis

and hydration of lipophilic xenobiotics, rendering them water soluble to make easier excretion from the body.

ii) Regulation of phase II drug metabolizing enzymes

PXR also regulates the expression of Phase II conjugating enzymes like UDP-glucuronosyl transferases (UGTs), sulfotransferases (SULTs) and glutathione S-transferases (GSTs) (Xu et al., 2005). Phase II enzymes add polar moieties to the xenobiotics/endobiotics to enhance their hydrophilicity and making them susceptible for elimination through biliary and/or urinary excretion (Wang et al., 2002). Among these conjugating enzymes, UGTs (Hu et al., 2014) and SULTs (Falkner et al, 2001) are the main transcriptional targets of ligand-activated PXR. Rifampicin, phenytoin, phenobarbital and carbamazepine-activated PXR were shown to induce UGT1A1 among UGTs family (Sugatani et al., 2005). PXR activation by PCN in mice was found to activate SULT1A1, SULT2A1 and SULT5A1 (Alnouti and Klaassen, 2008). However, activation of SULTs isoforms by activated hPXR remains poorly established. PAPS (3'-Phosphoadenosine 5'-phosphosulfate) acts as a donor molecule from which STD (a member of SULTs family) takes sulfonyl group and transfers it to the hydroxyl or amino group of the xenobiotics to generate sulfate or sulfamate conjugates (Sonoda et al., 2002). This is the way in which STD detoxifies lithocholic acid by adding sulfate moiety to this bile acid. Lithocholic acid is very toxic among all bile acids, and its retention in liver can damage the organ and can also progress into cirrhosis which is lethal (Sonoda et al., 2002). In some situations, conjugation reactions can activate a parent drug/phase I metabolites to a reactive metabolite, which may be toxic (Xu et al., 2005). It was reported for GSH conjugation (catalyzed by GSTs) to an electrophillic group of xenobiotics in generating reactive intermediates.

iii) Regulation of phase III transporter proteins

Like phase I and phase II biotransformation enzymes constituting the drug metabolizing component of ‘drug metabolism and disposition machinery’, phase III transporter proteins which dispose off the xenobiotics/endobiotics from cellular environment, also get up-regulated by ligand-activated PXR. Among the efflux transporters, MDR1 (P-glycoprotein), multidrug resistance associated proteins (MRP2, MRP3, MRP4, MRP5) belongs to ABC (ATP binding cassette) transporter family and are regulated by activated-PXR (Schrenk et al., 2001; Mills et al., 2004).

Organic anion transporter polypeptides (OATPs) are also transmembrane proteins and primarily considered as influx transporters. OATPs belong to the solute carrier superfamily and encoded by SLCO gene family. They are also considered as a therapeutic target in cancer treatment due to the uptake of anti-cancer drugs (Liu and Li, 2014). OATP1B1 and OATP1B3, members of OATP1B subfamily are shown to regulate the expression of PXR target genes by regulating the uptake of PXR ligands due to their broad ligand specificity (Meyer zu Schwabedissen and Kim, 2009). Reciprocally, SLCO1A2/OATP1A2, SLCO1B1/ OATP1B1 and SLCO1B3/OATP1B3 are OATP genes which are regulated by PXR (Ihunnah et al., 2011).

Role of PXR in maintaining physiological homeostasis

Though PXR has been well-studied as a xenobiotic receptor for regulating 'xenobiotic detoxification and elimination', emerging evidences also implicate PXR in regulating physiological homeostasis and are described below.

i) In bile acid homeostasis

Bile is synthesized and secreted from hepatocytes into the intestine to aid in the digestion and absorption of lipids and vitamins (Ma et al., 2008). Lecithins, bile acids, bile pigments and bicarbonate ions are the constituents of bile. Among these components, bile acid is a key player in cholesterol elimination, bile secretion from liver and emulsification of lipid and lipid soluble vitamins inside the gut. Bile acid is the natural detergent synthesized from cholesterol catabolism in hepatocytes. Bile acid also acts as ligand for many NRs including FXR, LXR, HNF4α, VDR, PXR and CAR (Li and Chiang, 2017). Accumulation of bile acids in liver under the pathological condition of cholestasis is proven to be fatal. Similarly, accumulation of lithocholic acid has also been found as hepatotoxic (Staudinger et al., 2001). PXR appears to be involved in regulating the synthesis, metabolism and transport of bile acids. PXR activation is shown to down-regulate CYP7A1 (cholesterol 7α-hydroxylase), a rate limiting enzyme for synthesis of bile acid, therefore affectsthe biosynthesis of bile acids (Staudinger et al., 2001). PXR activation is also reported to regulate genes involved in uptake of bile acids from sinusoidal blood into the hepatocytes including OATP2, a sulphotransferase. PXR also regulates the metabolism of bile acids by inducing the genes for hydroxylation and sulfation like CYP3A4 (CYP3A11 in mice) and sulfotransferse respectively (Staudinger et al.,

2001). Biliary cirrhosis has slow progression and autoimmune disorder characterized by the inflammatory distructions of intrahepatic bile ducts, causing the accumulation of bile and other toxins in liver, leading to fibrosis and cirrhosis (Carey et al., 2015). Its clinical manifestation includes fatigue, pruritis, jaundice, osteoporosis and dyslipidemia etc. (Purohit and Cappell, 2015). A PXR activator rifampicin is used for the management of primary biliary cirrhosis (Ma et al., 2010). Clinical data raised ambiguity on the role of PXR in biliary system, as it has increased the bile acid level and also found as hepatotoxic after the treatment of rifampicin in humans (Galeazzi et al., 1980; Bachs et al., 1992; Prince et al., 2002). Therefore, further studies are required to resolve the ambiguity and ascertain the exact role of PXR in biliary system.

ii) <u>In bone homeostasis</u>

Vitamin K is a necessary cofactor for blood clotting and also plays an important role in bone homeostasis. Vitamin K family is comprised of three members K_1, K_2 and K_3 of 2-methyl-1, 4-naphthoquinones origin, among which K_2 is more effective in bone homeostasis (Ichikawa et al., 2006). Vitamin K_2 is prescribed in Korea, Japan and Thailand as a therapeutic agent to treat osteoporosis and fractures (Ichikawa et al., 2006; Azuma et al., 2010). Being one of the key players in bone homeostasis, Vitamin K_2 up-regulates the bone markers alkaline phosphatase, osteopontin, osteoprotegerin and matrix Gla protein (MGP). In PXR-/- mice, the expression of these bone markers get reduced (Azuma et al., 2010). Also, Vitamin K_2 is reported to bind and activate PXR to induce PXR target genes in osteosarcoma cell lines (Tabb et al., 2003). In reciprocal manner, PXR activators rifampicin and hyperforin are also reported to up-regulate bone markers HOS, MG-63 and Saos-2 in similar manner as of vitamin K_2 (Tabb et al., 2003). Fourteen common genes were found to be up-regulated by both vitamin K_2 and rifampicin in osteoblastic cells including PXR target genes tsukushi (TSK), matrilin-2 (MATN2) and CD14 antigen (Ichikawa et al., 2006). Functional cross-talk between PXR and vitamin K_2 results into the collagen assembly and collegen accumulations in osteoblastic cells (Ichikawa et al., 2006). Conceivably, these reports suggest the activation of PXR by vitamin K_2 to intensify the extracellular matrix formation in osteoblastic cells.

iii) In vitamin D metabolism and bone disorders

Vitamin D is required for calcium absorption, bone mineralization and bone formations. Rickets in children and osteoporosis which later progressed into osteomalacia in adults are disease manifestations of vitamin D deficiency. Vitamin D deficiency is also associated with cardiovascular diseases, cancers, rheumatoid arthritis and type I diabetes etc (Holick, 2004). Vitamin D2 and D3 are two forms of vitamin D, where D3 is more effective than D2 in humans (Ma et al., 2008). Liver metabolizes vitamin D (uptake from sunlight or oral supplements) into 25 (OH) D3, which is the principal circulating form of vitamin D. The 25(OH) D3 is further metabolized into 1, 25 (OH) $_{2D3}$ by the enzyme CYP27B1 in proximal tubule of nephron (Bikle, 2012). To elicit its functions 1, 25 (OH) $_{2D3}$ binds with its high affinity receptor VDR (Vitamin D receptor). After ligand binding, VDR heterodimerizes with RXR (Retinoid X Receptor) and binds with the vitamin D response element present in its target genes (Pascussi et al., 2005). Renal CYP24 (a mitochondrial enzyme) is responsible for converting 1, 25 (OH) $_{2D3}$ into inactive metabolite 1, 24, 25-trihydroxyvitamin D3 and 24, 25-dihydroxyvitamin D3, where the latter one decreases the conversion of 25(OH) D3 into 1, 25 (OH) $_{2D3}$ (Ma et al., 2008). CYP24 (25-hydroxyvitamin D_3-24-hydroxylase) is reported to get up-regulated by ligand-activated PXR therefore, serving as a PXR target gene. PXR shares ~60% amino acids similarity in DBD and ~37% similarity in LDB regions with VDR. Being ~37% similar in LBD, both receptors respond against common ligands lithocholic acid and its derivatives (Pascussi et al., 2005). Similarly, due to ~60% homology in DBD, ligand-activated VDR binds with the response element of PXR in PXR target genes like CYP3A4, CYP2B6, CYP2C9 and iNOS etc (Pascussi et al., 2005). There exists a cross-talk between PXR and VDR for sharing cis-acting elements. Therefore, drug-induced PXR has been implicated in up-regulation of CYP24, causing vitamin D deficiency and osteomalacia (Pascussi et al., 2005). Similar to PXR-mediated cross-transactivation and induction of CYP24, 1, 25 (OH) $_{2D3}$ and lithocholic acid-activated VDR are also reported to up-regulate P-glycoprotein (PXR target gene) expression by binding with its response element in LS174T (human colorectal adenocarcinoma cell line) (Tachibana et al., 2009). CYP3A4, predominantly regulated by PXR, is also shown to be regulated by VDR, as both receptors recognize and bind with same response element motifs DR3 and ER6 present in the XREM and proximal promoter of CYP3A4 gene (Pavek et al., 2010).

iv) PXR in lipid metabolism and hepatic steatosis

Though PXR is primarily involved in 'DMD' regulation, it also plays a key role in regulating lipid homeostasis. Hepatic steatosis is characterized by the accumulation of triglycerides in hepatocytes, caused by impaired synthesis and breakdown of simple triglycerides. Lipid homeostasis is regulated by balance between the synthesis and catabolism of triglycerides in liver. Over-expression of PXR in mice has resulted in increased accumulation of triglycerides in liver. This accumulation was the cumulative effect of increased influx of free fatty acids, lipogenesis and inhibition of β-oxidation genes by activated PXR. PXR-mediated lipogenesis is independent of LXR-mediated and SREBP-1c (sterol regulatory element-binding protein) dependent mechanism. There is up-regulation of free fatty acid transporter CD36, involved in lipogenesis and other lipogenic genes like stearoyl-CoA desaturase-1 (SCD-1) and long chain free fatty acid elongase (Lee et al., 2008). Ligand-activated PXR regulates fatty acid translocase CD36 expression directly as well as indirectly by activating PPAR-γ. In this manner, it had promoted the uptake of free fatty acids in mice. PXR also promotes lipid storage by inhibiting β-oxidation genes like PPAR-α and 3-ketoacyl-CoA thiolase, involved in the catabolism of fatty acids. CD36 acts as a target gene for PXR as it horbors DR-3 motif in its promoter's response element to bind with PXR (Zhou et al., 2006).

Role of PXR in cancer

Cancer is one of the leading causes of death worldwide and responsible for one in four deaths in US (Chen et al., 2007; Vadlapatla et al., 2013). Though PXR is well-studied for regulating 'drug detoxification and elimination', the exact role of PXR in cancer development and progression remains still elusive. PXR is reported to up-regulate the anti-apoptotic genes like BIRC2, BAG3 and MCL-1, while down-regulate pro-apototic genes like BAK-1 and TP53 genes (Zhou et al., 2008). PXR has shown differential behavior for regulation of apoptosis in carcinogenesis. Genetic (by over-expressing PXR) and pharmacological activation (ligand-mediated activation) of PXR is suggested as anti-apoptotic in HepG2 (liver hepatocellular carcinoma cells), HCT116 (human colon cancer) and LS180 (human intestinal colon adenocarcinoma) cells (Robbins and Chen, 2014). While opposite behavior of PXR is observed in endometrial and breast cancer tissues, where PXR is reported to promote apoptosis (Masuyama et al., 2007; Verma et al., 2009). This differential behavior of PXR could

be attributed to difference in tissue microenvironment and presence of different ligands (Robbins and Chen, 2014). Recently, our laboratory has reported the down-regulation of PXR and associated components of 'DMD machinery' in hepatic cancer and also the higher PXR level in reducing the tumorigenic potential (Kotiya et al., 2016).

Chemotherapy is one of the most common treatment regimens for cancer patients where clinical efficacy gets compromised by resistance of cells towards this therapy. Multidrug resistance (MDR) is the major concern for failure of chemotherapy in cancer patients. Chemoresistance is mediated by increased biotransformation and efflux of structurally and functionally dissimilar chemotherapeutics by up-regulated 'DMD' machinery. The up-regulated DMD leads to the decreased accumulation and fast elimination of those chemotherapeutics from cells (Vadlapatla et al., 2013). PXR is a key xenobiotic receptor known for regulating 'DMD' machinery involved in all aspects of biotransformation, detoxification and efflux of drugs and xenobiotics, implicating a significant role of PXR in drug resistance to chemotherapeutic agents (Qiao E et al., 2013). PXR is also detected in breast (Miki et al., 2006), endometrial (Masuyama et al., 2007), ovarian (Gupta D et al., 2008), prostate (Chen et al., 2007), colon (Zhou et al., 2008), and oesophageal (Takeyama et al., 2010) cancerous tissues at significantly higher level than normal tissues. In cancerous tissues, PXR expresses at higher level than normal tissues (Robbins and Chen, 2014). Two most important members of the 'DMD machinery' are CYP3A4 and ABC transporters (MDR1). CYP3A4 is responsible for metabolism of ~60% of prescription and non-prescription drugs, while MDR1 plays a key role in efflux from cellular niche (Robbins and Chen, 2014). Few reports have suggested the activation of PXR by chemotherapeutic agents. There are evidences of chemoresistance (by up-regulation of CYP3A4 and MDR1) after activation of PXR by one of its very well known ligand SR12813 (Huang et al., 2006; Chen et al., 2007) in prostate cancer cells PC-3. The involvement of PXR is further verified by shRNA-mediated PXR silencing, which has increased cells sensitivity for the treatment of taxol and vinblastine. To circumvent the PXR-mediated drug resistance, PXR antagonists or non-activators of PXR would proveto be beneficial.

Role of PXR in metabolic diseases

Diabetes mellitus is a chronic, progressive and complex metabolic disorder. It is characterized by hypoglycemia resulting from defect in either insulin secretion, action or both. The prevalence of this disease is increasing rapidly worldwide and is expected to have already affected ~552 million people across the world, according to International Diabetes Federation (IDF) (Alam et al., 2014). There are several types of diabetes (**Table II**) among which type I (T1DM) and type II (T2DM) are the most commonly recognized forms. T1DM is characterized by the autoimmune destruction of β-cells leading to absolute insulin deficiency and accounts for 5-10% cases of diabetes. T1DM is HLA associated, making diabetic people susceptible for other autoimmune disorders like Addison disease, thyroid disease, vitiligo etc. T2DM is caused by both the insulin resistance and impaired insulin secretion. Obesity and hypertension are major risk factors for T2DM. T2DM accounts for 90-95% cases of diabetes (American diabetes association, 2010). In T2DM cases, glucose homeostasis get disturbed as glucose output from liver is elevated. The reason behind the elevation is induction of gluconeogenic genes phosphoenol pyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) inside the liver. T2DM is associated with the complications of retinopathy, nephropathy, neuropathy, renal insufficiency, cardiovascular diseases and also risk factors for osteoporosis and osteoarthritis. The imbalance between the nutrient uptake and storage capacity activates stress related pathways which finally leads to the inflammation of peripheral tissues (Kaiser et al., 2014).

Glucose level in blood is determined by the balance between the level of insulin and glucagon (Hukkanen et al., 2014). In case of insulin resistance, a manisfestation of T2DM, glucose release increases from the liver as a result of up-regulation of gluconeogenesis genes like phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase). Ligand-activated PXR has been reported to play an anti-hyperglycemic role by inhibiting gluconeogenesis genes. Gluconeogenesis gene horbors HNF-4 binding sites in their promoters and HNF-4 is reported as a ‘master regulator’ of about 910 genes in hepatocytes and 658 in pancreatic islets (Odom et al., 2004). HNF-4 (a transcription factor) is involved in maintaining the glucose homeostasis by acting as an inducer of PEPCK and G6Pase genes (Bhalla et al., 2004). PGC-1 (peroxisome proliferator-activated receptor-γ

interacting co-activator) acts as a co-activator for different NRs including PXR and HNF-4, thus involved in HNF-4 mediated induction of these gluconeogenesis genes. Being an anti-hyperglycemic agent, PXR inhibits the gluconeogenesis genes by competing with HNF-4 for a common co-activator PGC-1, thereby not allowing HNF-4 to bind with the promoters of its target genes (gluconeogenesis genes) (Bhalla et al., 2004). Similar to HNF-4, FOXO1 (a forkhead transcription factor), FOXA2 (a winged-helix/forkhead transcription factor) (Nakamura et al., 2007) and CREB (cAMP response element-binding protein) (Kodama et al., 2007) also acts as an inducer of PEPCK and G6Pase genes (Kodama et al., 2004). In PXR transgenic mice, level of PEPCK and G6Pase genes were also found to be down-regulated, further corroborating anti-hyperglycemic nature of PXR (Gao and Xie, 2012). Likewise HNF-4, PXR binds with all the aforementioned TFs, and represses these TFs-mediated activation of gluconeogenesis genes. Down-regulation of these gluconeogenesis genes are also observed after the over-expression of PXR in mice (Zhou et al., 2006). Hepatic glucose transporter 2 (GLUT2) which transports glucose into the liver cells has also been reported to be down-regulated by PCN treatment (Rysa et al., 2013). On the contrary, PXR is reported to cause hepatic steatosis in transgenic PXR mice and ligand-activated PXR also exhibited same phenomenon in wild type mice (Zhou et al., 2006). Conversely, ligand-activated PXR is also reported to up-regulate PEPCK and G6Pase genes without binding with FOXO1. Ligand-activated PXR binds with phosphorylated SGK2 (serum/glucocorticoid regulated kinase 2) and stimulatesdephorphorylation at Thr 193 position of SGK2 by PP2C (protein phosphatase 2C). Unphosphorylated SGK2-PXR complex binds with PSRE (PXR-SGK2 response element) and IRS (insulin response element), present in the promoters of PEPCK and G6Pase genes, leading to PXR-mediated induction of these genes (Gotoh et al., 2015).

Table-II: Types of Diabetes

S.No.	TYPES OF DIABETES	CAUSES
1.	Type 1 diabetes Subtypes: 1A)Immune mediated 1B) Idiopathic	β-cell destruction, usually leading to absolute insulin deficiency
2.	Type 2 diabetes	Causes may range from predominantly insulin resistance with relative insulin deficiencyto a predominantly secretory defect with insulin resistance
3.	Other specific types	**A)**Genetic defects of β-cell function: Chromosome 12, HNF-1_ (MODY3); Chromosome 7, glucokinase (MODY2); Chromosome 20, HNF-4_ (MODY1);Chromosome 13, insulin promoter factor-1 (IPF-1; MODY4); Chromosome 17, HNF-1_ (MODY5);Chromosome 2, *NeuroD1* (MODY6); Mitochondrial DNA; Others **B)**Genetic defects in insulin action: Type A insulin resistance; Leprechaunism; Rabson-Mendenhall syndrome; Lipoatrophic diabetes;Others **C)** Diseases of the exocrine pancreas: Pancreatitis; Trauma/pancreatectomy; Neoplasia; Cystic fibrosis; Hemochromatosis; Fibrocalculous pancreatopathy; Others **D)**Endocrinopathies: Acromegaly; Cushing's syndrome; Glucagonoma Pheochromocytoma; Hyperthyroidism;Somatostatinoma; Aldosteronoma; Others **E)** Drug or chemical induced: Vacor; Pentamidine; Nicotinic acid; Glucocorticoids; Thyroid hormone; Diazoxide; β-adrenergic agonists; Thiazides; Dilantin; γ-Interferon 11. Others **F)**Infections: Congenital rubella; Cytomegalovirus; Others **G)**Uncommon forms of immune-mediated diabetes:

		"Stiff-man" syndrome; Anti-insulin receptor antibodies; Others **H)**Other genetic syndromes sometimes associated with diabetes: Down syndrome; Klinefelter syndrome; Turner syndrome; Wolfram syndrome; Friedreich ataxia; Huntington chorea; Laurence-Moon-Biedl syndrome; Myotonic dystrophy; Porphyria; Prader-Willi syndrome; Others
4.	Gestational diabetes	

(*Adapted and modified fromAmerican Diabetes Association:Diabetes Care, 2010)*

There are conflicting reports regarding the role of PXR in diabetes. In some cases PXR has been reported to repress the gluconeogenic genes in liver, thus suggested as an anti-hyperglycemic. The proposed mechanism behind these genes down-regulation are interaction of activated PXR with some transcription factors like FOXO1, HNF-4 and CREB (cAMP response element-binding protein) which generally binds with gluconeogenic genes like phosphoenol pyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase). Therefore, these transcription factors would no more be available to bind with their target gluconeogenic genes. While other reports concluded the role of PXR in promoting diabetes because, lipin-1 which plays an important role in insulin resistance is also a regulatory target gene of PXR. There are other compiled data also which conclude the same pro-diabetic role of PXR but had proposed PXR to be involved in hepatosteatosis. Since PXR inhibits the genes for β-oxidation of lipids and ketogenesis like 3-hydroxy-3-methylglutarate-CoA synthase 2 (HMGCS2) and carnitine palmitoyltransferase 1A (CPT1A) genes while induces the genes for lipogenesis leading to hepatic steatosis, thus leading to diabetes. However exact role of PXR in diabetes is still obscure (Hukkanen et al., 2014). It is also reported that metformin reduces the expression of CYP3A4 by inhibiting PXR (Krausova et al., 2011).

PXR in inflammation and inflammatory bowel disease

Inflammatory bowel disease (IBD) is the inflammatory disease of gastrointestinal tract (Cheng et al., 2012) and is characterized by chronic inflammation of intestinal mucosal cells. Environmental genetic factors are etiological for development of IBD (Hanauer, 2006). Principal constituents of IBD are ulcerative

colitis (UC) and Crohn's disease (CD). The integrity of intestinal epithelial barrier gets compromised upon infection with any pathogens which leads to inflammatory responses and progressed further as IBD. Symptoms of IBD are weight loss, diarrhea, abdominal pain, rectal bleeding and altered intestinal crypt structure. Reciprocal cross-talk between a central player of inflammatory pathways NF-κB and PXR has been reported. Activation of PXR by its ligands inhibits the activity of NF-κB and vice versa (Zhou et al., 2006). PXR appeared to play a protective role in alleviating the pathogenesis of IBD. A mouse model with wtPXR and PXR−/− have been generated and IBD was induced experimentally by treating with DSS (dextran sulfate sodium). PCN (a well-known ligand of mPXR) treatment has diminished the pathology of IDB in wtPXR but not in PXR−/− mice. This result was due to the suppression of NF-κB target genes IL-10, IL-1β, TNF-α and iNOS by PCN-activated PXR (Shah et al., 2007). Rifaximin (rifampicin derived semi-synthetic antibiotic) has also shown disease alleviation by inducing PXR target genes via PXR activation in intestine, further exemplifying the protective role of PXR. Knockdown of PXR by siRNA nullified the soothing effect of rifaximin, firmly establishing its involvement in rifaximin-mediated anti-inflammatory effects in IDB (Cheng et al., 2010). It is known that activated PXR is able to reduce the activity of nuclear factor kappa B (NF-κB) (Gu et al., 2006). Similarly, activation of NF-κB by the inflammatory signals and infections reduces the 'drug metabolism and disposition' machinery. Binding of p65 (Rel A) subunit of NF-κB with PXR hetero-dimeric partner RXR has been proposed as a plausible reason for the suppression. This binding hinders the heterodimerization of PXR: RXR complex, and disallows PXR to bind with promoters of its target genes, resultingin decreased transactivation of these genes. Voluminous literatures have suggested PXR to play a protective role, thus act as a potential therapeutic target for the treatment of IBD.

PXR in drug-drug interactions (DDIs)

Pharmacokinetic drug-drug interaction is a phenomenon where presence of one drug can attenuate/enhance the metabolism of co-administered drugs, affecting its bio-availability/toxicity by inducing/ inhibiting CYP3A4 enzyme. In addition to drugs which had failed during their clinical and pre-clinical trials, there are still many drugs which proved successful in clinical trial but had to be withdrawn from the market because of their potential to cause drug-drug interaction (DDI). In 20-30% cases of

adverse drug reactions, drug-drug interactions were mainly found responsible (Kohler et al., 2000). Pharmacokinetic drug interactions can alter the ADME (absorption, distribution, metabolism and elimination) profile of co-administered drugs by modulating the components of 'drug metabolism and elimination' machinery. The components of this machinery act in a coordinated manner to biotransform and facilitate the elimination of small toxic molecules from the cellular milieu. Prescription of combination therapy is a common regimen during the treatment of diverse metabolic disorders and infectious diseases. In such combination therapies, one drug may modulate the expression of genes of 'DMD', influencing the metabolism of another co-administered drug. This leads to the decreased bioavailability or increased toxicity of the latter. PXR has been implicated in drug-hormone interactions, therefore affecting the homeostasis of endocrine system.

In PC-3 cells (human prostate carcinoma cells), when PXR is activated by treatment of one of its model ligand SR12813, then 'DMD' machinery gets up-regulated and consequently eliminates the anticancer drugs vinblastin and paclitaxel. Sensitivity of PC-3 cells for both of these anticancer drugs became normal after silencing of PXR by shRNA (Wang et al., 2014). Ketoconazole a known CYP3A4 inhibitor when given in combination with docetaxel has shown diminished acvivity of CYP3A4 causing toxic level of the latter to accumulate. Conversely, bioavailability of erlotinib (a tyrosine kinase inhibitor) got compromised when co-administered with rifampicin (Harmsen et al., 2000). Few more reported examples of low plasma level of drugs co-administered with rifampicin are cyclosporine, erythromycin and oral contraceptives (Li, 2001). Rifampicin enhanced the toxicity of acetaminophen when co-administered, by inducing CYP3A4 (Wang et al., 2014). Rifampicin-mediated DDIs were reported to be caused by CYP450s, which was reported first in 1972 by Remmer (Chen and Raymond, 2006). Now induction of CYP3A4 among CYP450s by rifampicin is better understood. Rifampicin is known as prototypical activator of PXR and receptor has now been well-established major regulator of CYP3A4, and both co-express abundantly in liver.

Among the phase I components of the 'DMD' machinery', CYP3A4 is responsible for metabolism of >50% of medicinally active compounds (Harmsen et al., 2000). Induction of CYP3A4 by these compounds not only promotes their self elimination but also accelerates the metabolism of co-administered drugs. Evaluating drug-drug interactions (DDIs) has now become a major safety concern during drug

discovery and development processes. There are several *in vitro* assays available to screen the PXR activation potential of xenobiotics which would reflect the increased induction of CYP3A4. These assays include promoter-reporter assays, ligand binding assays, hepatocyte induction assays, temperature-dependent circular dichroism (TdCD), automated ligand identification system (ALIS) etc. (Xiao et al., 2011). Sometimes, DDIs appeared beneficial to prevent the harmful side effects of co-administered drugs (Wang et al., 2014). Similar to erlotinib, AUC of anti-cancerous drugs ifosfamide and imatinib are reported to get decreased when given in combination with rifampicin (Harmsen et al., 2000). Imatinib efficacy decreased when co-medicated with anti-depressant St John's Wort (SJW) (Harmsen et al., 2000).

Many of the DDIs not only involve CYP3A4 but also MDR1 (coding p-glycoprotein), as both enzyme and protein show wide range of substrate specificity and also share common substrates (Chen and Raymond, 2006). One example of common substrate and inducer of CYP3A4 and MDR1 is rifampicin, a chemotherapeutic agent to treat tuberculosis. Both of these genes code for enzyme and protein of 'DMD' machinery and also regulated by a common xenosensor PXR. Any drug, if possess the ability to activate or inhibit the 'DMD' machinery, can show DDI when co-administered with other drugs (Chen and Raymond, 2006). Rifampicin had shown DDIs by modulating other CYPs also. The cholesterol lowering effects of simvastatin (a substrate of CYP3A4) got reduced when combined with rifampicin. Similarly, effectiveness of warfarin, an anti-coagulant and rosiglitazone (an anti-diabetic drug) both act as substrate of CYP2C9, were reduced when given in combination with rifampicin (Chen and Raymond, 2006). Rifampicin had also increased the toxicity of isoniazid (an anti-tuberculosis agent) by inducing CYP3A4 when prescribed under combination therapy for TB (Chen and Raymond, 2006). Rifampicin had lowered the bioavailability of an immunosuppressant cyclosporine (acts as a substrate of CYP3A4) by inducing CYP3A4 (Pichard et al., 1996). Combination therapy is a commonly employed in cancer chemotherapy.

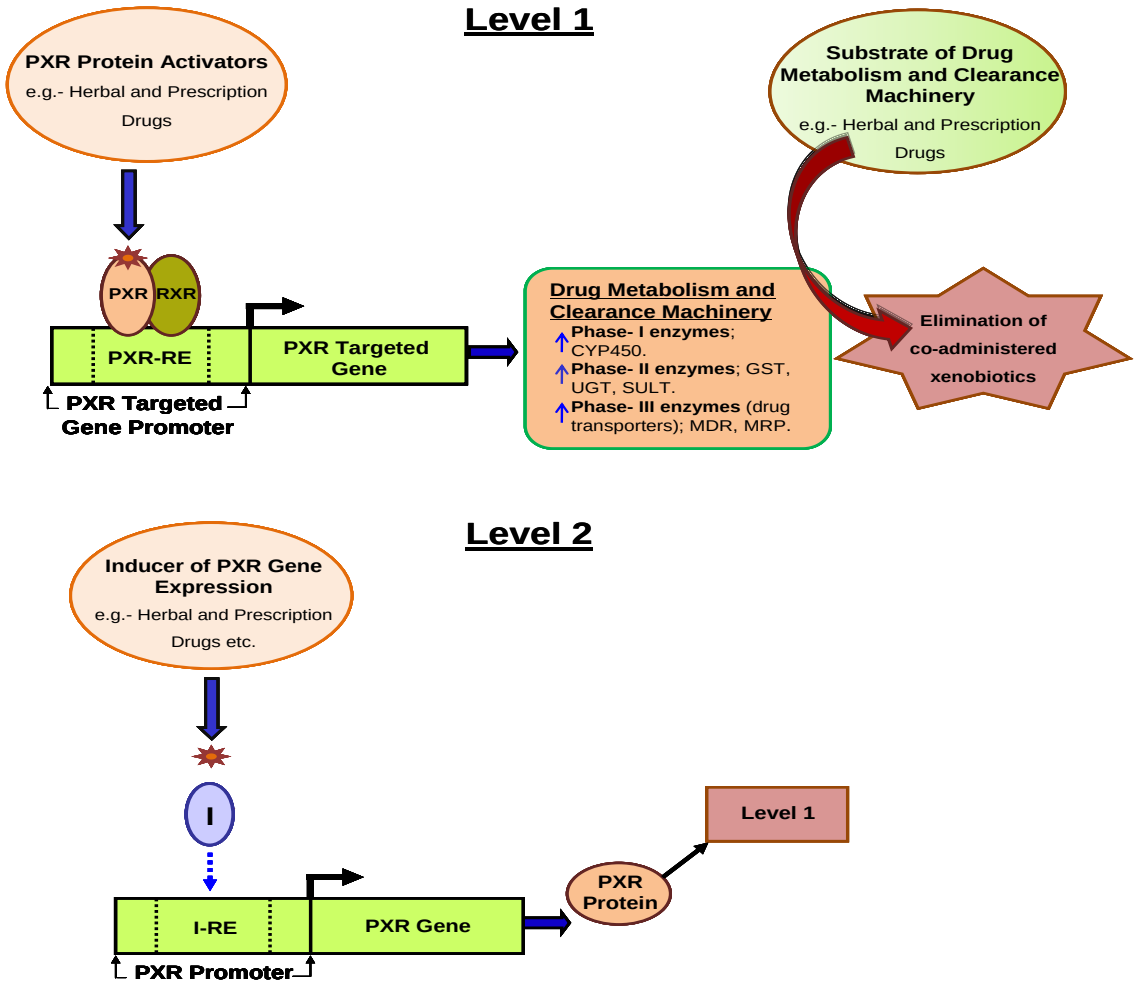
Many of the chemotherapeutic agents have narrow therapeutic index, thus small changes in their ADME profile could be life threatening (Harmsen et al., 2007). PXR, a modulator of 'DMD' senses and binds with large spectrum of xenobiotics, thus able to affect the metabolism of co-medicated drugs in DDIs (Wang et al., 2014). In such an anticancer combination therapy, PXR activation is reported to cause drug

resistance (Chen, 2010). Paclitaxel is such a kind of anti-neoplastic agent. Several reports have emphasized about the DDI of herbal anti-depressant, St John's wort with various co-administered drugs. This drug has enhanced the metabolism of; i) HIV protease inhibitor indinavir, lamivudine, nevirapine; ii) the immunosuppressant cyclosporine; iii) anticancer drug imatinib, irrinotecan; iv) oral contraceptives norethindrone, ethinyl estradiol and v) anti-inflammmatory agent ibuprofen, fexofenadine (Di et al., 2008) when co-administered with them, by inducing PXR-dependent CYP3A4 (Moore et al.,2004; Murphy, 2005). In case of cancer patients who are under the prescription of concurrent medicines, ~58% among them are suffering the consequences of DDIs (Jonsson-Schmunk et al., 2018). There are plentiful examples of DDIs. For example in 1998, an anti-hypertensive and anti-anginal drug mibefradil was withdrawn due to serious drug-drug interaction. Similarly, an anti-viral drug soruvidine has shown DDI with an anti-tumour pro-drug 5-fluorouracil (5-FU) and enhanced the metabolism of the latter. Therefore, soruvidine was withdrawn from Japanese market within one year of its launch (Li, 2001). When non-histamine and anti-sedating drug terfenadine was given in combination with ketoconazole it has shown fatal side effect of cardiotoxicity because of enhanced level of terfenadine in blood plasma. Therefore, terfenadine was removed from world wide market. Drug toxicity arosed because of increased toxic level of a particular drug than required concentration in the plasma, is the fatal outcome of inhibitory DDIs. So, in order to avoid the severe side-effect of DDIs, PXR-mediated CYP3A4 induction assays should be included during pre-clinical stages.

Screening for PXR activation/induction to predict drug-drug interaction

There are various *in vivo* and *in vitro* screening approaches to evaluate the therapeutic efficacy and safety of drugs. In general, novel molecules or compounds are screened/evaluated by using various approaches but these are either cell-free ligand binding assays (fluorescence polarization), not mimicking the cellular environment or cell-based two-hybrid assays which are able to predict the therapeutic behavior of drugs inside the cells, but not reliable enough, as it is carried out with only a portion of target protein of interest so could not represent same structure and functionality of target protein of interest. Also we can not identify the agonist or antagonist by following these ligand binding assays. In order to screen drugs and predict DDI and induction of CYP3A, high-throughput and robust cell-based reporter

assays has been developed. PXR-mediated DDIs and therapeutic failure during clinical trial can be prevented by evaluating the PXR activation potential of drugs during. Thereafter, small molecules can be modified accordingly or co-medicated with PXR antagonists without affecting their cellular activities (Wang et al., 2012). Now-a-days, for high-throughput screening of drugs *ex vivo* cell-based trans-activation assays have gained more attention because of being less time taking process, low cost, reproducibility of results and better adaptability for high-throughput screening strategies (Pinne and Raucy, 2014). In such a cell-based assay, liver cell lines are used to transfect with PXR expression vector and PXR-responsive promoter of its target gene fused with luciferase enzyme coding construct. Despite its benefits of representing the physiological cellular niche, reproducibility of results could be compromised because of variability in the expression of proteins during each and every step of transient transfections performed. To overcome the variability issues, cell-based assays with stable transfection has been considered as better choice which is also cost effective and more reliable than transient transfection based assays (Kim and Eberwine, 2010). In this context, a cell-based screening approach to investigate the safety of clinical drugs or xenobiotics at the dual level of PXR can be monitored by using two type of stably engineered cell lines generated in our lab (i) stable cell line HepXREM to screen drugs at PXR protein level (ii) stable cell line Hepx-497/+43 to evaluate at PXR-promoter level by transcription assays (**Figure 5**).



(Adapted from Negi et al., 2018)

Figure 5: **Schematic illustration of response of drugs, xenobiotics and endobiotics on PXR protein (Level 1) and PXR-promoter (Level 2).** *PXR heterodimerizes with its partner RXR after binding with ligands like prescription drugs, xenobiotics and endobiotics. This PXR: RXR complex binds to the promoters of PXR targeted genes (coding for components of drug metabolism and disposition machinery) harboring PXR response element. This results in enhanced expression of Phase I, II and III components of this machinery. As a consequence, a fast elimination of therapeutic drugs will be undertaken if these molecule(s) act as activator(s) of PXR. This up-regulated machinery consequently enhances the metabolism of not only the activators but also the co-administered drugs, xenobiotics and endobiotics leading to undesired effects (Level 1). These drug molecules and endobiotics may also act as inducers of PXR-promoter alone or by modulating certain unknown DNA binding protein(s). As a result of binding of inducer with PXR-promoter, PXR protein level get increased (Level 2). Increased expression of PXR protein leads to enhanced up-regulation of detoxification machinery (as depicted in Level 1).*

Level 1

PXR activation by prescription drugs, xenobiotics/endobiotics can be assayed which may also be helpful in eliminating the posibility of drug-drug interactions during treatment regimen. For screening at this level, HepXREM stable cell has been generated in our laboratory. In this cell, expression plasmid for PXR and a commonly used CYP3A4 promoter-reporter construct i.e. XREM-luciferase have been stably integrated in HepG2 cells.

Level 2

Drugs, xenobiotics and endobiotics that are capable of modulating PXR-promoter activity, thereby up-regulating/down regulating PXR protein expression level, can be assayed to identify and eliminate the possibility of drug-drug, drug-herbal and herbal-herbal interactions to exclude poor therapeutic benefits to the patient. Hepx-497/+43 cell line was generated to screen at this level, by stable transfection of PXR-promoter (promoter region -497/+43) in HepG2 cells.

MATERIALS AND METHODS

MATERIALS

General Laboratory Chemicals

Product Name	Company	Cat. No.
Agar	Himedia, India	RM301
Ampicillin	Himedia, India	RM645
Acetic Acid	Merck, India	60006325001730
Acrylamide	Sigma, St. Louis, MO, USA	A3553
Agarose	Sigma, St. Louis, MO, USA	A9539
Ammonium persulphate	Sigma, St. Louis, MO, USA	A3678
β-Mercaptoethanol	Sigma, St. Louis, MO, USA	M7522
Brilliant Blue G 250	Qualigens Fine Chemicals, India	10401
Bromophenol Blue	Himedia, India	RM117
BSA	Himedia, India	RM105
Calcium Chloride	Sigma, St. Louis, MO, USA	22231-3
Charcoal Stripped FBS	PAN Biotech, GmbH, Germany	P30-2301
Chloroform	GR Merck, India	S13SF53306
Coomasie Brilliant Blue R-250	Himedia, India	RM344
Diethyl pyrocarbonate	Sigma, St. Louis, MO, USA	D5758
DMEM (high glucose)	Sigma, St. Louis, MO, USA	D7777
DTT	Sigma, St. Louis, MO, USA	D9163
EDTA disodium salt	Sigma, St. Louis, MO, USA	E5513
Escort III	Sigma, St. Louis, MO, USA	L3037
Escort IV	Sigma, St. Louis, MO, USA	L3287
Ethanol	Merck, Germany	1009830511
Ethidium bromide	Himedia, India	RM813
FBS	PAN Biotech, GmbH, Germany	3302
Formaldehyde	Ranbaxy, India	F0070
Formamide	Qualigens Fine Chemicals, India	24015
Glycerol	Qualigens Fine Chemicals, India	15455
Glycine	Sigma, St. Louis, MO, USA	G8898
Hoechst 33258	Sigma, St. Louis, MO, USA	86140-5
Hydrochloric Acid	Rankem, India	H0070
Hydrogen Peroxide	Rankem, India	H0120
Isopropanol	Rankem, India	P0790

Kanamycin	Himedia, India	RM210
Lipofectamine-2000	Invitrogen Life Tech., Carlsbad CA	11668019
Luria Broth Powder	Himedia, India	M575
Magnesium chloride	Sigma, St. Louis, MO, USA	M8266
Methanol	Qualigens Fine Chemicals, India	43607
MOPS	Sigma, St. Louis, MO, USA	M1254
N, N'-Methylene-Bis-Acrylamide	Sigma, St. Louis, MO, USA	M7279
OPTI-MEM	GibcoR, USA	22600-050
Orthophosphoric acid	Qualigens Fine Chemicals, India	29905
p-Coumaric Acid	Sigma, St. Louis, MO, USA	C9008
PBS	Sigma, St. Louis, MO, USA	D-5652
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
PSA	Himedia, India	A002A
Ribonuclease A	Sigma, St. Louis, MO, USA	R6513
Sodium Acetate	Sigma, St. Louis, MO, USA	S-2889
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
TEMED	Sigma, St. Louis, MO, USA	T9281
TRI reagent	Sigma, St. Louis, MO, USA	T9424
Triton X-100	Sigma, St. Louis, MO, USA	T8787
Trizma base	Sigma, St. Louis, MO, USA	T6066
Tween-20	Sigma, St. Louis, MO, USA	P5927

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
Corning® Costar® cell culture plates 6-well	Corning, NY, USA (Sigma)	CLS3506
Corning® Costar® cell culture plates 12-well	Corning, NY, USA (Sigma)	CLS3513
Corning® Costar® cell culture plates 24-well	Corning, NY, USA (Sigma)	CLS3526
Corning® tissue-culture treated culture dishes 35 mm	Corning, NY, USA (Sigma)	CLS3430165
Corning® tissue-culture treated culture dishes 60 mm	Corning, NY, USA (Sigma)	CLS3430166
Corning® 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 100 mm	Tarson, Kolkata, India	460095

Protein and DNA standard size markers

Product Name	Company	Cat. No.
Prestained Protein Marker	Fermentas Interanational Inc., Canada	SM0671
1Kb DNA Size Standard	Fermentas Interanational Inc., Canada	SM0311
100bp DNA Size Standard	Fermentas Interanational Inc., Canada	SM0241
v50bp DNA Size Standard	Fermentas Interanational Inc., Canada	SM0373

Primary and secondary antibodies

Product Name	Company	Cat. No.
Anti-human PXR rabbit polyclonal antibody	Generated in our laboratory (Saradhi et al, 2005)	-
Anti-human CYP3A4 rabbit polyclonal antibody	Sigma, St. Louis, MO, USA	SAB1400065
Anti-human β- actin rabbit polyclonal antibody	Generated in our laboratory	-
Anti-His rabbit monoclonal antibody	Cell Signaling	12698
Anti-rabbit IgG HRP	Sigma, St. Louis, MO, USA	A0545

Standard Kits

Product Name	Company	Cat. No.
GenElute™ Gel Extraction Kit	RBC	1X23501
Luciferase assay	Promega, Madison, WI, USA	E1501
Plasmid DNA extraction mini prep prep	MDI Ambala, INDIA	MIPK50
cDNA synthesis	Thermo Scientific	K1631
Wizard®GenomicDNApurification	Promega, Madison, WI, USA	A1120

Enzymes

Product Name	Company	Cat. No.
BamHI	NEB, England	R0136S
Calf Intestinal Phosphatase (CIP)	NEB, England	M0290S
NotI	NEB, England	R3189S
Phusion High-Fidelity DNA polymerase	NEB, England	M0530S
RNasin ribonuclease inhibitor	Promega, Madison, WI, USA	N21111
T4 DNA Ligase	Fermentas International Inc., Canada	EL0015
Taq DNA Polymerase	NEB, England	M0273L
Syber green	GeneX	RT-SY2x.005

Miscellaneous materials

Product Name	Company	Cat. No.
Developer	Kodak, India	4908216
Disposable filter paper (0.22µ and 0.45µ)	MDI Ambala, India	CNXX0901XXXX104
dNTP set	Fermentas International Inc., Canada	R0181
Fixer	Kodak, India	4908232
Nylone membrane	MDI Ambala, India	SNNPZ
Parafilm	Tarson, Kolkata, India	380020
PVDF Membrane	MDI Ambala, India	SVF
Salmon sperm DNA (SS DNA)	Agilent Tech., USA	201190
Thiazolyl Blue Tetrazolium Bromide (MTT)	Sigma, St. Louis, MO, USA	M5655
Whatman Filter Paper 3 MM	Whatman, England	3030917
Whatman Filter Paper No.1	Whatman, England	100125

List of mammalian expression plasmids used in this study

Construct name	Nature of the construct	Source
CYP3A4	Mammalian expression vector encoding CYP3A4 protein	
MDR1-Tk-Luc (p-7975/7013-Tk-Luc)	Promoter-reporter expression plasmid encompassing a luciferase gene and a promoter region of the human MDR1 gene	Oliver Burk, Dr. Margarete Fischer-Bosch-Institute of Clinical Pharmacology, Germany
pcDNA3-CAR1	Mammalian expression vector coding for hCAR protein	Oliver Burk, Dr. Margarete Fischer-Bosch-Institute of Clinical Pharmacology, Germany
CYP2B6-PBREM-Luc	Promoter-reporter expression plasmid containing a luciferase gene and a promoter / enhancer region (PBREM) of the human CYP2B6 gene	Dr. Hongbing Wang (Division of Molecular Pharmaceutics, School of Pharmacy, University of North Carolina at Chapel Hill, NC 27599-7360)

XREM-Luc	Promoter-reporter expression plasmid containing a luciferase gene and a promoter / enhancer region of the human cytochrome P450 3A4 (CYP3A4) gene	C. Liddle, University of Sydney at Westmead Hospital, Australia
pCMV-β galactosidase	Mammalian expression vector encoding β-galactosidase cloned in pSV vector	Jeff Staudinger, Department of Pharmacology and Toxicology, University of Kansas, USA
pSG5-PXR	Human PXR-1 gene sequences cloned into pSG5 mammalian expression vector	S. A. Kliewer, University of Texas Southwestern Medical Center, Dallas, USA
RFP-hPXR	Mammalian expression vector encoding hPXR cloned at EcoRI and BamHI site of DsRed-Express-C1vector	Dash et al., 2017
GAL4-SRC1 VP16-hPXR FR-Luc	Mammalian two-hybrid expression vector for protein-protein interaction study. GAL4-responsive luciferase reporter gene vector used in mammalian two-hybrid assay	Rana et al., 2016
GAL4-CREB	Mammalian two-hybrid expression vector for protein-protein interaction study. cAMP response element binding protein ligated with GAL4 DBD	Prof. Ugo Moens, Department of Biochemistry, Institute of Medical Biology, University of Tromsø, N-9037 Tromsø, Norway
pG5E1b-Luc	Promoter-reporter expression plasmid harboring the GAL4 binding element in its promoter fused with lusiferase enzyme encoding gene	Prof. Ugo Moens, Department of Biochemistry, Institute of Medical Biology, University of Tromsø, N-9037 Tromsø, Norway
pcDNA3.1/HisC-JNK1-α1	Mammalian expression vector encoding hJNK1-α1 MAPK cloned at BamHI and NotI site of pcDNA3.1/HisC vector	Generated in this study

Flag-p38MAPK	Mammalian expression vector encoding p38-MAPK	Prof. Moorthy Anbalagan, School of Biosciences and Technology, VIT University,Vellore, Tamil Nadu, India
shRNA-hPXR	MISSION® shRNA against hPXR	Sigma, St. Louis, MO, USA
shRNA-p38 MAPK shRNA-JNK1/2 MAPK	shRNA constructs against hp38MAPK and JNK1/2 MAPK	Prof. Angel R. Nebreda Molecular Partners AG, Zürich, Switzerland
UGT1A1-Luc	Promoter-reporter expression plasmid containing a luciferase gene and a promoter / enhancer region (-3484/3194) of the human UGT1A1 gene	Generated in this study

List of cell lines used and their characteristics

Name	Origin	Characteristics
COS-1	Monkey Kidney	African green monkey kidney cell line
HepG2	Human Liver	Human hepatoma derived cell line
HEK293T	Human Kidney	Human kidney epithelial cells
HepXR	Human Liver	Human PXR stably integrated in HepG2
HepXREM	Human Liver	Human PXR and XREM-Luc stably integrated in HepG2
Hepx-497/+43	Human Liver	Human PXR-promoter region -497/+43 stably integrated in HepG2
LS180	Human colon	human colorectal adenocarcinoma cell line

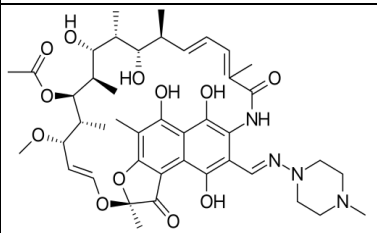
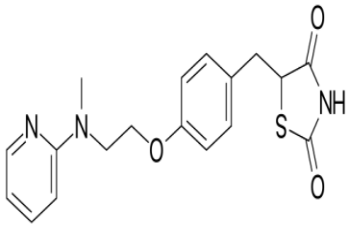
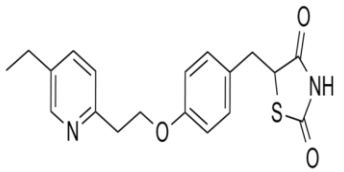
List of primers used in Real-Time PCR for indicated genes

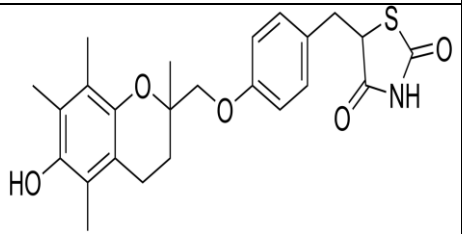
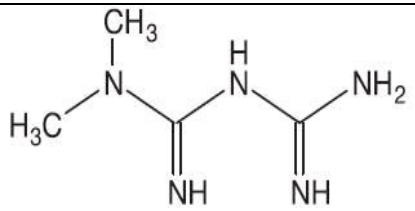
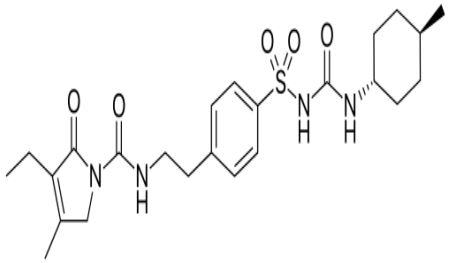
S.NO.	Primers	Direction	Sequence (5'-3')
1.	β-ACTIN	Forward	CCACACTGTGCCCATCTACG
		Reverse	GTGGTGGTGAAGCTGTAGCC
2.	CYP3A4	Forward	GGGGCCTTTGTCAGAACTAGAAT
		Reverse	CTAAACAATGGGCAAAGTCACAG
3.	UGT1A1	Forward	GTGACTTTGTGAAGGTTACC
		Reverse	TCCTGGGATAGTGGATTTTG
4.	MDR1	Forward	TGATGCTGCTCAAGTTAAAGG
		Reverse	CTTCAGTAGCGATCTTCCCA

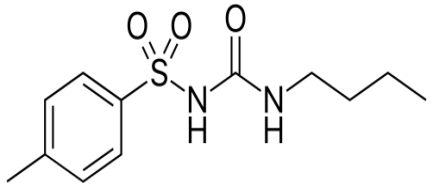
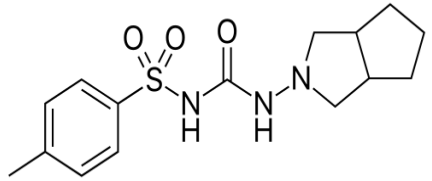
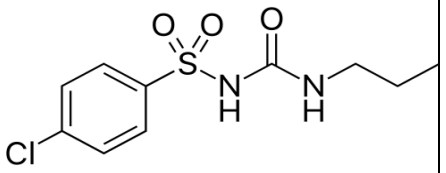
Anti-diabetic drugs, ligands and inhibitors

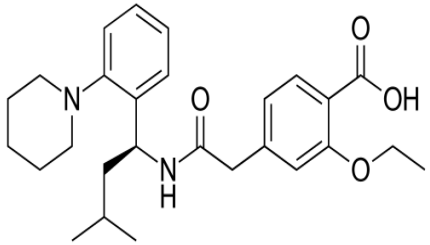
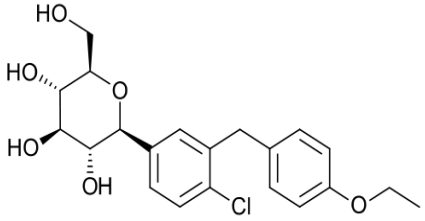
Name	Company	Cat. No.
8-Br-cAMP	Sigma, St. Louis, MO, USA	B5386
Chlorpropamide	Sigma, St. Louis, MO, USA	PHR1284
CITCO	Sigma, St. Louis, MO, USA	C6240
Dapagliflozin	Santa Cruz, Biotechnology, CA, USA	sc-364481
Dorsomorphin	Sigma, St. Louis, MO, USA	P5499
FR180204	Sigma, St. Louis, MO, USA	SML0320
Gliclazide	Sigma, St. Louis, MO, USA	PHR1288
Glimepiride	Santa Cruz, Biotechnology, CA, USA	sc-203058
GW9662	Cayman Chmicals	70785
H89 dihydrochloride	Sigma, St. Louis, MO, USA	B1427
MDL-12330A	Sigma, St. Louis, MO, USA	M182
Metformin hydrochloride	Sigma, St. Louis, MO, USA	PHR1084
Pioglitazone hydrochloride	Sigma, St. Louis, MO, USA	E6910
Repaglinide	Sigma, St. Louis, MO, USA	R9028
Rifampicin	G Biosciences	RC-191
Rosiglitazone	Sigma, St. Louis, MO, USA	R2408
SB203580	Tocris, UK	1202
SP600125	Tocris, UK	1496/10
Tolbutamide	Sigma, St. Louis, MO, USA	T0891
Troglitazone	Santa Cruz, Biotechnology, CA, USA	sc-200904

Table III: List of anti-diabetic drugs used in the present study. The drugs are described with their type, category, structure and mode of action.

S. No.	DRUG	TYPE OF DRUG	CATEGORY	STRUCTURE	MODE OF ACTION
1.	Rifampicin	Antituberculosis drug	Agonist of PXR		Increases transcriptional activity of PXR by promoting SRC-1 recruitment at the target gene promoter.
2.	Rosiglitazone	Antidiabetic drug (Withdrawn)	Thiazolidinedi-one		Increases the glucose uptake by peripheral organs.
3.	Pioglitazone	Antidiabetic drug (Withdrawn)	Thiazolidinedi-one		Increases the glucose uptake by peripheral organs.

S. No.	DRUG	TYPE OF DRUG	CATEGORY	STRUCTURE	MODE OF ACTION
4.	Troglitazone	Antidiabetic drug (Withdrawn)	Thiazolidinedi-one		Increases the glucose uptake by peripheral organs
5.	Metformin	Antidiabetic drug (In use)	Biguanide		Promotes glucose uptake by peripheral organs.
6.	Glimepiride	Antidiabetic drug (In use)	Sulfonylurea		Promotes the secretion of insulin from beta cells in pancreas.

S. No.	DRUG	TYPE OF DRUG	CATEGORY	STRUCTURE	MODE OF ACTION
7.	Tolbutamide	Antidiabetic drug (In use)	Sulfonylurea		promotes the secretion of insulin from beta cells in pancreas.
8.	Gliclazide	Antidiabetic drug (In use)	Sulfonylurea		promotes the secretion of insulin from beta cells in pancreas.
9.	Chlorpropa-mide	Antidiabetic drug (In use)	Sulfonylurea		promotes the secretion of insulin from beta cells in pancreas.

S. No.	DRUG	TYPE OF DRUG	CATEGORY	STRUCTURE	MODE OF ACTION
10.	Repaglinide	Antidiabetic drug (In use)	Meglitinide		Promotes insulin secretion from beta cells in pancreas
11.	Dapagliflozin	Antidiabetic drug (Newly Approved)	SGLT2 Inhibitor		Inhibits glucose re-absorption from proximal tubule.

Preparation of bacterial competent cells

DH10β strain of *E. coli* cells were made competent by $CaCl_2$ method, as mentioned in Sambrook et al., 1989. First, the *E. coli* cells were streaked on LB agar plate to obtain single colonies. After 12-16 hours of streaking, a single colony was inoculated in 5ml of LB medium and grown overnight at 37ºC with vigorous shaking at 250 rpm. Then 300µl of this overnight culture (1% inoculum) was added into 30 ml of LB medium and grown at 37ºC in shaker, until the O.D. at 600nm reaches 0.3-0.4. Then culture was centrifuged at 4,000 rpm for 10 min at 4ºC. The supernatant was discarded. The pellet was re-suspended in 15 ml of freshly prepared ice-cold $CaCl_2$ solution (100 mM) and incubated on ice for 30 min. cells were centrifuged again at 4,000 rpm for 10 min at 4ºC, and pellet was re-suspended in 1/10 of the original volume of ice cold 100 mM $CaCl_2$ (1.5 ml) and kept on ice for 2 hr. Finally, ice-cold glycerol was added to the final concentration of 15% and stored in 100µl aliquots at -80ºC till further use.

Bacterial transformation

For bacterial transformation 100µl of competent cells (*DH10β*) were used. Competent cells were immediately thawed on ice after taking them out from -80ºC. Around 50-100ng of DNA was added and incubated on ice for 30 min by tapping at every 10 min. The cells were then given heat shock at 42ºC for 90s and immediately kept on ice for 5 min. Then 1 ml of LB was added to the tube and incubated at 37ºC in shaker for 1 hr. After 1 hr, 100µl of cells were plated on LB agar plates containing appropriate antibiotics and grown for 12 to 16 hr at 37ºC in bacterial incubator. After that, single colony was inoculated in 5ml of LB with antibiotic and kept in shaker for another 12-16 hr. After that, plasmid was isolated using the MDI mini prep plasmid isolation kit.

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

Stocks for most of the drugs were prepared in DMSO and ethanol (1:1) or in recommended solvent. Drugs were kept in dessicator at 4˚C, while drug solutions were stored at -20°C.

Mammalian cell culture

All mammalian cells were cultured and routinely maintained in complete DMEM supplemented with 10% FBS, 100µg/ml of penicillin, 100µg/ml streptomycin and 0.25µg/ml amphotericin. The cultures were incubated in a humidified incubator at 5% CO_2 and 95% air atmosphere at 37°C.

Mammalian cell counting

For mammalian cell culture, cell counting was done to seed accurate number of cells as per experimental requirement. Equal volumes of 0.4% trypan blue stain and a uniform cell suspension were mixed and allowed to stand for 5 min at room temperature. Trypan blue/cell mix (approximately 10µl) was pipetted at the edge of the cover-slip and allowed to run under the cover slip in haemocytometer. Trypan blue is a vital stain and its entry is excluded from live cells. Live cells appear colourless and bright (refractile) under phase contrast microscope, whereas dead cells stain blue and are non-refractile. Viable (live) and dead cells were counted in one or more large corner squares under phase contrast microscope and the cell counts were recorded. The cell numbers were calculated by the formula given below.

Cell number per ml = Average number of cells in one large square x dilution factor x 10^4

Where, dilution factor is usually 2 (1:1 dilution with trypan blue), but may need to further dilute (or concentrate) the cell suspensions. 10^4 is conversion factor.

Cell viability analysis by MTT assay

MTT assay was performed to evaluate the possible cytotoxicity of anti-diabetic drugs. This assay is a colorimetric based assay which measures the cytotoxic effect in terms of number of metabolically active cells. Only the metabolically active cells are capable of generating reducing equivalents like NADH and NADPH by using the endogenous dehydrogenase enzyme which act on a yellow coloured MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole] and converts it into a purple coloured precipitated formazan in the mitochondria.

HepXREM cells were seeded at ~50% confluency in 96-well culture plates with complete DMEM medium containing 5% steroid-stripped serum without antibiotics. Next day, cells weretreated with different concentrations of drugs for 24 hr. After 24 hr, medium of each well was replaced with 100µl DMEM only (without

serum and antibiotics). MTT (1:10 dilution of the 5mg/ml stock in PBS) was added to this medium in each wells and further incubated at 37°C for 3 hr. Thereafter, the media was removed and a purple precipitate was clearly visible. A 100µl isopropanol was added (to dissolve the precipitate) to each well and further incubated at 37°C for 1 hr. At the end of incubation period, plate cover was removed and absorbance was measured at 570 nm with reference wave length at 630 nm. Absorbance value for control (with solvent treatment only) was plotted as 100% and sample value lower than control was treated as less cell proliferation or higher if values were more than 100%.

Liposome-mediated transient transfections

HepG2 cells were seeded at ~80-90% and ~60-70% of confluency in case of Lipofectamine 2000 and Escort III respectively, a day before transfection according to their manuals. Cells were seeded in 12-well culture plate in complete DMEM having 10% FBS and antibiotics. Medium of cells was changed with optiMEM 30 min prior to transfection. In two different microfuge tubes, 50µl of optiMEM was added. In one of the microfuge tube, 500-700ng of the total plasmid DNA was added while, in another microfuge tube 2.5µl of trasfection reagent (Liofectamine 2000/ Escort III) /well of 12-well plate was added and incubated for 5 min at RT. Plasmid encoding for β-galactosidase enzyme was also included in total plasmid concentration for each well of 12-well plates. When required, carrier DNA was added to make concentration of plasmidsequal in each well. After that, content of both the microfuge tube were mixed by a few gentle pippettingand kept for 30-45 min at RT for DNA–liposome complex formation. After the complex formation period, this mix was added dropwise in each well swirled gently and kept in incubator at 37ºC for 6-8 hr and 12-16 hr for Lipofectamine 2000 and Escort III respectively. After completion of transfection period, this media was replaced with complete DMEM having 5% steroid-stripped serum without antibiotics. In the same replenished medium drug was subsequently added. Cells were further kept for 24 hr in 37ºC incubator. After drug treatment period, cells were harvested and processed for luciferase assay.

In case of stably transfected cell lines, HepXREM and Hepx-497/+43-luc, they were seeded in 24-well plate in 5% steroid-stripped serum without medium. After 20-24 hr of seeding, drug was added into this medium and incubated for further 24 hr. After 24 hr, cells were harvested for luciferase assay.

Luciferase reporter gene assay or transcriptional assay

In order to perform promoter-reporter assay, cells were harvested after the drug treatment for a specified period. Luciferase assay was done, following the manufacturers protocol (Promega, Madison, WI, USA). Firstly, medium of the cells was removed and cells were washed with PBS twice. To lyse stable cells, 60µl reporter lysis buffer was added, while 100µl of this buffer was added for transiently transfected cells in each well of 24-well/12-well plates respectively and was kept on ice for 10-15 min. After lysis, cells were scraped by using a rubber policeman. Scraped cells were transferred into microfuge tube and centrifuged at 10,000 rpm for 8 min at 4ºC. In different set of microfuge tubes, 25µl of luciferin (substrate of luciferase enzyme) were aliquoted. In this aliquoted luciferin, 5µl of the lysate (supernant from lysed cells) was added and luminescence was measured in the TD-20/20 DLReady™ luminometer (Turner Designs). The luminometer was programmed to perform a 3 second pre-measurement delay followed by a 15s measurement period for each reporter assay.

Individual luciferase activity of samples were normalized with their respective β-gal value in transient transfections and plotted as luc/β-gal values. While, in case of stable cell lines, individual luciferase activity was normalized with the total protein concentration in lysates. Protein amounts were quantitated by Bradford's method and luc/µg protein values for reporter luciferase are plotted (Schagat et al., 2007). The luciferase activity was expressed as 'relative luciferase activity'. Relative value of luciferase activity for the control was considered as 1.

Determination of β-galactosidase activity and normalization of the luciferase value

To determine the β-galactosidase activity, β-galactosidase assay buffer was prepared by adding 1M sodium phosphate buffer (pH 7.4), 1M $MgCl_2$, β-mercaptoethanol and O-Nitrophenyl-beta-galactopyranoside (substrate of β-galactosidase enzyme).A 50µl ofassay buffer per tube was added in different microfuge tube for each sample. In this buffer, 50µl of the same lysate, which was initially used for luciferase assay were added. This mix was incubated overnight at 37ºC to develop faint yellow color. After that, the contents of these microfuge tubes were transferred in flat 96-well plate and absorbance of these samples were recorded at 415nm using a microplate reader (Bio-Rad, CA, USA).

Isolation of total RNA from cultured mammalian cells

Isolation of total RNA from cultured cells was performed with TRI REAGENT^{TM} (a mixture of guanidine thiocynate and phenol in a monophase solution). LS180 cells were cultured in 60 mm culture plate at ~60% confluency. Next day, cells were given the treatment of drugs for 24 hr. After the treatment period, cells were washed twice in PBS and lysed in 500µl of TRI reagent. Cells were allowed to stand for 5 min at RT after homogenization and lysis. Then, 200µl of chloroform was added to homogenized samples and vortexed vigorously for 15s and allowed to stand for 10 min at RT. Subsequently, samples were centrifuged at 12,000 rpm for 15 min at 4°C. The colourless upper aqueous phase having total RNA was transferred to a fresh tube. The RNA was precipitated by the addition of 200µl of isopropanol to the aqueous phase, mixed and allowed to stand for 10 min at RT followed by centrifugation at 12,000 rpm for 10 min at 4ºC. Supernatant was decanted and the RNA pellet was washed with 1 ml of 75% ethanol. The samples were centrifuged at 10,000 rpm for 5 min at 4ºC. RNA pellet was air dried, while supernanat was discarded. Completely dried RNA pellet was dissolved in appropriate volume (20µl) of DEPC (diethylpyrocarbonate) treated water with mixing at 55-60ºC for 10 to 15 min. The RNA samples were stored at -80ºC for further use.

Quantitative Real-Time PCR

Total RNA was isolated by TRI reagent after drug treatment for 24 hr. DNase I treatment was given in order to eliminate genomic DNA contamination. Reverse transcription of 5µg of total RNA into cDNA was performed by using First stand cDNA synthesis kit following the manufacturer's recommended protocol. Relative quantitation of CYP3A4, UGT1A1 and MDR1 mRNA expression was performed using specific primer sets. The expressions of these genes were measured by SYBR Green PCR Master Mix using 7500 Real-Time PCR System (Applied biosystem, Foster City, CA, USA). PCR amplification conditions were, step i)activation at 50°C for 2 min; step ii) initial denaturation at 95°C for 10 min;step iii) 40 cycles of amplification including denaturation at 95°C for 30 sec, annealing and amplification both at 60°for 1 min. After the complete amplification, dissociation stage was also included. β-Actin, a house-keeping gene was also amplified, which served as endogenous control. The Ct values of target genes were normalized with the Ct value of their respective endogenous controls. The relative gene expression (untreated Vs treated) were calculated by comparative threshold method using the formula; ΔCt = Ct (target gene of interest) – Ct (endogenous control); ΔΔCt (target gene of interest) = ΔCt (target gene in treated sample) – ΔCt (target gene in vehicle treated sample) and the fold change of mRNA = $2^{-\Delta\Delta Ct}$, which indicates the mRNA level of the corresponding transcript in relation to that in the control samples. Data were analyzed in Applied Biosystems SDS v2.0 software using auto threshold and auto baseline settings.

Isolation of genomic DNA

LS180 cells were grown in 60 mm plate at ~60% confluence for 24 hr in DMEM having 10% FBS and antibiotics. After 24 hr, cells were harvested for Genomic DNA isolation using Wizard^{R} genomic DNA purification kit, according to the manufacturer's protocol (Promega, Madison, WI, USA). Briefly, cells were washed twice with PBS and scraped in 1 ml PBS, then transferred to a microfuge tube. Microfuge tube containing cells were centrifuged at 5,000 rpm for 5 min to pellet the cells. Supernatant was discarded and pellet was mixed with 600µl of nuclei lysis solution by repetitive pipetting. A viscuous solution appeared. A 3µl of RNase solution was added to the viscuous lysate and mixed by inverting the tube 3-6 times, followed by incubation at 37°C for 15 min in waterbath. After removing tube from

water bath, lysate was cooled at room temperature. A 200µl of protein precipitation solution was added and vortexed for 15-20 sec. A clump of proteins were visible after vortexing. Lysate was centrifuged at 10,000 rpm for 8 min at room temperature to precipitate the proteins. Supernanatant was transferred to a clean 2 ml microfuge tube containg 600µl of isopropanol (room temperature). This solution was gently mixed by inverting the tube until the thread like DNA became visible. The solution was centrifuged at 10,000 rpm for 5 min at room temperature. A pellet of DNA became visible. Supernatant was discarded and pellet was washed with 600µl of isopropanolby inverting the tube 6-8 times. Solution was again centrifuged at 10,000 rpm for 5 min at RT. Supernatant was carefully removed and DNA pellet was air dried for 15 min at room temperature. A 200µl of DNA redydration solution was added and DNA pellet was dissolved at 65°C for 1 hr. Dissolved genomic DNA was stored at 4-6°C till further use.

Agarose gel electrophoresis

Agarose gel electrophoresis was performed as mentioned in Sambrook et al (1989). To separate plasmid and genomic DNA samples, 1% agarose was prepared in TAE buffer (40mM Tris-Acetate, 1.0mM EDTA, pH 8.0), boiled and then cooled at RT. Semiquantitative PCR products were resolved on 2% agarose gel. After cooling, DNA visualizing dye, ethidium bromide (0.5µg/ml) was added just before casting the gel on the gel tray. Samples were mixed with DNA gel loading buffer (in one-sixth volume) and loaded onto the wells. The electrophoresis was performed at 5V/cm in TAE buffer and the DNA was visualized on an UV transilluminator.

Preparation of lysate from the cultured mammalian cells for western blotting

After removing the media, cells were washed twice with PBS. Cells were then mechanically detached from the surface in PBS, using a cell scraper and collected in a microfuge tube. The microfuge tube was centrifuged at 5,000 rpm for 5 min to pellet the cells. Supernatant was discarded and cell pellet was lysed in lysis buffer (20mM Tris pH 7.6, 0.5mM DTT, 1mM EDTA, 1mM PMSF, 0.1% NP-40 and protease inhibitor cocktail) and incubated on ice for 30 min with intermittent tapping. After incubation for half an hr, 5M NaCl was added to reach the final concentration of 400mM with further incubation on ice for 30 min. Further, cells were centrifuged at

10,000 rpm for 8 min at 4°C and supernatant (lysate) was collected in another microfuge tube and stored at -20°C. Protein concentration of cell lysate was estimated by Bradford reagent and proceeded for western blot analysis.

Cloning of JNK1-α1 MAPK from cDNA and generation of pcDNA3.1/HisC-JNK1- α1 construct

CDS sequence against human JNK MAPK (MAPK8) transcript variant JNK1-α1 has been identified from NCBI. Forward primer 5'-CTTAAAGCCAGTCAGGCAAGGGA-3' and reverse primer 5'-GTCAGGGATCTT TGGTGGTGGA-3' were designed to amplify this region of 1155 nucleotides coding for JNK1-α1 MAPK. The forward and reverse primers harbor restriction enzyme sites for BamHI and NotI respectively. To amplify this target sequence from cDNA library, LS180 cells were seeded in 60 mm plates in complete DMEM with 10% FBS with antibiotics. On the following day, cells were washed with PBS and total RNA from cultured cells was isolated and cDNA was prepared in the same manner as mentioned *vide supra* in RNA isolation method. Procedure was slightly modified as cDNA was prepared by using JNK1 MAPK specific primers instead of OligodT. JNK1 MAPK was again PCR amplified from this cDNA by using gene specific primers to enrich the gene. After initial denaturation at 95°C for 3 min, amplification was conducted for 30 cycles of denaturation at 95°C for 45s, annealing at 61°C for 45s and extension at 72°C for 1 min. Final extension was carried out at 72°C for 10 min. Amplified fragment was extracted from gel by using RBC gel extraction kit after running in 1% agarose gel. Amplified fragment of 1155 bp and pcDNA3.1/His C vector were double digested with BamHI and NotI enzymes in separate microfuge tubes. Digestion was performed in 50µl of reaction volume containing 1µg each of the PCR amplified fragment and pcDNA3.1/His C vector. After completion of restriction digestion for 4 hr both the insert and vectors were eluted in Milli-Q water from 1% agarose gel elution. Subsequently, both the insert and vector were ligated by using T_4DNA ligase enzyme at 16°C for 20 hr in water bath. Using 10µl of ligation product *DH10β* competent cells were transformed and incubated at 37°C under ampicillin selection for 16 hr. Single bacterial colony was grown further for plasmid isolation. Purified plasmid was again digested with both the restriction enzymes to confirm the presence of 1155bp insert in the plasmid. Sequencing of the plasmid was done to further verify the presence of insert in frame with His-tag.

Molecular Modelling and Docking studies

The protein structure of apo-hPXR-LBD was retrieved from the protein data bank (PDB ID: 1ILG). The missing residues in the three-dimensional structure were modelled using MODELLER9V2. The PXR protein was energy minimized in GROMACS 4.5.3 package using the GROMOS 96 force field. The box dimensions ensured that any protein atom was at least 1.5 nm away from the wall of the box with periodic boundary conditions, solvated by simple point charge (spc) water molecules. NaCl counterions were added to satisfy the electro-neutrality condition. Energy minimization was carried out using the steepest descent method. The compounds were retrieved from the PubChem compound database in 3D SDF format. Further, Open Babel software is used to convert the 3D SDF format to 3D mol2 format. In case of Rifampicin, 3D structure was not available in PubChem compound database (3D conformer generation is disallowed since too many atoms), so we retrieved the 2D SDF format and converted into 3D mol2 format using Open Babel. The active site of the PXR is retrieved from the information available in PXR 3D complex structure (PDB ID: 1SKX). In silico docking is performed using GOLD docking program which generates large number of conformations for each ligand before docking. GOLD uses a genetic algorithm to explore the full range of ligand conformational flexibility with partial flexibility of the protein. The ligands showing maximum interactions with the protein were plotted using the program LIGPLOT.

Live cell imaging by fluorescense Microscopy

For PXR translocation study, COS-1cells were seeded in 35 mm plate to ~60% confluency in complete DMEM containing 10% FBS with antibiotics. Next day, the cells were transfected with DNA-liposome complex of Escort IV and RFP-PXR plasmid in optiMEM I. Following 12 hr transfection period, cells were treated with drugs in DMEM supplemented with 5% steroid-stripped serum without antibiotics for 24 hr. After drug treatment period, Hoechst dye was added at least two hours before to facilitate the visualization of the nucleus followed by imaging under a Nikon upright fluorescence microscope model 80i equipped with water immersion objectives and connected to a cooled-CCD digital camera (model Evolution VF, Media Cybernetics, USA). For subcellular localization purposes we counted 100 cells under different experimental conditions. When the receptor fluorescence was exclusively or predominantly nuclear it was considered as ‘N’. When it was

exclusively or predominantly cytoplasmic or uniformly distributed between nucleus and cytoplasm it was considered as 'C'.

Statistical analysis

Most of the experiments were done at least 3 times in duplicates and values represent the means ± SE of three independent experiments. Unpaired Student's t-test and analysis of variance (ANOVA) were performed for statistical analysis. Asterisks/apostrophes (*, ** and ***/ ', ″ and ‴) signify values that differed significantly from the control experiments with p-value less than 0.05, 0.01 and 0.001 respectively ($p<0.05$, $p<0.01$ and $p<0.001$).

CHAPTER I

Evaluation of selected anti-diabetic drugs on nuclear receptor PXR platform and associated components of detoxification machinery

Background

Nuclear Receptors (NRs) are ligand-modulated, DNA binding transcription factors with 48 members identified in human genome. All NR members share a common evolutionary conserved modular structure including centrally located and highly conserved DNA binding domain (DBD). DBD contains two highly conserved zinc fingers and a P-box. The P-box is involved in receptor dimerization and provides response element binding sequence specificity in the corresponding target genes of NRs. At the N-terminus of DBD, a highly variable N-terminal domain (NTD) is present, which harbors constitutively active region named activation function 1 (AF-1) that acts in ligand-independent manner. Towards the C-terminus of DBD is a moderately conserved ligand binding domain (LBD). LBD contains ligand binding sites and transcriptional activation domain AF-2 which acts in ligand-dependent fashion. LBD is connected with DBD through a less conserved hinge region (D domain) which contains nuclear localization signal (NLS) (Robinson-Rechavi M et al., 2003). Binding of a ligand to LBD exposes hydrophobic pocket of helix-12 and this conformational change leads to the recruitment of co-activators followed by up-regulation of the target gene's expression (Lazar, 2011).

PXR is one of the members of NR superfamily. PXR acts as a 'xenosensor' and plays a protective role against myriads of harmful chemicals whether endogenous (steroids, lithocholic acid etc.) or exogenous (therapeutic drugs, dietary supplements, endocrine disruptors etc.) by detoxifying and eliminating them from the cellular environment. In response to ligands, PXR heterodimerizes with RXR and binds to its response elements present in the target gene promoters. Furthermore, for induction of target genes, PXR recruits co-activators (SRC-1, PBP etc.), chromatin remodeling complexes and histone modifiers after binding with DNA response elements (Ma et al., 2008). This cascade of protein recruitment leads to transcriptional response. PXR appears to be a potent inducer of those genes which are actively involved in 'drug detoxification and elimination' (Goodwin et al., 2002; Kliewer et al., 2002). The components of detoxification machinery which it activates are i) Phase I (CYP3A4, CYP3A23, CYP3A11, CYP2B6, CYP2B9, CYP2C55, CYP2C8, CYP2C9, CYP2C19, CYP1A and CYP3A7 enzymes) (Koki et al., 2007) involved in oxidation, reduction, hydrolysis and hydration of lipophilic xenobiotics, rendering them water soluble; ii) Phase II [glutathione S-transferases (GSTs), UDP-

glucuronosyltransferases (UGTs), sulfotransferases (SULTs) enzymes] (Sonoda et al., 2002) which further increases the xenobiotic polarization and solubility; and iii) Phase III [multidrug resistant proteins (MDR1, MDR2), multidrug resistance associated protein 2 (MRP2) and the organic anion transporter polypeptide 2 (OATP2) transporter] (Staudinger et al., 2001) that finally efflux the noxious chemicals out of the cell. Alongside the induction of 'drug metabolism and disposition', PXR is also documented to be involved in several pathological conditions like cancer, inflammatory bowel disease, hepatic steatosis and a few other metabolic diseases etc. (Wang et al., 2012).

Diabetes is one of the complex metabolic disorders, defined by hyperglycemia in blood plasma. As of 2014, the number of people affected by diabetes are around 382 million and is expected to increase up to 590 million by 2035 (Fugita et al., 2014). There are two types of diabetes mellitus, type I (T1DM) and type II (T2DM). T1DM is a chronic condition characterized by the destruction of insulin producing β-cells and responsible for ~5-10% cases of diabetes. T2DM (~90-95%) is characterized by hyperglycemia resulting from insulin resistance at initial stage, followed by deficiency of insulin due to autoimmune destruction of β-cells in later stages. In case of T2DM, glucose homeostasis gets disturbed as glucose output from liver increases. The reason for this increase is induction of gluconeogenesis genes like glucose-6-phosphatase (G6Pase) and phosphoenol pyruvate carboxykinase (PEPCK) inside the liver.Retinopathy, nephropathy, renal insufficiency, cardiovascular diseases and cerebro-vascular diseases are the pathological consequences of T2DM. Around 60% of the type II diabetic patients die because of myocardial infarction (Kaiser et al., 2014). T2DM is influenced by genetic as well as with environmental factors. Obesity is the major risk factor for the development of T2DM. The imbalance between the nutrient uptake and storage capacity activates stress related pathways which finally leads to the inflammation of peripheral tissues (Kaiser et al., 2014).

There are various oral as well as injectible formulations available to cope up with T2DM. Among oral medications, the one in current use are sulfonylureas (tolbutamide, glimepiride etc.) which stimulate the secretion of insulin from β-cells in pancreas. Sulfonylureas (SUs) are in use since 1950s and work by inhibiting the ATP-dependent potassium channel, which leads to membrane depolarization and promotes

calcium uptake from calcium channels. Thus, SUs are considered as insulin secretagogue. Among the undesired effects of SUs are weight gain and hypoglycemia. Repaglinide also works as insulin secretagogue and is as effective as SUs but not associated with hypoglycemia. Due to short half-life, its dosing schedule is frequent (Silvio et al., 2002). Subsequently, in 1996 metformin came in fore that acts by inhibiting the hepatic gluconeogenesis.

Beside this, metformin also acts like insulin sensitizer by promoting the glucose uptake by peripheral tissues. The rarely found side effects associated with metformin are lactic acidosis, gastrointestinal stress, nausea and diarrhea in ~50% of patients. It works in both AMPK-dependant as well as independent manner to inhibit the hepatic gluconeogenesis. It is the first choice drug among all of the currently available drugs, as its benefits outweighs the side effects (Kaiser and Oetjen, 2014). Another insulin secretagogue is GLP-1, secreted from the intestinal L-cells in response to meal ingestion. Short half-life of around two minute makes it unsuitable as an anti-diabetic drug. It is cleaved by dipeptidylpeptidase 4 (DPP-4) making it short-lived. In market, mutated versions of GLP-1 are available which are not cleaved by DPP-4 and named as 'exenatide'. Liraglutide, another version of GLP-1 is also available with masked DPP-4 cleavage site. In order to let the GLP-1 work in native form, DPP-4 inhibitors were introduced in market with name 'gliptins' (sitagliptin, saxagliptin, vildagliptin etc.). GLP-1 slows down gastric emptying, thus inhibits weight gain and secretion of glucagon from α-cells of pancreas but associated with pancreatitis (Kaiser et al., 2014). In 1997, one of the members of thiazolidinedione (TZD) family, troglitazone was launched in US market. Members of TZD class of drugs also act as PPAR-γ ligands (Taylor et al., 2009). TZD inhibits hepatic gluconeogenesis, improve insulin sensitivity and also execute anti-inflammatory activities (Kaiser and Oetjen, 2014). Despite multitude of beneficial effects, there are also some serious side effects reported with some members of TZD group of anti-diabetic drugs, which raises safety concerns over their uses. Troglitazone was reported to cause hepatocellular injury and thus removed from the clinical practice (Lim et al., 2008). In 2012, another member of TZD, rosiglitazone was also banned as it was found to be associated with myocardial infarction (Nissen and Wolski, 2007; 2010). Pioglitazone proved to be a good choice among TZD and also in current use as it has very few side effects. However, its usage have come under scruitny over the

development of bladder cancer among diabetic patients (Zhu et al., 2012). Due to the development of bladder cancer, its usage has been banned in some countries, while in others it is prescribed under restricted conditions. Major safety concerns for TZDs are oedema due to renal absorption of sodium and water, heart failure, weight gain and bone fracture. There are mild to severe side effects associated with all the anti-diabetic drugs as aforementioned, which raises serious concerns about the usage of these medications. To overcome these side effects, a novel therapeutic drug class has been developed which is SGLT2 inhibitors. SGLT2, a membrane sodium glucose co-transporter is expressed particularly in proximal tubule of kidney. It inhibits glucose re-absorption from proximal tubule, thus maintains the glucose level of plasma. It comprised of dapagliflozin, canagliflozin, ipragliflozin, tofogliflozin and empagliflozin etc.

Canagliflozin was first approved and released in U.S. market in 2013. They are not associated with weight gain, hypoglycemia, cardiovascular risks, changes in blood pressure, lipid profile and liver dysfunction induced by fatty acids. Since it is a class of SGLT2 inhibitor, therefore can elevate the glucose level in urine, causing salt imbalance, genital and urinary tract infection (Fugita et al., 2014).

There are conflicting reports regarding the role of PXR in diabetes. Some reports suggest PXR as anti-hyperglycemic because of repression of gluconeogenesis genes in liver. According to such literatures, activated PXR interacts with some of the transcription factors like FOXO1 (Kodama et al., 2004), HNF-4 (Bhalla et al., 2004) and CREB (cAMP response element-binding protein) (Kodama et al., 2007) and inhibits their binding with gluconeogenesis genes like phosphoenol pyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase). On the contrary, other reports consider PXR as hyperglycemic which leads to diabetes (He et al., 2013). Interestingly, lipin-1 plays an important role in insulin resistance and also a target gene of PXR. Few other compiled data also favor the hyperglycemic nature of PXR but suggest PXR to be involved in hepatosteatosis (Zhou et al., 2008; Cheng et al., 2012). In such cases, PXR inhibits the genes for β-oxidation of lipids and ketogenesis likecarnitine palmitoyltransferase 1A (CPT1A) and 3-hydroxy-3-methylglutarate-CoA synthase 2 (HMGCS2) respectively. On the contrary, it induces the genes for

lipogenesis leading to hepatic steatosis. Hitherto, the exact role of PXR in diabetes is still obscure (Hukkanen et al., 2014).

CAR (Constitutive Androstane Receptor) is another member of NR superfamily which also acts as a 'xenosensor', shows ligand promiscuity like PXR. It also regulates the Phase I, Phase II enzymes and drug transporters (Phase III). PXR and CAR share some ligands and also regulate a few overlapping target genes of 'drug detoxification and disposition machinery'. Besides this, these two xenobiotic nuclear receptors also regulate many unique sets of genes. CAR preferentially binds with CYP2B6 over PXR (Wang and Chen, 2012), while PXR dominantly regulates CYP3A4. This cross-talk between PXR and CAR is responsible for the detoxification of ~80% of xenobiotics, thus activation of both of these xenosensor can severely affect drug-drug interactions (DDIs). So it is important to screen and evaluate drug molecules for CAR and PXR activation. If a drug is found to activate either one or both of these receptors then certain structural modification is required to overcome their activation. On the contrary, antagonist of CAR and PXR may be required to be administered along with the prescribed drugs (Wang and Chen, 2012).

An anti-diabetic drug metformin is reported to reduce the expression of CYP3A4 by inhibiting PXR (Krausova et al., 2011). However, several therapeutic drugs used in the treatments of T2DM have not been thoroughly examined for modulation of PXR. Investigation and identification of a multi-tier, cell-based drug screening approach on the nuclear receptor PXR platform may be able to explain why some drugs are clinically successful while others are not. Now-a-days, cell-based transactivation assays are used more often for high-throughput screening of drugs, which surmount the drawbacks of assays mentioned above (Pinne and Raucy, 2014). It is known that, in cell-based assays stable transfection is not only cost effective but also more reliable than transient transfection and primary human hepatocytes-based experimentation as it eliminates the variability issues associated with them (Kim et al., 2010; Jetten et al., 2016). Therefore, to overcome these variability issues, human liver cell derived stable cells have been generated in our laboratory (Negi S et al., 2018).

Activation of PXR not only enables this receptor to play an instrumental role in 'detoxification and elimination' of toxic xenobiotics/endobiotics, but also inflicts a

serious concern for drug-drug interactions (DDIs). Such DDIs could decrease the efficacy or increase the toxicity of co-administered drugs by altering the metabolism of small molecules that it senses in the cellular milieu. Therefore, it is advantageous to evaluate small molecules to assess their xenobiotic receptor activation/induction potential. In our study, we have utilized HepXREM stable cells to screen the the anti-diabetic drugs at PXR protein level, while modulators of PXR expression were identified by using PXR-promoter stable cell line Hepx-497/+43.

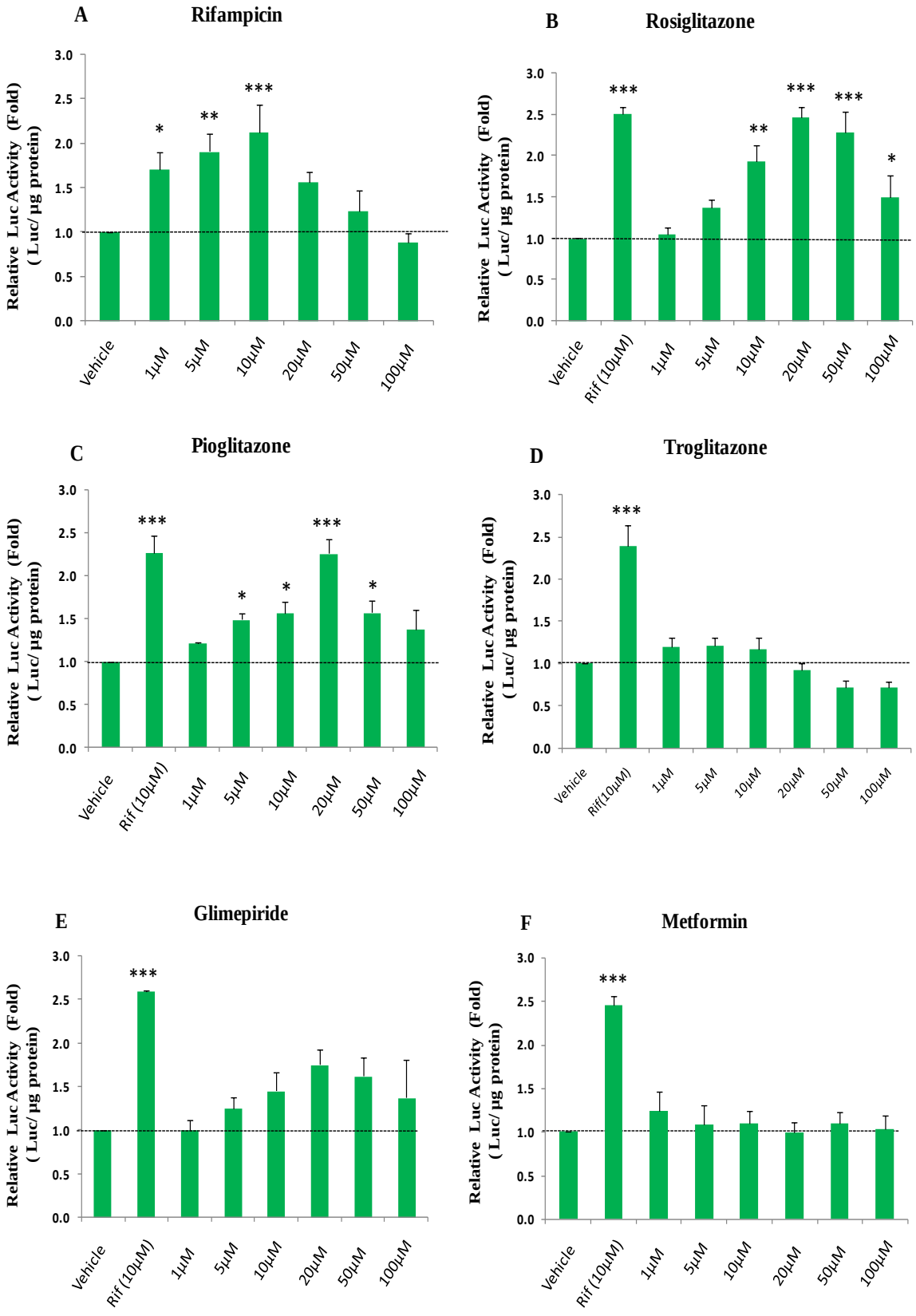
RESULTS

The present study focussed on the modulation (activation/induction) of PXR by certain classes of anti-diabetic drugs, using stable cell lines HepXREM and Hepx-497/+43. These stable cells have been proved to be suitable tools to investigate the metabolism rate of xenobiotics in PXR-dependent manner. The objective of this study was to utilize these stable cell lines to predict the CYP3A4 induction, using anti-diabetic drugs, which may illicit DDIs. Transactivation and expression level of components of 'drug metabolism and disposition' machinery were further studied using HepG2 cells and LS180 cells in the presence of anti-diabetic drugs. Similar approaches of luciferase-based assays were applied to examine the transactivation of another xenobiotic receptor CAR by transient transfections.Our result demonstrates that out of the ten selected clinical anti-diabetic drugs, only two from the redundant category had activated PXR and CAR.

Modulation of PXR transcriptionalactivity by anti-diabetic drugs

Nuclear receptor PXR is well-documented for sensing and responding against chemical insults by regulating 'drug metabolism and disposition (DMD) machinery' in liver. Downregulation or inhibition of 'DMD' machinery leads to the accumulation of small molecules which may be harmful. Conversely, up-regulation or activation of this machinery may cause reduced bioavailability/efficacy of small drug molecules. To address these issues, in the present study we have selected representatives of classical withdrawn (redundant), established and novel anti-diabetic drug molecules to examine the modulation of PXR transcriptional activity. Rosiglitazone, pioglitazone and troglitazone were selected from the redundant group. Established anti-diabetic drugs taken for study included metformin, glimepiride, repaglinide, tolbutamide,

chlorpropamide and gliclazide. Dapagliflozin is the novel one, selected for evaluation. First, we evaluated the PXR activation potential of these ten anti-diabetic drugs by using stable cell line HepXREM [stably integrated with PXR and most commonly used CYP3A4-promoter-reporter (XREM-Luc)]. These stable cells are derived from human liver cell line HepG2, generated and characterized in our laboratory and used to investigate PXR activation potential of drugs (Negi S et al., 2018). These cells were seeded in 24-well plates at ~40-50% confluency in complete DMEM having 5% steroid-stripped serum without antibiotics. After 20-24 hr, cells were treated with different drugs for 24 hr. Drugs were studied for their effect on PXR transcriptional activity at the final concentration of 1µM, 5µM, 10µM, 20µM, 50µM and 100µM. After 24 hr of incubation period, luciferase reporter activity was measured (**Figure 6**). Subsequent to normalization by total protein, the luciferase activity was represented as 'relative luciferase activity' in comparison to the vehicle treated cells. Rifampicin, a well-known potent agonist of human PXR (Jones et al., 2000) was used as a positive reference ligand for PXR transcriptional activity. Rifampicin showed maximum PXR transcriptional activity at 10µM, which is also in accordance with literatures. Both rosiglitazone and pioglitazone at 20µM concentration strongly increased PXR transcriptional activity similar to rifampicin. A gradual decrease in luciferase activity was observed with troglitazone after 20µM, which could be due to the cytotoxic effects. Dapagliflozin at its 100µM concentration was also found to be associated with reduced transcriptional activity, probably due to toxicity issue at this high concentration. While, repaglinide and gliclazide have shown increased luciferase activity at their 100µM concentrations (**Figure 6**).


A
Rifampicin
Relative Luc Activity (Fold)
(Luc/ µg protein)
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2.0
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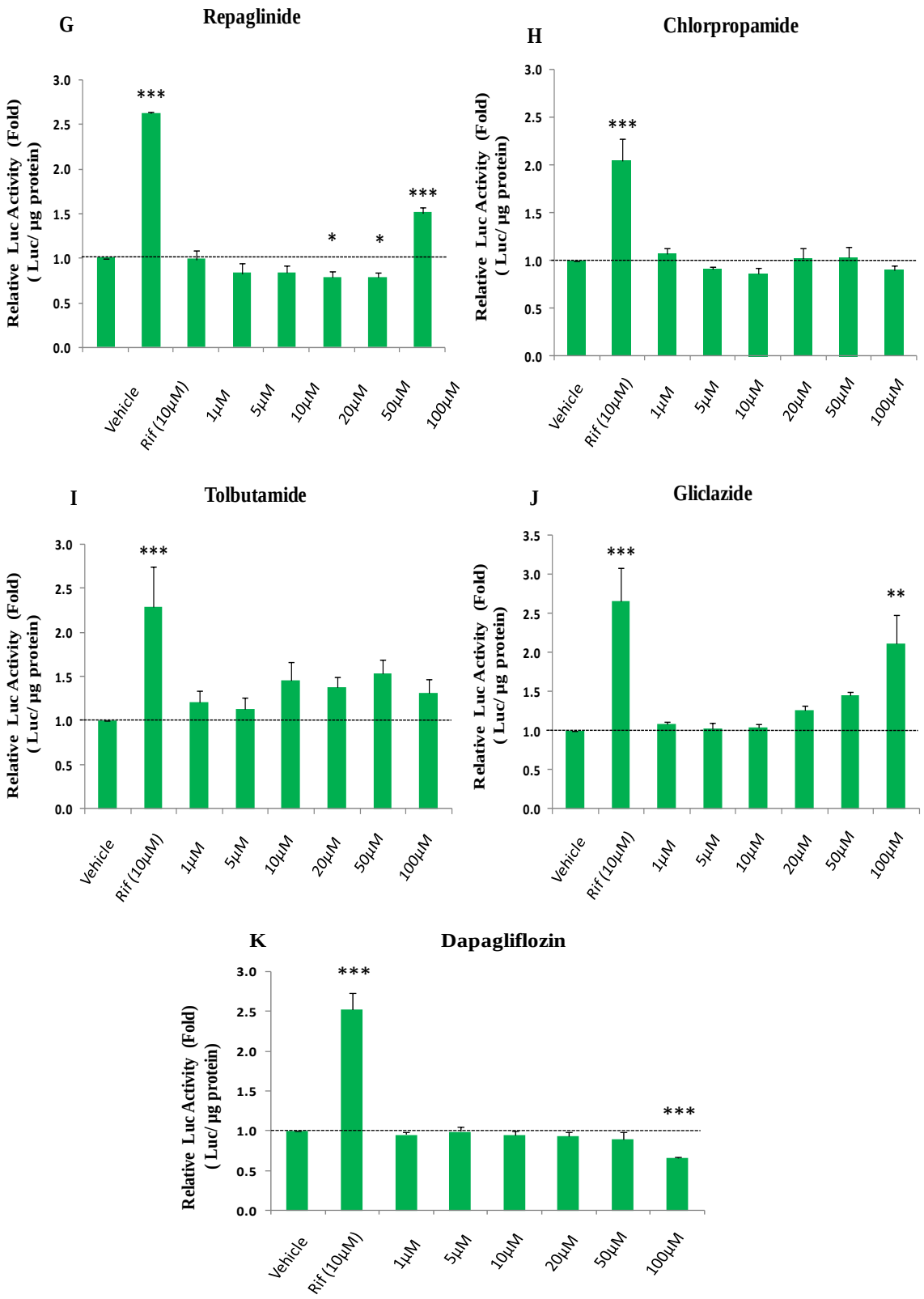


Figure 6: Dose-dependent effect of anti-diabetic drugs on the transcriptional activity of PXR in HepXREM stable cell line. *HepXREM cells (stably integrated with pSG5-PXR and XREM-Luc) were treated with drugs at their final concentration of 1µM, 5µM, 10µM, 20µM, 50µM and 100µMfor 24 hr.* ***(A)*** *Rifampicin* ***(B)*** *Rosiglitazone* ***(C)*** *Pioglitazone* ***(D)*** *Troglitazone* ***(E)*** *Glimepiride* ***(F)*** *Metformin* ***(G)***

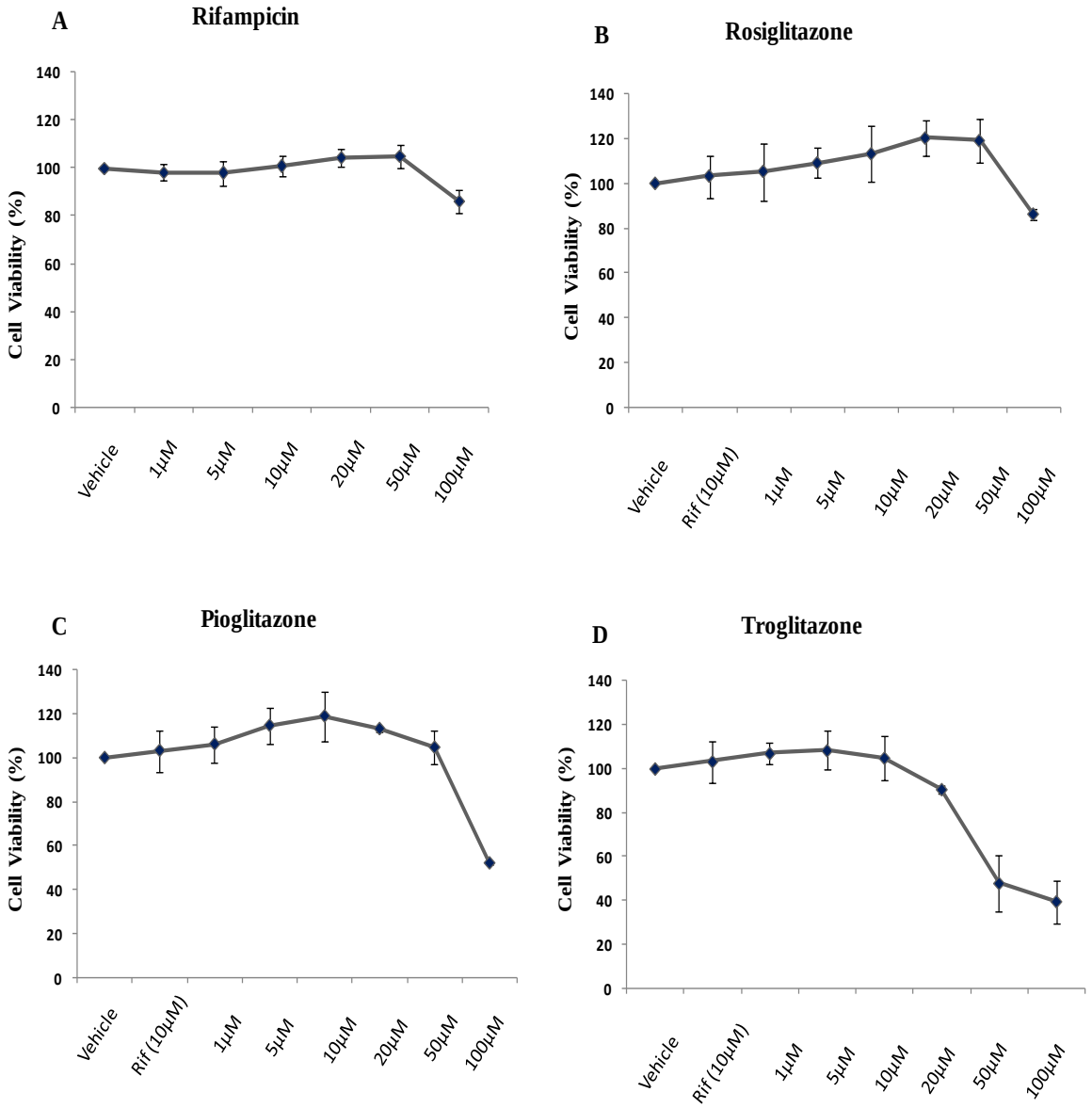
*Repaglinide **(H)** Chlorpropamide **(I)** Tolbutamide **(J)** Gliclazide **(K)** Dapagliflozin. Rifampicin, a well-known agonist of PXR, was used at 10µM. After 24 hr of treatment period, luciferase activities were determined and normalized with protein values. Luciferase activity is expressed as fold compared with control (DMSO:EtOH treated) cells. Data represent the mean ± SE of three independent experiments. Asterisks (*, ** and ***) signify luciferase values that differed significantly from the scores of corresponding controls (P<0.05, P<0.01 and P<0.001 respectively in Student's t-test).*

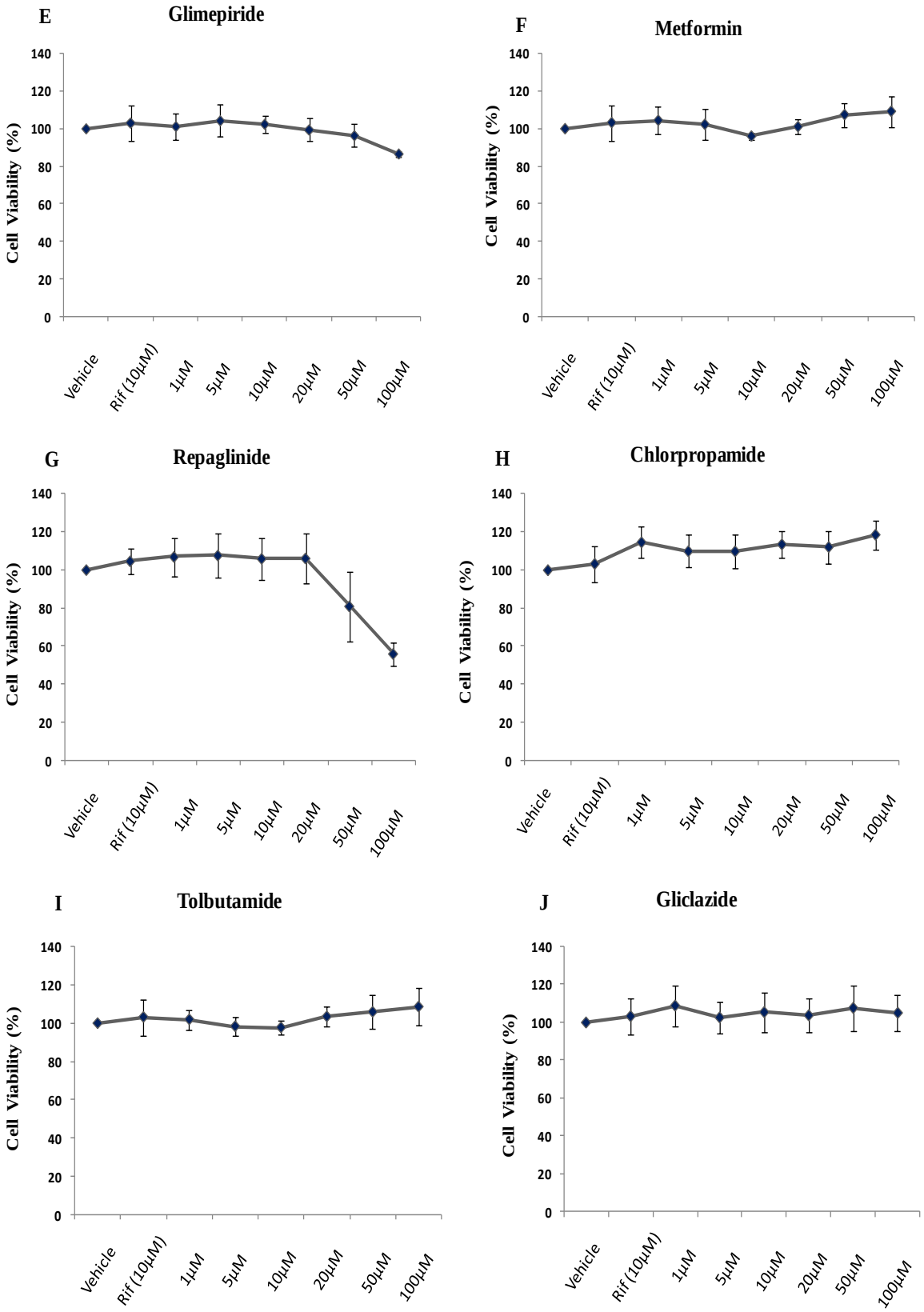
Effect of anti-diabetic drugs on cell viability

To visualize the effect of the selected concentrations of anti-diabetic drugs on cell viability, MTT assays were performed. MTT assay resolves any artifacts observed for the decreased luciferase activities examined in the previous section for PXR transactivation assay (**Figure 6**). For this assay, HepXREM cells were seeded in 96-well culture plate and treated with different drugs at their final concentration of 1µM, 5µM, 10µM, 20µM, 50µM and 100µM for 24 hr. After 24hr of incubation period, MTT assays were performed as described in 'Materials and Methods'. Rifampicin, rosiglitazone, pioglitazone, glimepiride and dapagliflozin appeared to decrease the cell viability by 13.77%, 13.87%, 47.80%, 13.21% and 17.51% at their 100µM concentrations (**Figure 7**). Repaglinide decreased the viability by 19.11% at 50µM and 45.15% at its 100µM concentrations. Troglitazone was found to be cytotoxic and drastically reduced the viability at its concentration ranging from 20µM to 100µM. At the 20µM, 50µM and 100µM concentrations of troglitazone viability decreased by 9.39%, 52.07% and 60.35% and this may be a plausible reason behind the decreased PXR transcriptional activity at the corresponding concentrations (**Figure 6**). Troglitazone was also reported to be hepatotoxic, causing hepatocellular injury, a reason for its withdrawal (Jaeschke, 2007).

As expected, most of the drugs examined in this study have shown some degree of cytotoxicity at their higher concentration of 50µM and 100µM. So, based on the results of PXR transactivation assay (**Figure 6**) and cell proliferation assay (**Figure 7**) optimum concentrations of each anti-diabetic drug were selected for further studies. Concentrations of anti-diabetic drugs, at which they have shown maximum PXR transcriptional activity without compromising cellular viability, were considered as optimum. Rosiglitazone and pioglitazone were the only anti-diabetic drugs which enhanced the PXR transcriptional activity at 20µM without causing cellular toxicity. While, the remaining anti-diabetic drugs did not influence the PXR

transactivation. Based on these observations, 20µM concentration was chosen to be optimum for rosiglitazone and pioglitazone and 10µM for the remaining anti-diabetic drugs.




E
Glimepiride
F
Metformin
G
Repaglinide
H
Chlorpropamide
I
Tolbutamide
J
Gliclazide
Cell Viability (%)
0
20
40
60
80
100
120
140
Vehicle
Rif (10μM)
1μM
5μM
10μM
20μM
50μM
100μM

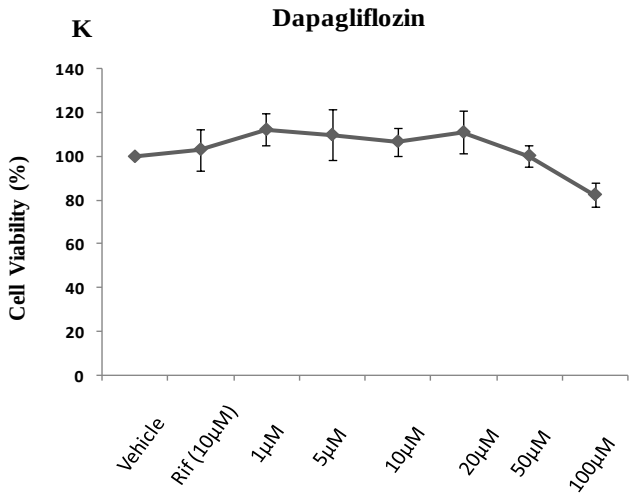
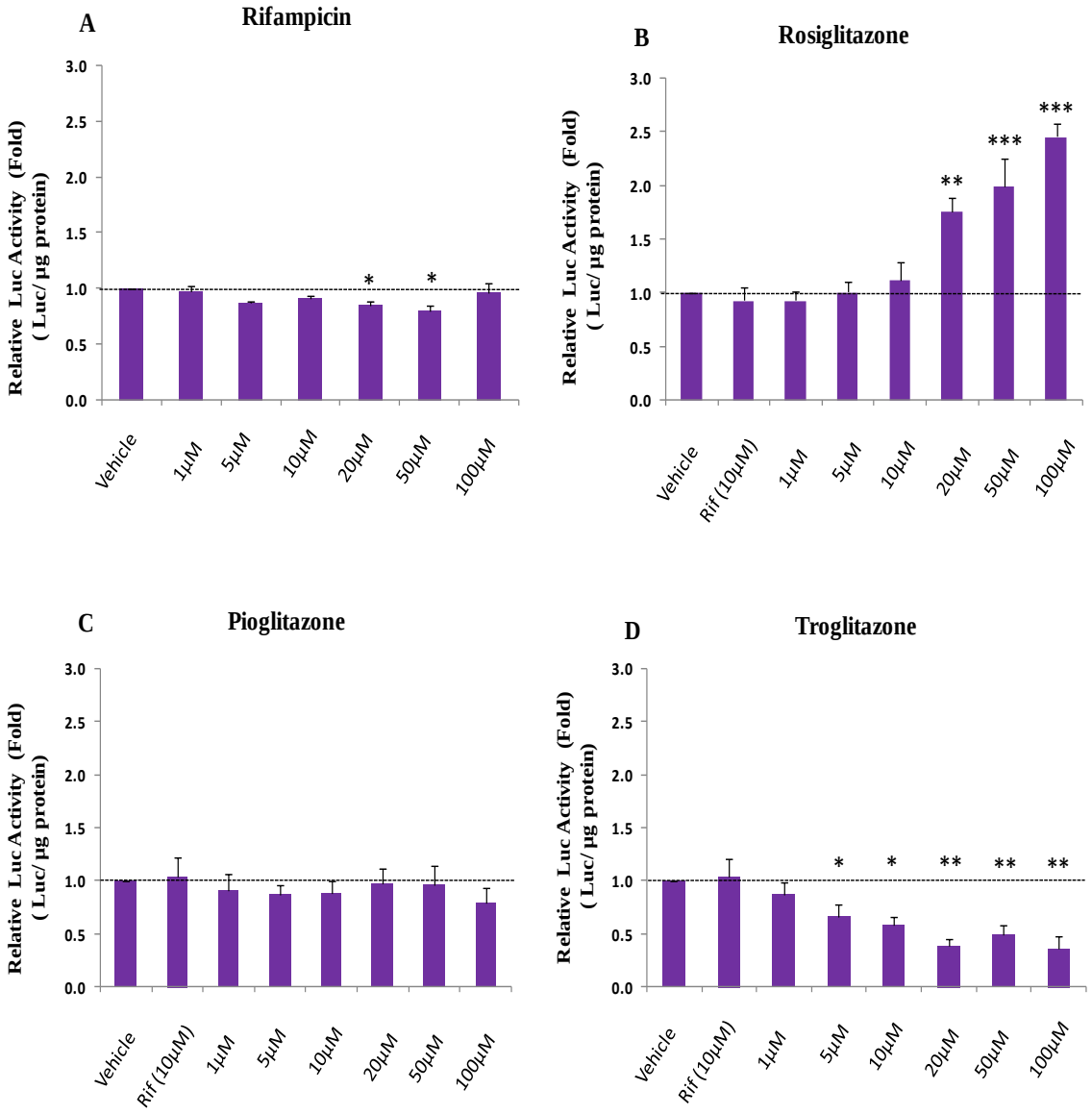


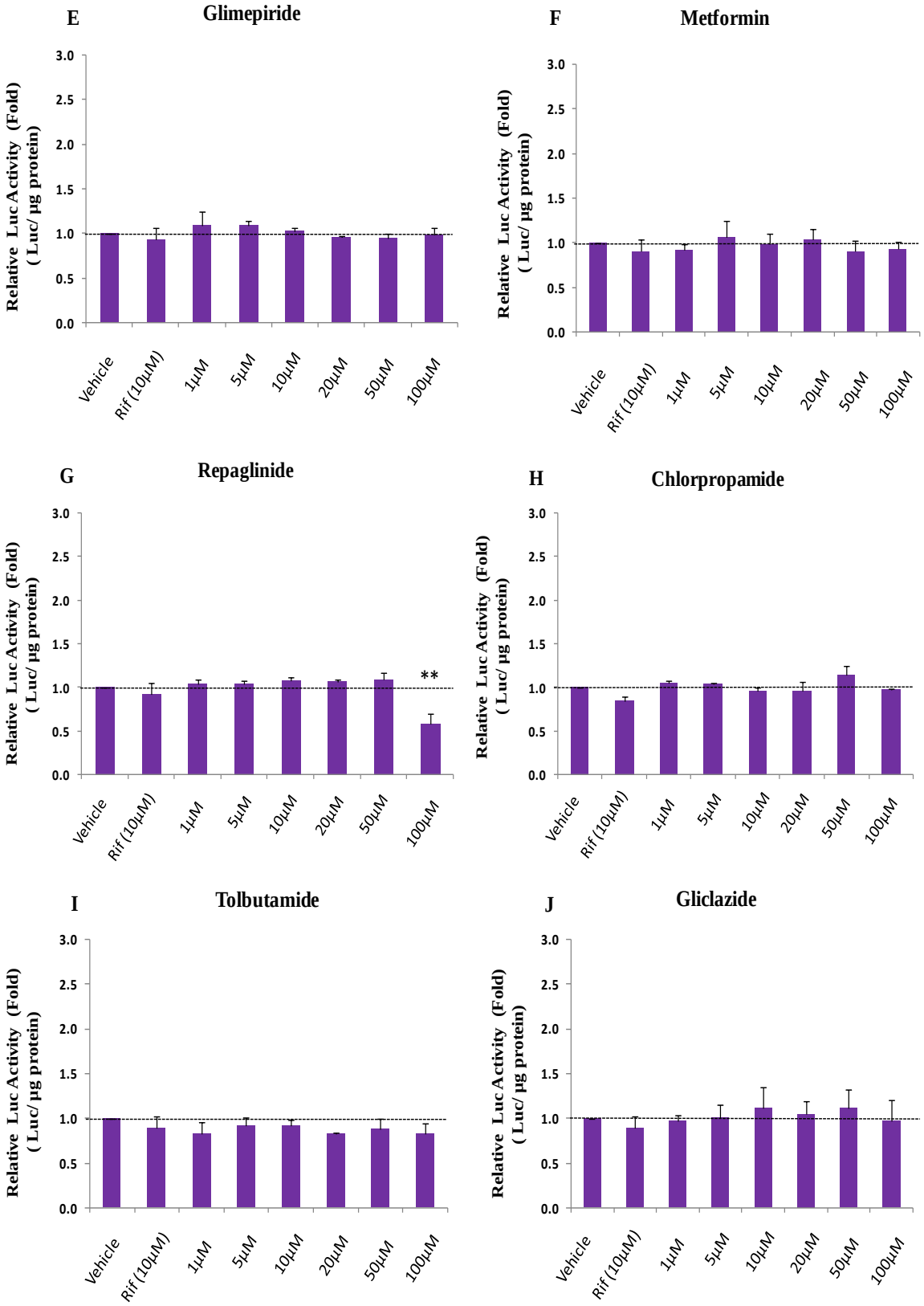
Figure 7: Dose-dependent effect of anti-diabetic drugs on cell viability. *HepXREM cells were seeded in 96-well plate and treated with 1μM, 5μM, 10μM, 20μM, 50μM and 100μM concentration of each of the anti-diabetic drugs for 24 hr.* ***(A)*** *Rifampicin* ***(B)*** *Rosiglitazone* ***(C)*** *Pioglitazone* ***(D)*** *Troglitazone* ***(E)*** *Glimepiride* ***(F)*** *Metformin* ***(G)*** *Repaglinide* ***(H)*** *Chlorpropamide* ***(I)*** *Tolbutamide* ***(J)*** *Gliclazide* ***(K)*** *Dapagliflozin. After the drug treatment period, MTT assay was performed. Data represent the mean ± SE of three independent experiments. Vehicle treated cells were considered as 100% viable and drug treated cells were compared with respect to the vehicle treated ones.*

Modulation of PXR-promoter activity by prospective anti-diabetic drugs

In the above study, the anti-diabetic drugs have been shown to activate PXR post translationally. This augmentation can also be seen when PXR expression is increased after treatment with these drugs. To examine whether these drugs are modulating the expression of PXR, stable cell line Hepx-497/+43 (stably integrated with PXR promoter-reporter construct -497/+43-luc) has been used. Hepx-497/+43 stable cells were seeded in 24-well plates in complete DMEM having 5% steroid-stripped serum without antibiotics and incubated in CO_2 incubator. Next day, the cells were treated with different drugs at the final concentrations of 1μM, 5μM, 10μM, 20μM, 50μM and 100μM for 24 hr. On completion of drug treatment, cells were processed for luciferase reporter assay. A gradual increase in the PXR-promoter activity was observed with rosiglitazone, where activity was augmented at 20μM, followed by 50μM and maximal at 100μM concentration. Dapagliflozin has also exhibited enhancement in PXR-promoter activity at 100μM concentration. While, at 100μM concentration, repaglinide has shown decreasein the PXR-promoter activity (**Figure 8**) apparently due to cellular toxicity (**Figure 7**). Troglitazone manifested

constant decrease in the luciferase activity in a dose-dependent manner from 1µM to 100µM. Likewise its effect on PXR transcriptional activity (**Figure 6**), diminished promoter-activity may be due to its cytotoxicity (**Figure 7**) and not actually related with decreased PXR-promoter activity (**Figure 8**).




E
Glimepiride
F
Metformin
G
Repaglinide
H
Chlorpropamide
I
Tolbutamide
J
Gliclazide
Relative Luc Activity (Fold)
(Luc/ µg protein)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Vehicle
Rif (10µM)
1µM
5µM
10µM
20µM
50µM
100µM
**

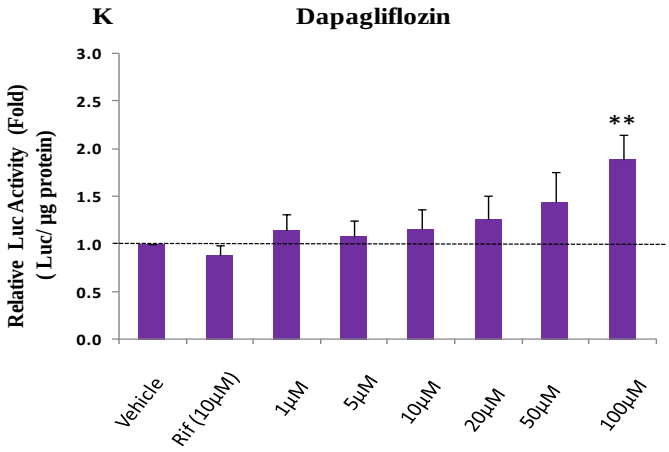


Figure 8: Dose-dependent effect of anti-diabetic drugs on PXR-promoter reporter activity. *Hepx-497/+43 cells (stably integrated with PXR-promoter reporter construct) were treated with 1µM, 5µM, 10µM, 20µM, 50µM and 100µM concentration of each of the anti-diabetic drugs for 24 hr.* ***(A)*** *Rifampicin* ***(B)*** *Rosiglitazone* ***(C)*** *Pioglitazone* ***(D)*** *Troglitazone* ***(E)*** *Glimepiride* ***(F)*** *Metformin* ***(G)*** *Repaglinide* ***(H)*** *Chlorpropamide* ***(I)*** *Tolbutamide* ***(J)*** *Gliclazide* ***(K)*** *Dapagliflozin. Rifampicin, a potent PXR agonist was used at 10µM. After the treatment period, cells were harvested and luciferase activity was measured and normalized with protein values. Luciferase activity is expressed as fold compared with control (DMSO: EtOH treated) cells. Data represent the mean ± SE of three independent experiments. Asterisks (*, ** and ***) signify luciferase values that differed significantly from the scores of corresponding controls (P<0.05, P<0.01 and P<0.001 respectively in Student's t-test).*

Induction of PXR expression in intestinal cells (LS180) by rosiglitazone and pioglitazone

From the previous experiment, it was perceived that rosiglitazone at 20µM and 50µM while, dapagliflozin at 100µM concentration manifest increased PXR-promoter activity in Hepx-497/+43 stable PXR-promoter-reporter cells (**Figure 8**). To further cross-examine the effect of these drugs on endogenous PXR protein level, we conducted our experiments in LS180 cells. LS180 acts as a model cell line for such studies as it is considered to express significantly higher PXR levels (Gupta et al., 2008; Harmsen et al., 2008). LS180 cells were propagated in 100 mm culture plates in DMEM with 5% steroid-stripped serum without antibiotics. Equal amounts of total protein (100µg) was run on 10% SDS-gel and detected with anti-PXR antibody.

No significant difference in protein level between drug-treated and vehicle-treated cells were observed **(Figure 9**). HepXR cells (having stable integration of PXR), was used as positive control for PXR.

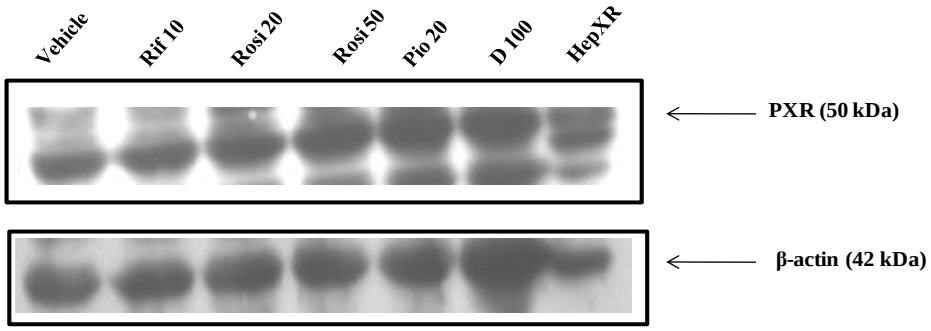


Figure 9: **Effect of anti-diabetic drugs on the PXR protein expression in LS180**. *LS180 cells were grown in 100 mm culture plates and the next day were treated with vehicle (control), 10μM of rifampicin, 20μM and 50μM of rosiglitazone, 20μM pioglitazone and 100μM of dapagliflozin and allowed to incubate for 24 hr. Following the treatment period, cell lysate were prepared and equal amounts of total protein samples were run on SDS-gel. Proteins were transferred to PVDF membrane and western blot was performed. β-Actin served as a loading control. Arrow indicates 50 kDa band of PXR and 42 kDa band of β-actin (an endogenous control).*

Induction of CYP3A4 expression in intestinal cells (LS180) by rosiglitazone and pioglitazone

From our preliminary experiments, both the drugs rosiglitazone and pioglitazone have been found to induce the CYP3A4 promoter-reporter construct via PXR activation (**Figure 6**). To further examine the effect of rosiglitazone and pioglitazone on the endogenous CYP3A4 expression level, CYP3A4 mRNA and protein levels were measured. To verify the change in CYP3A4 mRNA level, LS180 cells were seeded into 60 mm plates a day before drug treatment. Next day, the cells were treated with vehicle control, rifampicin (10μM), rosiglitazone (20μM) and pioglitazone (20μM) for 24 hr. After 24 hr, total RNA was isolated as described in 'Material and Methods' and quantitative real-time PCR was performed using human CYP3A4 primers and SYBR Green Master mix. The C(t) value of CYP3A4 was normalized with the C(t) value of endogenous control β-actin. We observed that rifampicin induced the CYP3A4 mRNA level up to 4-fold while rosiglitazone and pioglitazone induced the CYP3A4 expression 3.4-folds and 1.77-folds respectively (**Figure 10A**). Next, to see the change in the protein level of CYP3A4 after the

treatment of rosiglitazone and pioglitazone, LS180 cells were seeded and treated with the drugs following the same procedure as was done for measuring its mRNA level. We observed ~2.0 fold increase in the CYP3A4 protein (57 kDa) level by both rifampicin and rosiglitazone and 1.5-fold by pioglitazone as compared to vehicle alone (**Figure 10B, C)**. HEK cells, transiently transfected for CYP3A4 expression, served as positive control.

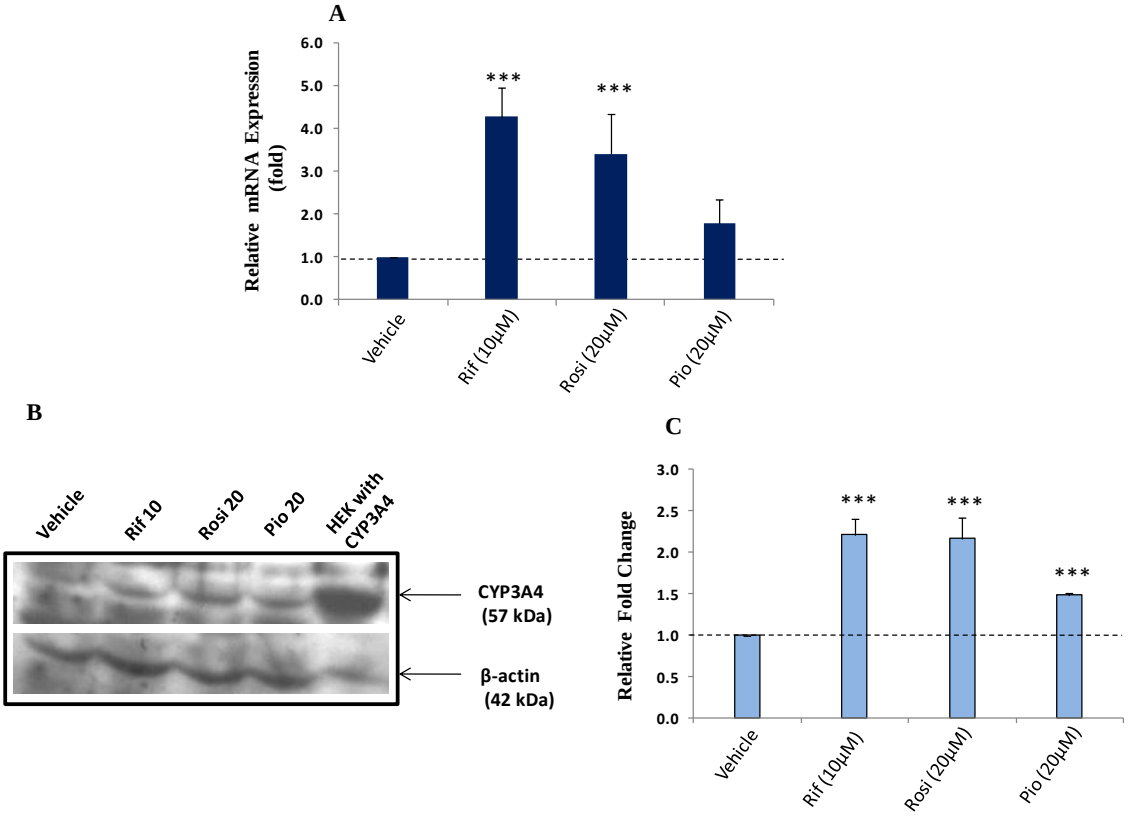


Figure 10: **Effect of anti-diabetic drugs on the CYP3A4 transcript and protein levels in LS180 cells**. *LS180 cells were grown in 60mm plates and treated with 10μM rifampicin, 20μM rosiglitazone and 20μM of pioglitazone for 24 hr.* ***(A)*** *After 24 hr, total RNA were isolated and mRNA levels were analyzed by quantitative real-time PCR. Data were normalized with β-actin.* ***(B)*** *Proteins isolated from cell extracts were separated on 10% SDS-PAGE. After separation, proteins were transferred on PVDF membrane and CYP3A4 was probed with polyclonal CYP3A4 antibody. β-Actin was used as loading control. Extracts from HEK cells transfected with CYP3A4 plasmid was used as positive control.* ***(C)*** *The relative fold change in the endogenous protein expression levels of CYP3A4 was quantified by densitometry. Individual protein values were normalized with β-actin and expressed as fold. Data represent the mean ± SE of three independent experiments. Asterisk *** signifies values that differed significantly from the scores of corresponding control (P<0.001 in Student's t-test).*

Cloning of UGT1A1 promoter and generation of UGT1A1-Luc construct

UGT1A1 is one of the main conjugating enzymes among phase II enzymes, regulated by PXR in response to drugs. UGT1A1 promoter region -3484/-3194 (290 bp) containing different xenobiotic receptor binding sites (Sugatani et al., 2008) (**Figure 11A**), was cloned into pGL3 promoter vector. Genomic DNA was isolated from LS180 cells by using Wizard^{R} genomic DNA purification kit according to the manufacturer's protocol (Promega, Madison, WI, USA). The isolated genomic DNA acted as template to amplify 290 bp region (-3484/-3194) of UGT1A1 minimal promoter region by PCR using the following specific primers

Forward- 5'-TTTAGACGCGTTACACTAGTAAAGGTCACTCA-3'

Reverse-5'-TAATACTCGAGCCCTCTAGCCATTCTGGA-3'

Chimeric UGT1A1 promoter-luciferase reporter construct was generated by digesting this amplified fragment with Mlu I and Xho I which was cloned into Mlu I and Xho I digested pGL3-promoter vector having compatible ends (**Figure 11B**). This construct was further verified by sequencing.

Up-regulation in the expression level of UGT1A1 gene

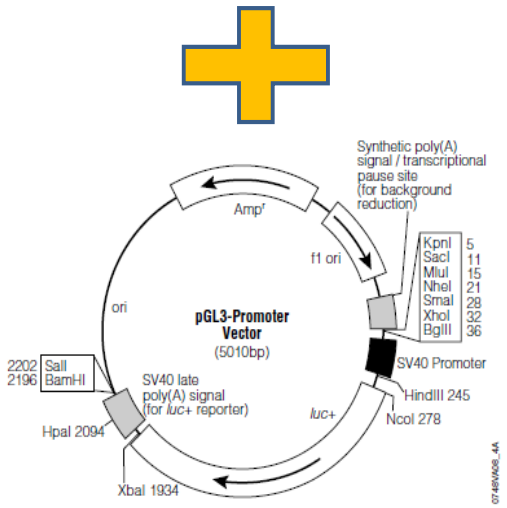
PXR also regulates the expression of Phase II drug metabolizing enzymes, including UDP-glucuronosyl transferases (UGTs), sulfotransferases (SULTs) and glutathione S transferases (GSTs) enzymes (Xu et al., 2005). UGTs, SULTs and GSTs contribute extensively to metabolism by catalyzing the addition of a UDP-glucuronic acid, sulphate conjugates and glutathione (GSH) moieties to endobiotics and xenobiotics (Bian et al., 2007). Addition of these polar molecules to the xenobiotics and endobiotics, enhance their cellular solubilities. Indeed, a major consequence of PXR- mediated Phase II metabolic enzyme regulation results into the metabolism and detoxification of bile acids, estrogens and xenobiotics (Xie et al., 2003). Like CYP3A4 (a major Phase I enzyme), conjugation reaction of Phase II biotransformations are mainly executed by UGT family members, and among them, UGT1A1 plays a crucial role in the metabolism of xenobiotics/endobiotics by catalyzing conjugation (Kiang et al., 2005). It has been reported that flavonoid chrysin leads to the induction of UGT1A1 gene (Yueh et al., 2003). So, to investigate the induction of UGT1A1 after the treatment of rosiglitazone and pioglitazone, HepG2

cells were co-transfected with PXR expression plasmid and construct having the UGT1A1-promoter region of 290bp ligated with luciferase enzyme coding gene (UGT1A1-luc). Subsequent to transfection, cells were treated with different experimental drugs for 24 hr, following which luciferase activity was determined. The transcriptional activity of UGT1A1-promoter was substantially increased by rosiglitazone and pioglitazone as compared to rifampicin (**Figure 12A**). Further, mRNA level of UGT1A1 was also examined after drug treatment, using UGT1A1 specific primers by real-time PCR. It was observed that rifampicin and rosiglitazone induced the UGT1A1 mRNA level by 2.5-fold while pioglitazone induced by 2.0-fold (**Figure 12B**).

A **UGT1A1 promoter (-3484/-3194) and binding site for xenobiotic receptor**

Mlu-

TACACTAGTAAAGGTCACTCAATTCCA(CAR)AGGGGAAAATGATTAACCAAAGAACATTCTAACGGTTCATAAAGGGTATTAGGTGTAA
TGAGGATGTGTTATCTCACCAGAAC(GRE1)AAACTTCTGAGTTTATATAACCT(CAR, PXR)CTAGTTACATAACCTGAAACCCGGAC
TTGGCACTTGGTAAGCACGCAATGAA(AhR)CAGTCATAGTAAGCTGGCCAAGGGTAGAGTTCAGTTTGAACA(CAR, PXR)AAGC
AATTTGAGAACATCAAAGGAAGTTTGGGGAACAGCA(GRE2)AGGGATCCAGAATGGCTAGAGGG-XhoI



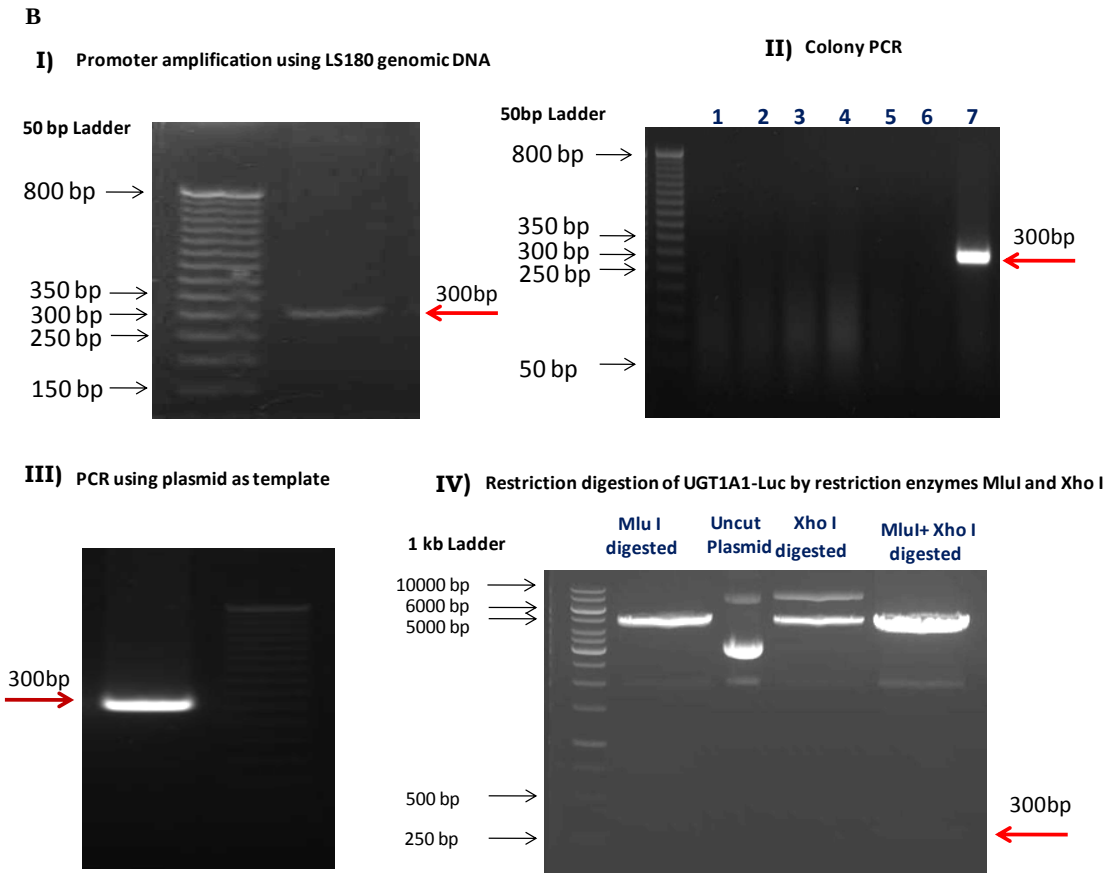


Figure 11: **Cloning of human UGT1A1 promoter region -3484/-3194 and generation of UGT1A1-Luc** ***(A)*** *UGT1A1 promoter region (-3484/-3194) harboring consensus response element motifs for different nuclear receptors has been shown. This 290bp region (-3484/-3194) of promoter was amplified from genomic DNA, digested with restriction enzymes and cloned into digested pGL3 promoter vector.* ***(B, I)*** *UGT1A1 290bp region was cloned by PCR amplification from LS180 genomic DNA. Size of amplicon increased to 300bp due to addition of few bases of restriction sites at the two ends. PCR amplified fragment and pGL3 promoter vector were digested with MluI and XhoI and proceeded for ligation in 20µl reaction volume for 16 hr. After ligation, competent E.coli DH10β bacterial cells were transformed with 10µl of ligation product.* ***(B, II)*** *Some colonies appeared after transformation and some were screened for 300bp amplicon of promoter by using promoter specific primers. One colony was found positive among seven colonies screened for insertion of 300bp promoter region.* ***(B, III)*** *Plasmid from positive colony was isolated and verified for the presence of 300bp promoter region by PCR amplification. The plasmid construct has shown the presence of insert (desired promoter) of 300bp amplicon size.* ***(B, IV)*** *Presence of insert in pGL3 promoter vector was further verified by restriction digestion using MluI and XhoI enzymes. A 300bp fallout after double digestion with MluI and XhoI, confirmed that UGT1A1-Luc has been generated.*

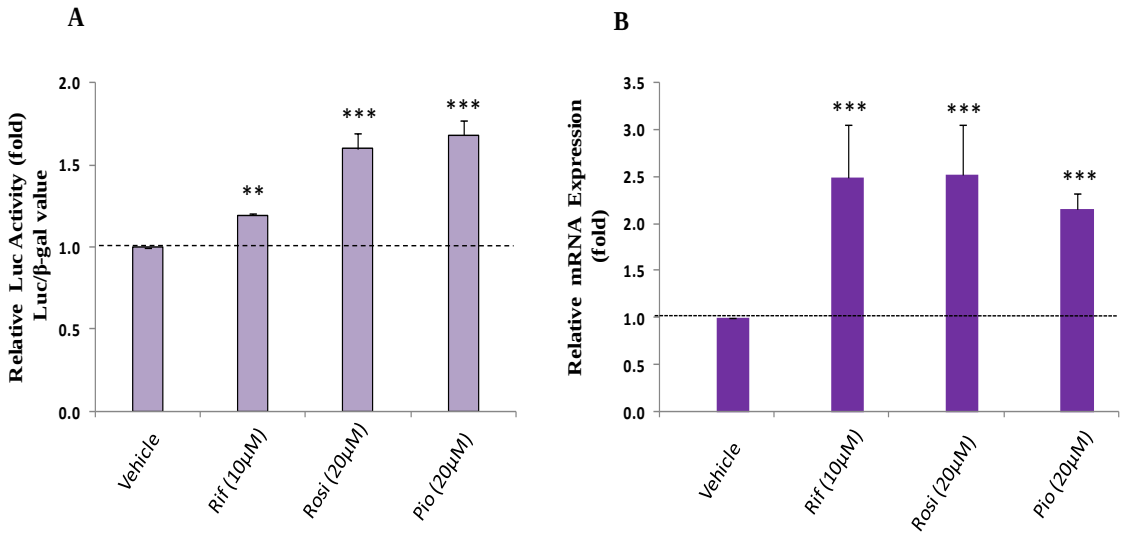


Figure 12: **Effect of anti-diabetic drugs on UGT1A1 expression level.** ***(A)*** *HepG2 cells were seeded and co-transfected with PXR expression plasmid and construct UGT1A1-luc in ratio of 1:6. For normalization, plasmid encoding β-gal enzyme was included at 120ng concentration/well of 12-well plate. After the transfection period, cells were treated with 10μM of rifampicin, 20μM of rosiglitazone and pioglitazone for 24 hr. After treatment, luciferase assay was performed and normalized with β-gal value.* ***(B)*** *LS180 cells were cultured in 60 mm plate and treated with 10μM of rifampicin, 20μM of rosiglitazone and 20μM of pioglitazone for 24 hr. After 24 hr, total RNA were isolated and mRNA was quantified by real-time PCR. Data were normalized with β-actin. Data represent the mean ± SE of three independent experiments. Asterisks (** and ***) signify values that differed significantly from the scores of respective controls (P<0.01 and P<0.001) in Student's t-test.*

PXR-mediated transactivation of MDR1-promoter

There are mounting evidences available claiming that PXR regulates Phase I, Phase II and Phase III components of the 'xenobiotic detoxification and elimination machinery'. PXR induced the expression of CYP3A4 (Phase I) and UGT1A1 (Phase II) enzymes of this machinery after getting activated by anti-diabetic drugs (**Figure 6, 10, 12**). Next, we examined the effect of the selected anti-diabetic drugs on MDR1 promoter-reporter mediated by activated PXR. To execute this experiment, HepG2 cells were seeded and transfected with pSG5-PXR expression plasmid and MDR1-promoter-reporter construct (p7975/7013-tk-luc) in a ratio of 1:8 in 12-well plates. After transfection, the cells were treated with optimal concentrations (20μM for rosiglitazone and pioglitazone and 10μM for other drugs) of all drugs and incubated for 24 hr. After the drug treatment period, cells were harvested and luciferase assay

was performed. Rosiglitazone and pioglitazone induced MDR1-promoter more than that of rifampicin (**Figure 13**).

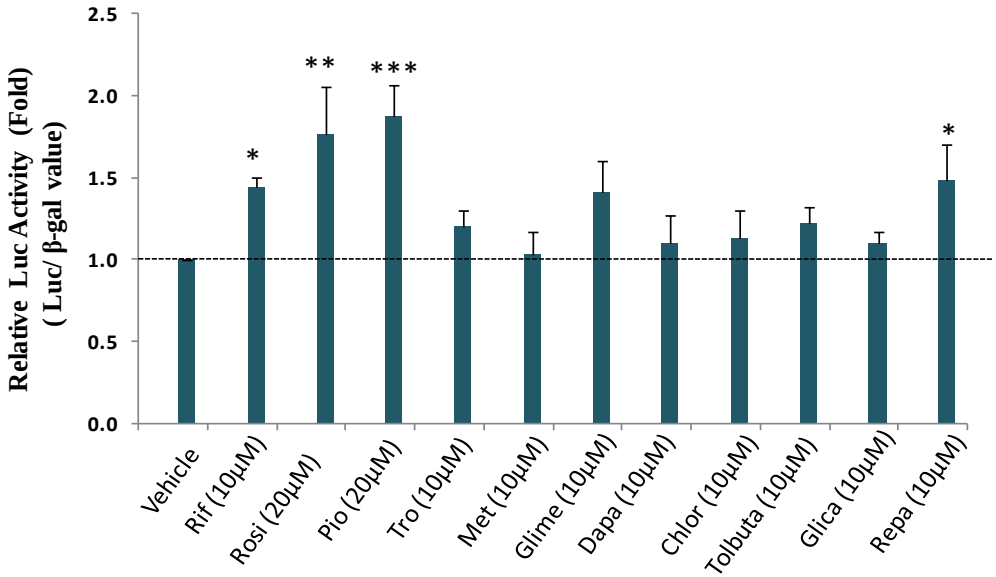


Figure 13: Effect of anti-diabetic drugs on PXR-mediated transactivation of MDR1-promoter-reporter (p-7975/7013/Tk-luc): *HepG2 cells were co-transfected with pSG5-PXR expression plasmid along with construct MDR1 (7975/7013)-Tk-Luc in ratio of 1:8. For normalization, plasmid encoding β-gal enzyme was included at 120ng concentration/well of 12-well plate. After the transfection period, cells were treated with specific concentrations of each of the anti-diabetic drugs for 24 hr. Except rosiglitazone (20μM) and pioglitazone (20μM), all the other drugs were used at 10μM. Rifampicin, a potent agonist of PXR was also used at 10μM. After 24 hr, the luciferase activity was determined and normalized with β-gal value. Data represent the mean ± SE of three independent experiments. Asterisks (*, ** and ***) signify luciferase values that differed significantly from the scores of corresponding controls (P<0.05, P<0.01 and P<0.001 respectively in Student's t-test).*

Induction of MDR1 mRNA expression in intestinal cells (LS180) by rosiglitazone and pioglitazone

Both of the redundant drugs rosiglitazone and pioglitazone had induced the MDR1-promote by modulating PXR in luciferase assay (**Figure 13**). Therefore, we focused our study primarily to rosiglitazone and pioglitazone. To further examine the effect of rosiglitazone and poglitazone on the MDR1 expression, endogenous expression levels of MDR1 mRNA were measured in LS180 cells (where PXR expression is higher). To see the change in MDR1 mRNA level, LS180 cells were seeded in 60 mm plates a day before drug treatment. Next day, the cells were treated

either with vehicle or with rifampicin (10µM), rosiglitazone (20µM) and pioglitazone (20µM) for 24 hr. After 24 hr, total RNA was isolated as described in 'Material and Methods' and quantitative real-time PCR was performed with SYBR Green using human MDR1 primers. We observed that rifampicin induced the MDR1 mRNA level up to 4-fold while rosiglitazone and piogllitazone induced the MDR1 expression up to 2-folds and 1.4-folds respectively (**Figure 14**).

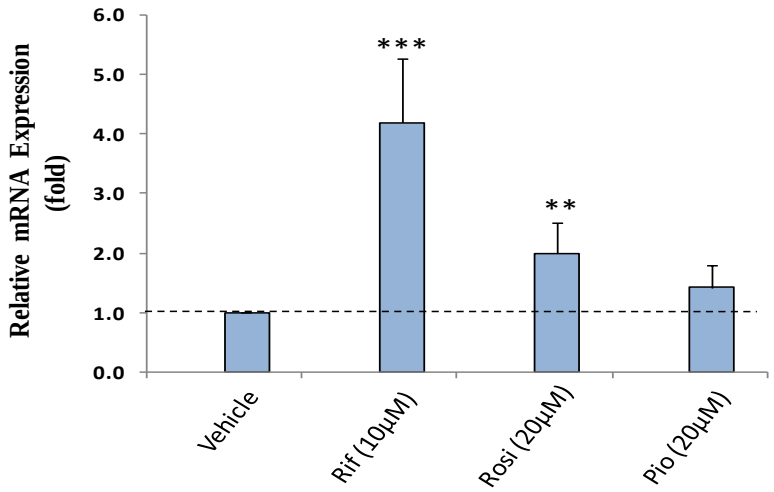


Figure 14: Effect of anti-diabetic drugs on the MDR1 mRNA level in LS180. *LS180 cells were cultured and treated with 10µM rifampicin, 20µM rosiglitazone and 20µM of pioglitazone for 24 hr. After treatment, total RNA was isolated and mRNA was analyzed by quantitative real-time PCR. Data were normalized with endogenous control β-actin. Data represent the mean ± SE of three independent experiments. Asterisks (** and ***) signify values that differed significantly from the scores of corresponding control (P<.01 and P<0.001 in Student's t-test).*

Modulation of CAR (Constitutive Androstane Receptor) transcriptional activity by anti-diabetic drugs

Constitutive androstane receptor (CAR), a member of the nuclear receptor superfamily, also acts as a transcriptional regulator of the 'DMD' machinery. CAR is another 'xenosensor' and also activates Phase I, Phase II and Phase III components of 'DMD' machinery like PXR. Though the primary function of CAR is regulation of DMD machinery, but it is also implicated in other physiological as well as pathophysiological conditions like obesity, diabetes and cancer by regulating energy homeostasis, insulin signaling pathways and cell proliferations (Mackowiak and Wang, 2016). CAR inhibits gluconeogenesis genes in case of diabetes (Dong et al., 2009). It is also reported that both PXR and CAR share some common ligands (Moore et al., 2000), as both of these receptors show ligand promiscuity. Owing to

these similarities, it was logical to examine whether these drugs also modulate CAR in a similar way as PXR. CAR is the principal transcriptional regulator of CYP2B6 gene (Maglich et al., 2003). CAR is known to bind at phenobarbital-responsive enhancer module (PBREM) lying in the enhancer region upstream of CYP2B6 proximal promoter. In order to examine the modulation of transcriptional activity of CAR by these drugs, transient transfections were performed in HepG2 cells. The cells were seeded in 12-well plate a day before transfection. On the following day,the cells were transfected with pcDNA3.1-CAR expression plasmid and CAR activated promoter-reporter construct, CYP2B6-Luc in the ratio of 1:6. After the transfection period, cells were treated with drugs for 24 hr. Rosiglitazone and pioglitazone were used at final concentration of 20µM while all others were used at their 10µM concentration. CITCO, a well known standard activator of CAR was used at 10µM according to the literatures (Huang et al., 2004). After 24 hr of treatment, cells were harvested for luciferase assay. It was observed that pioglitazone induces maximum transcriptional activity in CAR, while rosiglitazone activated CAR as potently as CITCO (**Figure 15A**). Since both CAR and CYP2B6 were co-transfected, there exists a possibility of induction of CYP2B6 by drugs without the involvement of CAR (**Figure 15A**). To rule out this possibility, CYP2B6 was either transfected alone or co-transfected with CAR in HepG2 cells. After transfection period, cells were treated with CITCO (10µM), rosiglitazone (20µM) and pioglitazone (20µM) and performed the luciferase assay (**Figure 15B**). The results were in agreement with the one observed in **Figure 15A**, confirming that drugs rosiglitazone and pioglitazone are working in CAR-dependent manner as they have not induced CYP2B6 alone (**Figure 15B**).

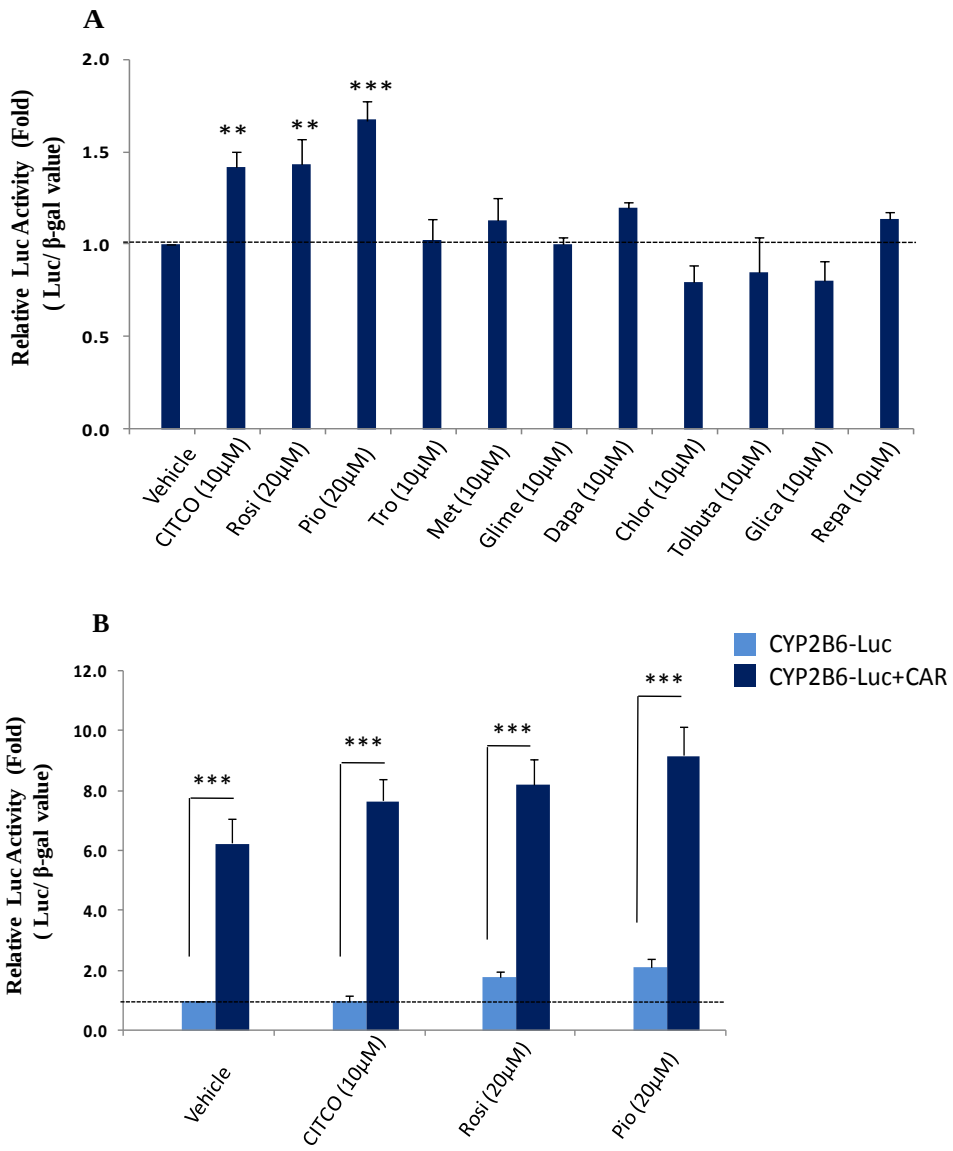


Figure 15: Effect of anti-diabetic drugs on CAR-mediated transactivation of CYP2B6-promoter reporter. *HepG2 cells were either co-transfected with expression plasmid pcDNA3.1-CAR and construct for CYP2B6 promoter-reporter in 1:6 ratio* ***(A), (B)*** *or transfected with CYP2B6 alone* ***(B)****. Plasmid encoding β-gal enzyme was co-transfected in all the wells for normalization. Similarly, carrier DNA was included to maintain equal concentration of total DNA in each well. After transfection period, drug treatments were given for 24 hr. Except for rosiglitazone (20μM) and pioglitazone (20μM) all other drugs were used at their 10μM concentration. CITCO was also used at 10μM. After 24 hr, luciferase activity was determined and normalized with β-gal value. Data represent the mean ± SE of three independent experiments. Asterisks (**and ***) signify luciferase values that differed significantly from the scores of corresponding controls (P<0.01, P<0.001) in Student's t-test.*

Transactivation of CYP2B6 by PXR after treatment of anti-diabetic drugs

CYP2B6 is a key member of the CYP450 family, which also catalyzes oxidative metabolism of medicinal compounds similar to CYP3A4. It has been found to be responsible for the detoxification of ~25% of xenobiotics, ranging from some anti-cancerous drugs, anesthetic drugs, drugs for central nervous system to anti-retro viral drugs (Xie et al., 2001). Though CYP3A4 and CYP2B6 are regulated predominantly by PXR and CAR respectively, these target genes are also reciprocally regulated by PXR and CAR both (Faucette et al., 2006). CYP2B6 response element harbours DR-4 type NR1 and NR3 elements which are symmetrically recognized by CAR and PXR both (Faucette et al., 2006). CAR preferentially binds with CYP2B6 than CYP3A4 responsive elements, while PXR recognize both (CYP3A4 and CYP2B6) response element modules and binds with equal affinity (Faucette et al., 2006). Rifampicin-activated PXR is also known to bind with and transcriptionally induce CYP2B6 gene (Wang et al., 2003). Following these lines of evidences, we also evaluated corresponding PXR-mediated transactivation of CYP2B6 under the influence of anti-diabetic drugs. For this purpose, HepG2 cells were co-transfected with expression plasmid pSG5-PXR and construct CYP2B6-Luc (CYP2B6-promoter-reporter). After transfection, cells were treated with drugs for 24 hr. All the experimental drugs used at their 10µM concentrations except rosiglitazone and pioglitazone, which were used at 20µM. Subsequently, luciferase activity was measured (**Figure 16A**). PXR-mediated transactivation of CYP2B6 was observed highest for pioglitazone, followed by rosiglitazone and then by rifampicin (**Figure 16A**). In order to avoid interference from cellular factors in transactivating CYP2B6 (i.e. in absence of PXR), CYP2B6 was transfected alone, with PXR as well and carried on in similar manner as in **Figure 15 B** for luciferse assay (**Figure 16B**). This exemplified the observation that enhancement in luciferase activity associated with CYP2B6 was only because of activation of PXR by selected drugs and not due to the involvement of another cellular factors (**Figure 16B**).

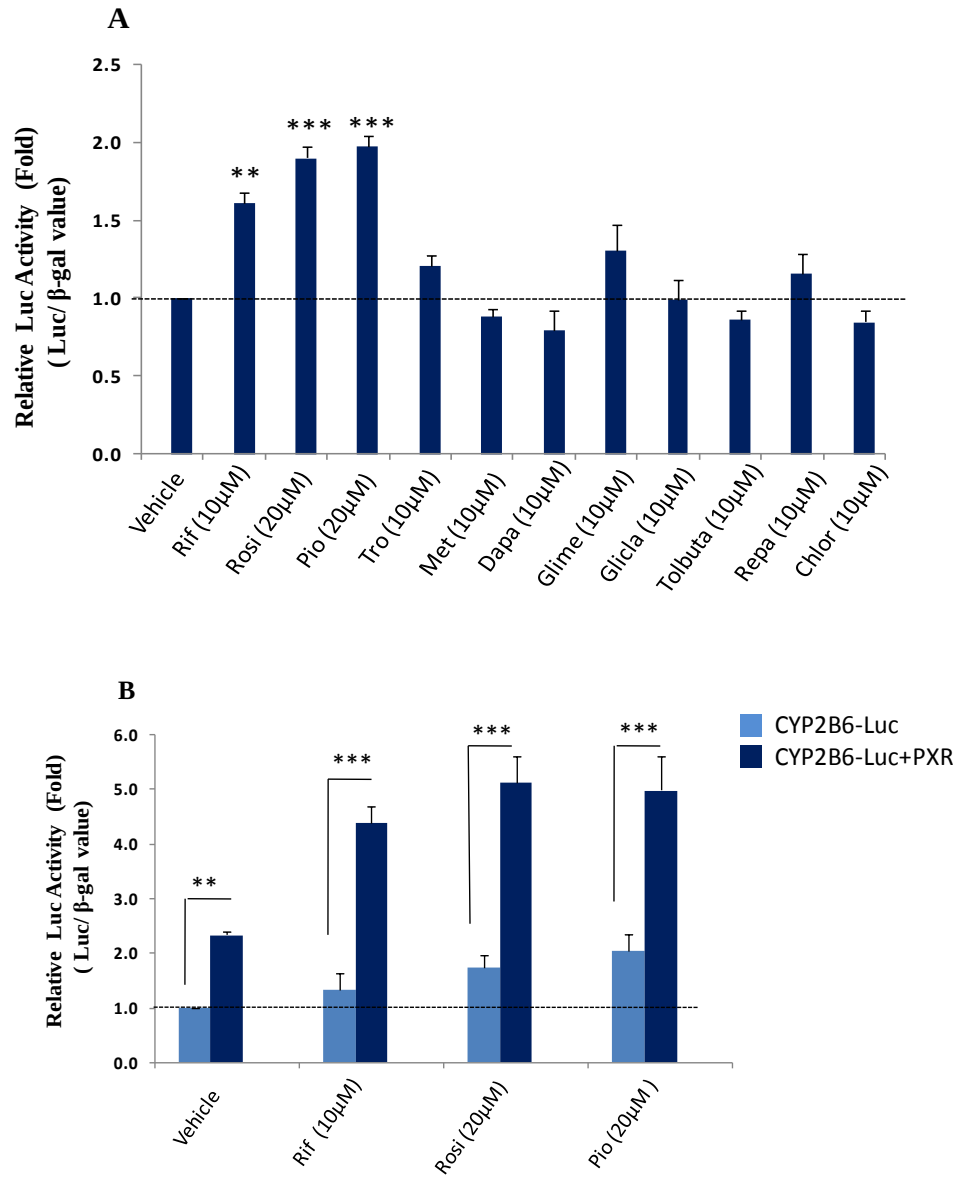


Figure 16: PXR-mediated transactivation of CYP2B6 by anti-diabetic drugs.
HepG2 cells were either co-transfected with expression plasmid pSG5-PXR and construct for CYP2B6-promoter reporter (CYP2B6- Luc) in 1:6 ratio ***(A), (B)*** *or transfected with CYP2B6-Luc alone* ***(B)*** *along with β-gal plasmid and carrier DNA. After transfection, the cells were treated with rosiglitazone and pioglitazone at 20μM and other anti-diabetic drugs at 10μM for 24 hr. Rifampicin, a potent ligand of PXR, was also used at 10μM. After that, cells were harvested and luciferase activity was measured and normalized by β-gal value. Data represent the mean ± SE of three independent experiments. Asterisks (** and ***) signify luciferase values that differed significantly from the scores of corresponding controls [(P<0.01and P<0.001) respectively in Student's t-test].*

Effect of anti-diabetic drugs on CAR-mediated induction of CYP3A4

PXR is the predominant regulator of CYP3A4 which plays an important role in the clearance of more than 50% of xenochemicals including prescription drugs. CAR is another xenobiotic sensor which also ensures protection from toxic insults by regulating hepatic clearance machinery that act in a co-ordinated fashion. As elaborated in aforementioned section about CYP2B6 regulation by PXR, CAR is also known to cross-transactivate CYP3A4 (Faucette et al., 2006). These researchers observed that CAR asymmetrically regulates CYP3A4, unlike PXR which indiscriminately transactivates CYP3A4 and CYP2B6 both. CAR exhibits high affinity for NR1 and NR2 (DR4-type) elements within the promoter of its prototypical target gene CYP2B6. On the contrary, CAR has less affinity for ER-6 motif present in the proximal promoter of CYP3A4 (pPXRE) gene (Faucette et al., 2006). Conversely, the affinity of CAR for DR-3 motif present in distal XREM (dPXRE) region (-7836/-7208) of CYP3A4 promoter is higher than the proximal motif ER-6 (Goodwin et al., 2002; Faucette et al., 2006). In 2002, Goodwin and group reported that, to transactivate CYP3A4 by CAR, the lesser affinity motif ER-6 (present in the proximal promoter) is required to work in co-operative manner with distal DR-3 motif. This functional crosstalk between both the sister xenobiotic receptors PXR and CAR have allocated them a feature for sharing some common set of genes coding for 'drug metabolism and transport' proteins. The reciprocity of PXR and CAR to activate CYP2B6 and CYP3A4 enables enhanced metabolism of xenobiotics by 80%. Following these evidences of their interplay, we performed experiments to assess the effect of these selected anti-diabetic drugs on the CYP3A4 transactivation by CAR.

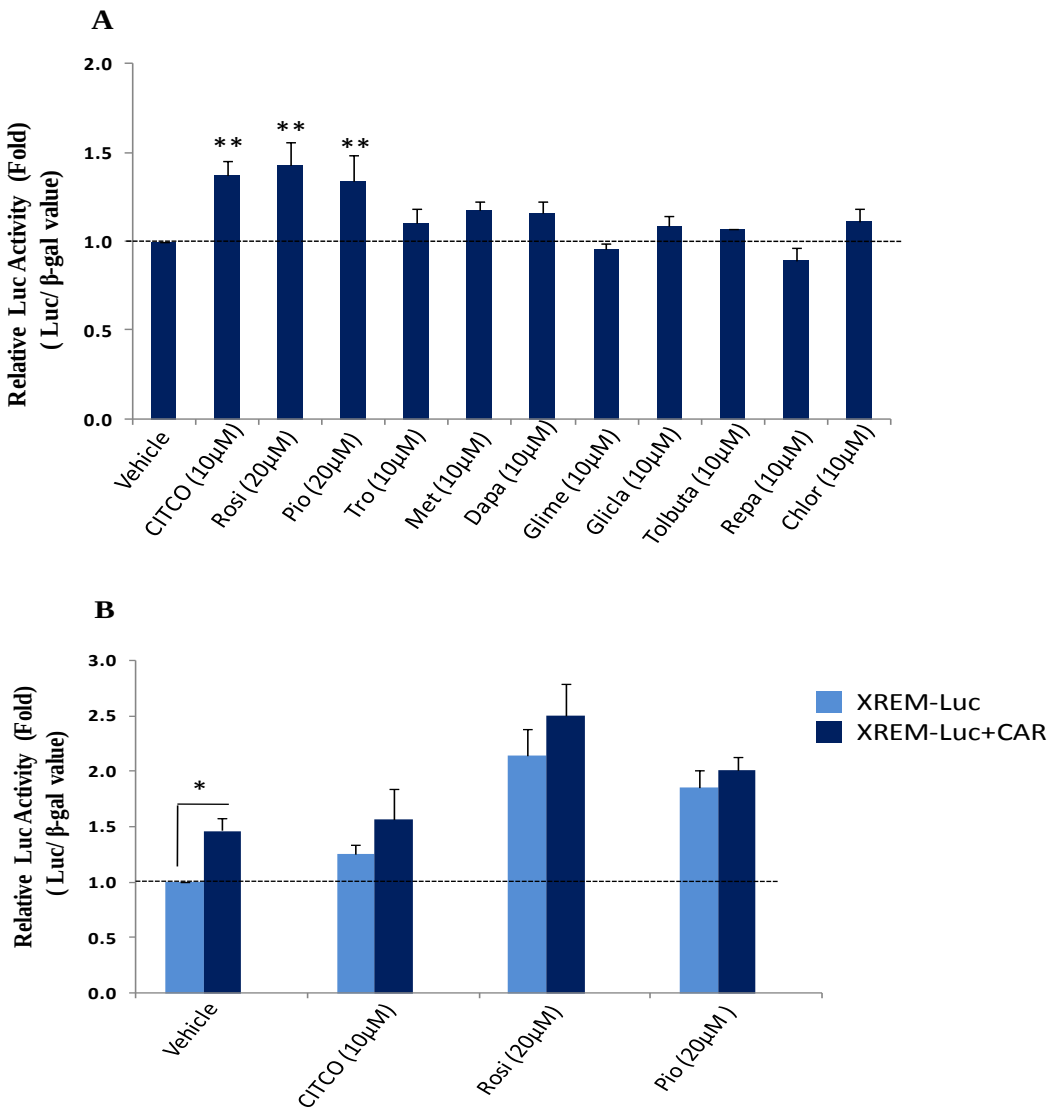


Figure 17: CYP3A4-promoter transactivation by CAR after anti-diabetic drug treatment. *HepG2 cells were either co-transfected with expression plasmid pcDNA3.1-CAR and construct for XREM-Luc in 1:6 ratio* ***(A), (B)*** *or transfected with XREM-Luc alone* ***(B)****. In addition, plasmid encoding for β-gal enzyme (for normalization) and carrier DNA (to keep equal concentration of total plasmid in each well) were also co-transfected. After completion of transfection period, the cells were treated with rosiglitazone and pioglitazone at 20μM and other anti-diabetic drugs at 10μM for 24 hr. CITCO, a ligand of CAR was used at 10μM. After 24 hr, luciferase activity was measured and normalized by β-gal value.Data represent the mean ± SE of three independent experiments. Asterisks (* and **) signify luciferase values that differed significantly from the scores of corresponding controls (P<0.05 and P<0.001 respectively in Student's t-test).*

Expression plasmid for CAR along with CYP3A4-promoter-reporter constructs (XREM-Luc) were co-transfected in HepG2 cells, in the ratio of 1:6. Drug concentrations used were same as in PXR and CYP2B6 transactivation assay (**Figure 16**). CITCO, a ligand and activator of CAR was used at 10µM concentration. After 24 hr of drug treatment, luciferase assay was performed (**Figure 17A**). Rosiglitazone, pioglitazone and CITCO (a well-known activator of CAR) have shown increased transactivation of CYP3A4 (**Figure 17A**). To find whether the enhancement in CYP3A4-promoter-activity is due to activation of CAR or modulation of other factors, we performed similar experiment by transfecting with CYP2B6 alone. Except this inclusion, rest of the experimental set up and steps were same. We observed that unlike PXR-dependent cross-transactivation of CYP2B6, transactivation of CYP3A4 is not a CAR-dependent event. Basal activity of CYP2B6 gets enhanced by rosiglitazone, pioglitzazone and reference ligand CITCO even in the absence of CAR (**Figure 17B**). This suggests the possible involvement of other cellular factors (like PXR).

DISCUSSION

The human body has developed a defense system to prevent the accumulation of endogenous (bile acids, steroids, cholesterol metabolites, neurotransmitters etc.) as well as exogenous (xenobiotics, dietary constituents and clinical drugs etc.) small molecules at toxic levels. This task is accomplished by 'drug metabolism and disposition (DMD) machinery' which entail Phase I, Phase II enzymes and Phase III transporter proteins. The components of this machinery act in a coordinated manner to biotransform and facilitate the elimination of small toxic molecules from the cellular milieu. PXR acts as a major transcriptional regulator of the 'DMD' machinery. Prescription of combination therapy is a common regimen during the treatment of diverse metabolic disorders and infectious diseases. In such combination therapies one drug may modulate the expression of genes of 'DMD', influencing the metabolism of another co-administered drug. This leads to decreased bioavailability or increased toxicity of the latter. There are plentiful of examples in literature reporting PXR as a key mediator of drug-drug, herb-drug and food-drug interactions (Negi et al., 2008; Prakash et al., 2015). One among the reported is St John's wort (herbal drug) showing drug-drug interactions (DDIs) with wide range of drug molecules. It has shown drug-drug interactions with immune-suppressant

cyclosporine and sirolimus, with cytotoxic drugs doxorubicin, etoposide, paclitaxel, vinblastin and with cardiovascular drugs digoxin, amiodarone as well as with indinavir, ritonavir, saquinavir etc. (anti-HIV drugs) decreasing the efficacy of these co-administered drugs (Tirona et al., 2006; Negi et al., 2008). PXR activation has been proposed in aforementioned cases of drug-drug interactions associated with St John's wort (Moore et al., 2000; Ernst, 2002). Due to severe consequences of drug-drug interactions with ketoconazole, drug terfenadine has been withdrawn from clinical practice. Similarly, mibefradil showing DDIs was also withdrawn (Eddershaw et al., 1999). Probability of failure of any medicine in co-medication therapy is high if one of the used agents influences the ADME (absorption, distribution, metabolism and excretion) of other medications leading to drug-drug interactions (Pal et al., 2006). In case of sulfonylurea and nateglinide class of anti-diabetic drugs, CYP2C9 (among the phase I drug metabolizing enzymes) has been shown to affect their metabolism. While up-regulation of CYP2C8 has been associated with altered metabolism of repaglinide and TZD class of oral anti-diabetic agents (Tornio et al., 2012). In type II diabetic cases as well, multidrug therapy is a common practice. For example TZD are prescribed additively with sulfonylurea and metformin.

Based on the information available in the aforementioned instances, we selected a class of anti-diabetic drugs (novel, established and withdrawn). These were then systematically evaluated at PXR level to determine if this screening platform can predict the success or failure of a drug. We utilized the HepG2 derived stable cell lines, HepXREM and Hepx-497/+43 generated in our laboratory to screen the drugs at dual level of PXR protein and its own promoter. The HepXREM cell line is used to investigate PXR activation potential of any drugs, while Hepx-497/+ 43 cells are used to assess PXR gene induction behavior of drugs. We selected 10 anti-diabetic drugs to examine their PXR activation/induction potential and likelihood of induction of 'DMD' machinery. In our preliminary experiment we have evaluated the dose-dependent effect of anti-diabetic drugs on the transcriptional activity of PXR at the concentrations of 1µM, 5µM, 10µM, 20µM, 50µM and 100µM. The transactivation findings in HepXREM stable cells suggest that out of the ten selected anti-diabetic drugs, rosiglitazone and pioglitazone were highly potent in activating PXR like its standard ligand rifampicin, at their 20µM concentration.

Rosiglitazone and pioglitazone transactivated PXR as potently as rifampicin. Repaglinide and gliclazide transactivated PXR moderately. Further, dose-dependent effects of all of the selected anti-diabetic drugs were evaluated on the viability of HepXREM cells. On the basis of outcome of PXR transactivation assay and cell viability, concentrations of all the anti-diabetic drugs at which they have shown maximum PXR activation without affecting cell viability, were selected as optimum. Except rosiglitazone and pioglitazone for which 20µM was selected as optimum, rest of the drugs were considered for further experiment at their 10µM concentration. All the selected TZD members (rosiglitazone, pioglitazone and troglitazone) also act as PPAR-γ ligands. Unlike to rosiglitazone and pioglitazone, another member of TZD class, troglitazone did not activate PXR and was withdrawn for inflicting cytotoxicity. Rosiglitazone and dapagliflozin enhanced PXR-promoter activity in cell line Hepx-497/+43.

Induction and involvement of CYP3A4 is primarily responsible for oxidative metabolism of ~60% of clinical drugs, which could lead to drug-drug interactions with co-medicated drugs, and therefore resulting in therapeutic failure (Zhou et al., 2005). There are several examples of drugs like St John's wort, carbamazepine, phenytoin, topiramate, rifampicin that induce CYP3A4 in PXR-dependent manner (Johannessen et al., 2010). DDIs change the drugs pharmacokinetic behavior leading to undesirable failures (Mizuno et al., 2003). PXR has now been proposed as the main transcriptional regulator of CYP3A4. Any small molecule that activates PXR-protein or induce PXR gene is likely to fail in clinical settings. Thus, screening of drugs for their potential to induce/activate PXR appears to be important in early stages of drug discovery processes. In this context, using HepXREM stable cell line we have directly demonstrated that the PXR-dependent CYP3A4 induction by the withdrawn TZD drugs (i.e. rosiglitazone and pioglitazone) is the plausible reason of their clinical failure. Interestingly, when CYP3A4 mRNA expression after the treatment of the same drugs was quantified by real-time PCR, the claim was supported. In the LS180 intestinal cells rifampicin has shown maximum PXR-dependent induction of CYP3A4 mRNA, followed by rosiglitazone and then by pioglitazone. Similar patterns of induction of CYP3A4 protein levels have also been observed by these TZD members. LS180 acts as a model cell line for such studies as it is considered to express significantly higher PXR levels (Gupta et al., 2008; Harmsen et al., 2008). Among the

phase II conjugating enzymes the UGT1A1, a member of UGTs family, plays a major role in increasing the hydrophilicity of small molecules. We also evaluated UGT1A1 induction by the same drugs. There was a significant activation of UGT1A1 promoter-reporter construct along with induction of mRNA by rosiglitazone and pioglitazone.

MDR1 (Phase III transporter) causes efflux of a broad range of structurally diverse and low affinity xenobiotics/endobiotics (Mizuno et al., 2003). This broad range substrate specificity appears to be responsible for drug-drug interactions during co-medication therapy. Induction of MDR1-promoter via PXR is shown by both the TZDs treatment and is also validated by up-regulation in the MDR1 mRNA level. Overall, our findings suggest that rosiglitazone and pioglitazone are highly potent activators of PXR and its target genes of 'DMD' machinery, thereby causing undesirable clinical consequences and their subsequent withdrawal.

CAR is a closely related to PXR and is another 'xenosensor' which also regulates Phase I (CYP2B6, CYP2C9, CYP2C19 etc.), Phase II (UGT1A1, SULT2A1, GSTA1 etc.) and Phase III (ABCB1, OATP2, MRP-1 etc.) genes involved in biotransformation and transport of endogenous and exogenous compounds. PXR and CAR control the expression of some overlapping sets of genes for 'DMD' machinery (Chang, 2009). Among Phase I enzymes, CYP2B6 was reported as a prototypical target gene of CAR, while CYP3A4 was known to be mainly regulated by PXR. The PXR and CAR are also known to cross transactivate CYP2B6 and CYP3A4. Taken together, induction of the respective target genes of PXR and CAR are responsible for the metabolism of ~80% of the prescription drugs. Therefore, activation of both of these xenosensors may lead to undesirable DDIs. Hence, pre-evaluation ofsmall drug molecules for the modulatory effects on PXR and CAR and development of common antagonists for PXR and CAR can resolve these safety concerns and treatment failures due to the harmful DDIs (Wang et al., 2012; Chai et al., 2016). Keeping the ligand promiscuous nature of CAR like PXR in view, we also evaluated CAR activation potential of selected anti-diabetic drugs as well as the cross-transactivation of CYP2B6 and CYP3A4 by PXR and CAR respectively. CAR-mediated transactivation of CYP2B6 (Phase I) has been shown to be increased byrosiglitazone and pioglitazone. PXR-mediated cross-transactivation of CYP2B6 promoter was found to be enhanced by rosiglitazone and pioglitazone .While there

was no effect of any of the drugs on the cross-transactivation of CYP3A4 by CAR. This suggests that PXR binds to the promoters of CYP3A4 and CYP2B6 with equal affinity while, CAR appears to have differential responses and selective activationof CYP2B6 over CYP3A4 by small molecules.

Rosiglitazone and pioglitazone, the withdrawn category of anti-diabetic drugs, are shown to transactivate and induce the expression of CYP3A4, UGT1A1 and MDR1 by activating PXR. Likewise, these two TZDs also possess CAR activation potential. In view of the observations made herein, it appears that these two drugs i.e. rosiglitazone and pioglitazone have failed clinically for causing myocardial infarction and bladder cancer respectively that may also be attributable to PXR and CAR activation. Their PXR and CAR activation behavior may have added to their clinical failure.

Therefore, it is advisable that when advocating for novel small therapeutic molecules, these molecules must be screened and validated for their nature as activators of PXR and/or CAR. Usually complete profiling of every drug is done before their launch in the market on the basis of different parameters. One of the important parameter among those is MDR1 and CYP450s induction. CYP450s are the Phase I detoxification component and MDR1 is the elimination component of the 'drug detoxification and elimination machinery'. Drugs are screened to examine whether they activate an important member of CYP450 family, CYP3A4 or not. It is reported that ~50% xenobiotics are detoxified by CYP3A4. This detoxification is enhanced upto 80% if CYP2B6 along with CYP3A4 is considered. If any drug activates both CYP2B6 and CYP3A4 then it would cause the severe drug-drug interactions (DDIs). Because of DDIs, another drug given in combination with these inducers will also be eliminated faster and their efficacy would be compromised. Drugs are also screened to assess whether they are substrate of MDR1. But no screening is done at the level of PXR and CAR, the major regulators of CYP3A4 and CYP2B6 respectively. Our study suggests that, had the PXR activation potential of rosiglitazone and pioglitazone been examined before the human trial these would never have been in the market at first place.

CHAPTER II

Mechanisms of PXR activation by anti-diabetic drugs

Background

There are growing bodies of evidences that xenobiotics can modulate the transcriptional activity of PXR directly, by binding with the receptor and indirectly, by altering certain kinds of signaling pathways which introduce post-translational modifications in PXR (Pondugula et al., 2009; Staudinger et al., 2011). NRs are apparently known to be activated in non-liganded manner by a variety of kinases in response to different stimuli (Rochette-Egly, 2003). Post-translational modifications (PTMs) of various NRs are also reported to play an important role in regulating their functions. These PTMs include phosphorylation, acetylation, sumoylation, ubiquitination, methylation, myristoylation, ADP-ribosylation and isoprenylation (Anbalagan et al., 2012) and are divided into two categories; reversible and irreversible PTMs. Reversible modifications add certain chemical groups (phosphate, acetyl) to the specific amino acid like serine, threonine and tyrosine, while in irreversible modifications specific proteins or polypeptide sequences are added, as in case of sumoylation and ubiquitination. Recently, some reports have highlighted the association between PTMs of certain NRs and disease progression in diabetes and cancer etc. (Anbalagan et al., 2012). Reported post-translational modifications of PXR are, phosphorylation, sumoylation, ubiquitination and acetylation (Smutny et al., 2013).

Mostly NRs are phosphorylated at their A/B region of NTD. This region is supposed to contain the consensus sequences to be recognized by proline-dependent kinases like CDKs, AkT-PKB (protein kinase B) and MAPKs (MAP-kinases) (Chang and Karin, 2001; Pearson et al., 2001; Rochette-Egly, 2003). Among the NRs which get phophorylated at their AF-1 region by p38 MAPK are AR, ER-α, ER-β, PR, PPARs and RARs (Rochette-Egly, 2003). RXR-α gets phosphorylated by JNK MAPK at its AF-1 domain (Adam-Stitah et al., 1999). RA (retinoic acid)-dependent phosphorylation of AF-1 region by p38 MAPK has been shown to regulate the transcriptional activity of RAR-γ. Like AF-1, LBD is also reported to get phosphorylated in ligand-independent manner. ER-α and RXR-α have been shown to get phosphorylated by tyrosine kinases, while RARs gets phophorylated by PKA at their LBD (Rochette-Egly, 2003). Similarly, NRs are also phosphorylated at DBD. ER-α gets phophorylated by PKA, while RAR-α and VDR by PKC at their DBD region (Rochette-Egly, 2003). Unlike to the phenomenon of phosphorylation of LBD

leading increased transcriptional activity, phosphorylation of DBD is associated with decreased activity of NRs (Rochette-Egly, 2003). The decreased transactivation is reported for VDR (PKC), RAR-α (PKC) and ER-α (PKA). PKC-mediated phosphorylation of VDR does not allow the receptor to bind with its response element, thus transcriptional activity get abolished (Hsieh et al., 1993). Heterodimerization of RAR-α with RXR gets affected after phosphorylation by PKC due to the decreased binding affinity of DBD (Delmotte et al., 1999).

PXR negatively regulates lipid metabolism, gluconeogenesis and inflammation directly by ligand binding and also indirectly by cross-talking with signaling pathways (Staudinger et al., 2011). Staudinger et al (2011) suggested that PXR exhibits its maximal transcriptional activity due to the integration of ligand-dependent activity and non-ligand based activation of signaling pathways causing PTMs of PXR. Forskolin, a diterpine and derivative of plant *C. forskohlii* of Indian origin, is shown to induce XREM-Luc. This plant has been used as an Ayurvedic medicine for a variety of diseases including heart disease, respiratory disease, hypothyroidism and also inhibit platelet aggregation (Ammon and Müller, 1985). Though forskolin is well-known to activate PKA by stimulating adenylate cyclase, its role extend beyond this, as forskolin also acts in cAMP and PKA-independent manner and behaves as a ligand of mouse PXR and thus, induced CYP3A11 in hepatocytes (Ding and Staudinger, 2004). PKA activation has been found to increase the co-activator interaction with PXR, to enhance PXR activity. They found that activation of PKA by an analog of cAMP (8-Br-cAMP) increased the interaction between human PXR and co-activator SRC-1 in CV-1 cell line. From this experiment they have proposed that PKA pathway and ligand-dependent PXR transactivation act in co-ordination with each other to fine tune the transcriptional activity of PXR (Ding and Staudinger, 2004).

Similar to aforesaid kinases which are well-described for phosphorylating several NRs and modulating their transcriptional activity, AMP-activated protein kinase (AMPK) is also known to regulate the functions of NRs via phosphorylation. AMPK senses the level of AMP and ATP inside the cell and is activated when AMP/ATP ratio increases inside the cells (Hardie et al., 1998). So, AMPK is known as a ‘metabolic master switch’ as it shuts off the anabolic pathways requiring ATP

like fatty acid and sterol synthesis, while turns on ATP generating catabolic pathways like fatty acid oxidation (Hardie et al., 1998; Viollet et al., 2006). AMPK gets activated in response to cellular stresses like reactive oxygen species, hypoxia, hyperosmolarity, ischemia, hypoglycemia etc. Transcriptional activity of PPAR-α is known under the control of AMPK (Sozio et al., 2011). AMPK has also been reported to negatively regulate the ligand-dependent LXR transcriptional activity (Yang et al., 2009). AMPK inhibits fatty acid synthesis by inhibiting SREBP-1c (sterol regulatory element binding protein-1c), a 'master regulator' of lipogenic genes in liver at transcriptional level (Yang et al., 2009). SREBP-1c encompasses LXR responsive element in its promoters. LXR ligand T0901317 has been shown to induce SREBP-1c promoter in LXR-dependent manner and this induction was reduced by activation of AMPK. However it remains ambiguous whether the suppression is due to phosphorylation of LXR (Yang et al., 2009). Nonetheless, the involvement of AMPK in the regulation of PXR transcriptional function is still not explicated.

MAPKs (mitogen-activated protein kinases) are one among principal kinases regulating a variety of aspects of cellular processes. There are six different types of MAPKs reported, among them ERK (ERK-1, 2), JNK (JNK-1, 2) and p38 (p38-α, β, γ, δ) prevail (Tanos et al., 2005). The cascade of MAPK pathways get initiated in response to environmental stimuli (growth factors, hormones, osmotic imbalance, heat shock, oxidative stress, UV-radiation etc.) and regulate the critical cellular processes like development, growth, differentiation and proliferations (Kato et al., 1997). SAPK term is prevalently referred for JNK and p38 MAPKs (Tanos et al., 2005). SAPKs are regulated by both cellular stress and physical stress like oxidative stress, osmotic shock, heat shock, UV-radiation, protein synthesis inhibitors etc. (Tanos et al., 2005). Glucocorticoid receptor (GR) has been reported to get phosphorylated by p38 MAPK to confront the stressors like UV-irradiation and oxidative stress and maintain homeostatic balance in both hormone (glucocorticoid)-dependent as well as independent manner (Galliher-Beckley et al., 2011). Likewise to GR, ER-α and ER-β have also been reported to get phosphorylated by protein kinases and execute their transcriptional functions (Zhang et al., 2006). MAPKs phosphorylate and enhance the transcriptional activity of ER-β, PPAR-α and AR by promoting the recruitment of co-activators (SRC-1 for ER-β, PGC-1 for PPRA-γ and ARA70 for AR) (Tremblay et al., 1999; Yeh et al., 1999; Barger et al., 2001).

Phosphorylation regulates a broad range of functions of NRs including receptor-ligand interaction, ligand-dependent activation of NRs, transcriptional activity, sub-cellular localization pattern, dimerization in case of non-steroidal NRs, DNA binding affinity and interactions with co-regulators etc. (Pondugula et al., 2009, Mackowiak and Wang, 2016). PXR is reported to undergo phosphorylation and has species specific effects of this event on PXR transcriptional activity (Wang et al., 2012). PXR activity was found to be mostly attenuated in rat and human primary hepatocytes that end up in down-regulation of CYP3A1 and CYP3A4 respectively after phosphorylation by protein kinase A. On the contrary, PXR-mediated up-regulation of CYP3A11 (ortholog of human CYP3A4) mRNA and recruitment of co-activators (SRC-1 and PBP) were observed in mice hepatocytes after stimulation of PKA signaling (Lichti-Kaiser et al., 2009; Staudinger et al., 2011; Smutny et al., 2013). Similarly, PKC (protein kinase C) phosphorylated PXR become unable to recruit co-activator SRC-1, but instead recruits co-repressor NCOR1, leading to decreased transcriptional activity of PXR (Staudinger et al., 2011). CDK2 (cyclin-dependent kinase 2) was also shown to repress CYP3A4 as a result of phosphorylation of PXR at S350 residue (Pondugula et al., 2009).

Conclusively, PKA, MAPKs and cyclin A-CDK2 are principal kinases among the protein kinases, involved in transducing external stimuli to nucleus. In summary, it is reported that ligand-independent activation of signaling kinases merges with ligand-dependent activation in order to impart the utmost transcriptional activity of NRs (Rochette-Egly, 2003).Thus, following such an immense literature, we attempted to find the mechanism of activation of PXR.From the previous chapter, anti-diabetic drugs untaken for this study, rosiglitazone and pioglitazone were observed to activate PXR and induce Phase I (CYP3A4), Phase II (UGT1A1) drug metabolizing enzymes and Phase III (MDR1) drug tansporter. This part will explorethe possible mechanisms of PXR activation under the influence of these drugs. To look for the mechanistic insight, both direct as well as indirect activation pathways were studied.

RESULTS

In light of these lieratures, direct ligand binding and indirect activation of certain signaling pathways are proposed as the mechanisms for activation of NRs in the presence of any small molecules.We also triedout both the mechanisms. *In silico* and *in vivo* experiments were conducted to look for the possibility of drug-PXR interaction. However, involvements of known signaling pathways affecting PXR transcriptional activity were studied by applying the specific inhibitors of suspected pathways. Modulation of other factors involvement in PXR activation were also ruled out, suggesting that the event of induction of Phase I, Phase II and Phase III components were only due to activation of PXR.

Examining the activation of PPAR-γ by anti-diabetic drugs

PPAR-γ is a member of NR superfamily and known as a key regulator of adipocyte differentiation, glucose and lipid metabolism (Sauer S, 2016). Its role in inducing fatty acid storage in adipocytesis well-established. PPAR-γ is predominantly expressed in adipose tissues, macrophages, inflammatory cells and at relatively lower levels in different cells (Braissant et al., 1996). Thiazolidinedione (rosiglitazone and pioglitazone) are well-established agonists of PPAR-γ (Tontonoz and Spiegelman, 2008; Sauer S, 2016). This receptor is responsible for insulin sensitizing and anti-inflammatory actions of TZDs. PPAR-γ transcriptional activity is enhanced by TZD, which act as its potent agonists by increasing the recruitment of co-activators to the receptor. Since long, PPAR-γ is also known to act as a target in management of T2DM (Sauer S, 2016). To execute its transcriptional activity, PPAR-γ heterodimerizes with RXR andbinds with the responsive elementsof its target genes.As already stated beforepioglitazone is commercialized under black box warning as there were some cases of bladder cancer reported after its usage. Risk of bladder cancer due to exposure of pioglitazone differs among different ethnicity (Kuo et al., 2014).

In Europe and North America, incidence of bladder cancer is highest in T2DM patients. There is a report, which suggests high expression of PPAR-γ in bladder cancer compared to normal urothelium (Suzuki et al., 2010). Thereafter, it was proposed that after ligand binding PPAR-γ modulates cell proliferation and differentiation leading to various other cancers (Tachibana et al., 2008). Therefore, it

was reasonable to examine whether PPAR-γ is acting as an intermediate factor for increased PXR transcriptional activity. Ligand property of TZDs for PPAR-γ tempted us to speculate that possibly there exists a cross-talk between PPAR-γ and PXR, due to the activation of PPAR-γ by rosiglitazone and pioglitazone. Activated PPAR-γ may be further enhancing PXR transcriptional activity. To detect the PPAR-γ dependent transactivation of PXR by both of the drugs, we have applied an established antagonist of PPAR-γ, GW9662. HepXREM stable cells were employed here. GW9662 was introduced in the cells at least 1 hr before the selected TZDs (rosiglitazone and pioglitazone) treatment. GW9662 was used at 1µM concentration as per reported in the literature (Seargent et al., 2004; Li et al., 2009). Drug treatment was given for 24 hr and thereafter, cells were processed for luciferase activity measurement. Rifampicin was used as a positive reference ligand for PXR in HepXREM cells. If this receptor was involved in the regulation of PXR, then inhibition of PPAR-γ could have led to the reduction in PXR transcriptional activity in HepXREM cells. There was no change in the PXR activity by the antagonist GW9662 in the absence of any TZDs. PXR transcriptional activity was enhanced by these TZDs and remained uninfluenced in the presence of antagonist GW9662 (**Figure 18**). This indicated that PXR gets activated by TZDs directly, without the involvement of PPAR-γ.Also, our speculation of cross-talk between PPAR-γ and PXR was ruled out.

Molecular Modelling and Docking studies

PXR is reported to be a promiscuous nuclear receptor due to its non-selective nature of binding with broad spectrum of ligands. X-ray crystallography has revealed that PXR-LBD is large and flexible, lined by mostly hydrophobic amino acids with a very few polar residues (Ekins et al., 2007). To find whether anti-diabetic drugs selected in our study, are behaving as a PXR ligands, molecular docking of these drugs with available crystal structure of PXR-LBD was done. For this docking purpose, human apo-PXR-LBD protein structure was retrieved from the protein data bank (PDB ID: 1ILG).

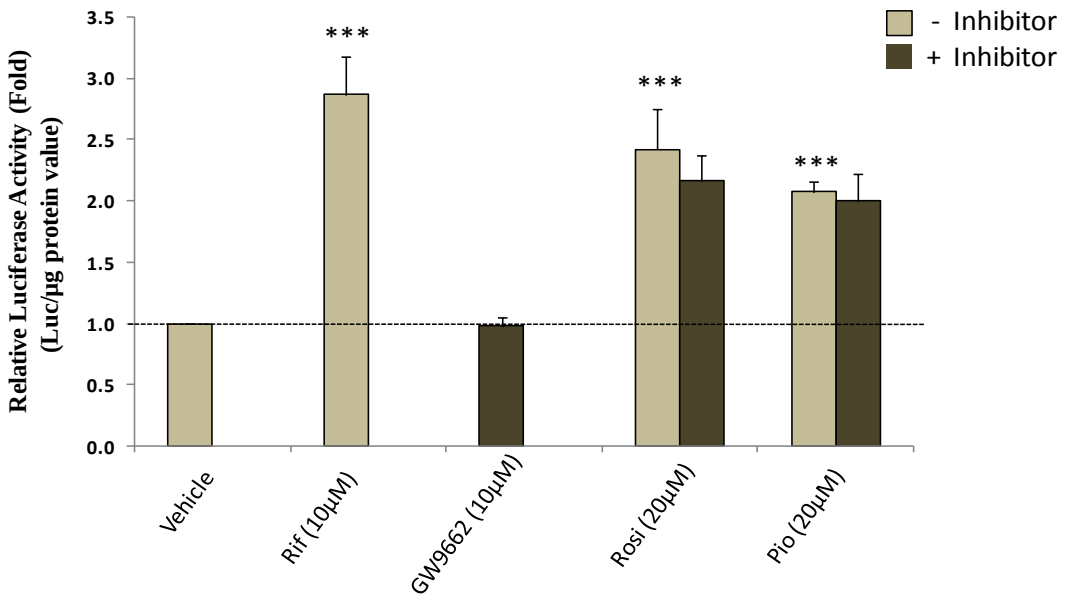
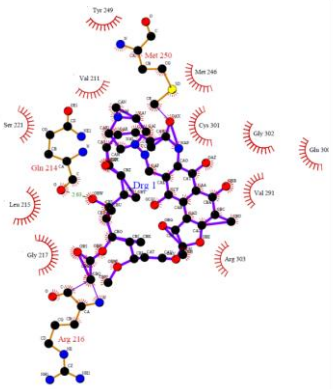


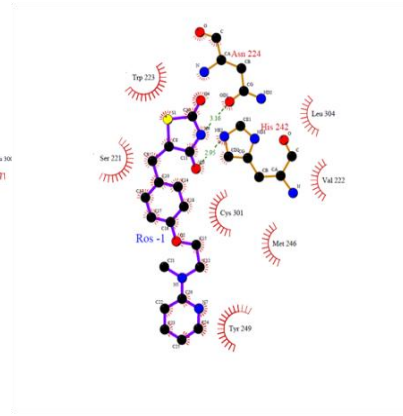
Figure 18: Effect of PPAR-γ antagonist GW9662 on PXR transcriptional activity in HepXREM stable cells. *HepXREM cells were seeded in 24-well culture plate in DMEM having 5% steroid-stripped serum without antibiotics. Next day, cells were given the drug treatment for 24 hr. Rifampicin was used at 10µM, GW9662 was used at 1µM, while rosiglitazone and pioglitazone were used at their 20µM. Following treatment period, cells were harvested and processed for luciferase assay. Data represent the mean ± SE of three independent experiments. Asterisk *** signifies luciferase values that differed significantly from the score of vehicle (P<0.001) treated cells in Student's t-test.*

Further, these 3D SDF format structures were converted into 3D mol2 format by using Open Babel software. *In silico* docking was performed using GOLD docking program as mentioned under 'Materials and Methods'. The ligands showing maximum interactions with the protein were plotted using the program LIGPLOT (**Figure 19**). Based on the binding affinity of drugs with apo-PXR-LBD, docking score was calculated (**Table IV**). Docking score value of glimepiride, pioglitazone, dapagliflozin, repaglinide, gliclazide and rosiglitazone were much higher than the rifampicin. Docking score value reflects the affinity of interaction between drugs and PXR. Therefore, it suggests that the anti-diabetic drugs possessing higher docking scores are binders of PXR.

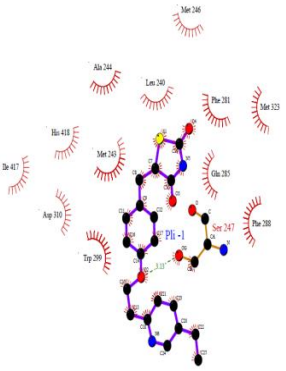
(A) Rifampicin



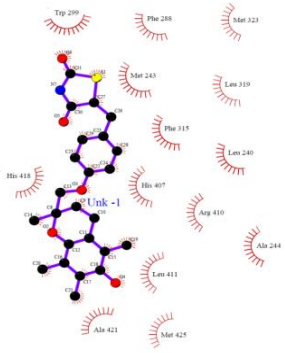
(B) Rosiglitazone



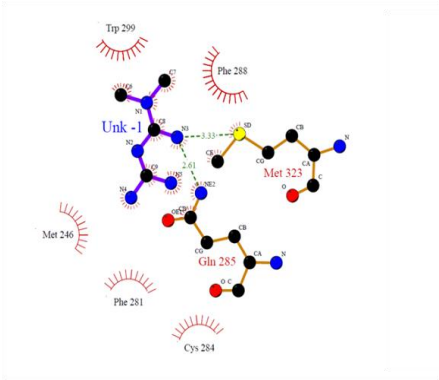
(C) Pioglitazone



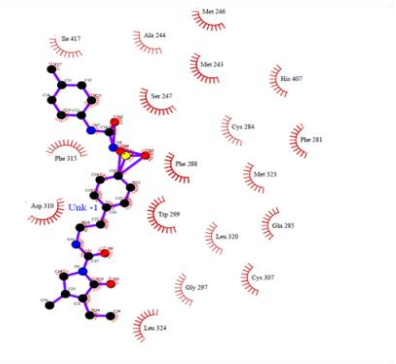
(D) Troglitazone



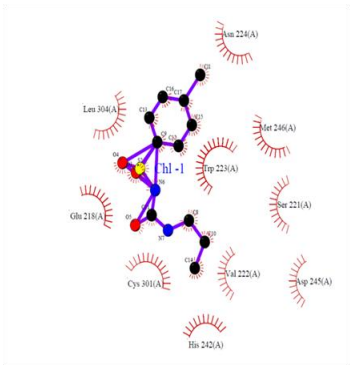
(E) Metformin



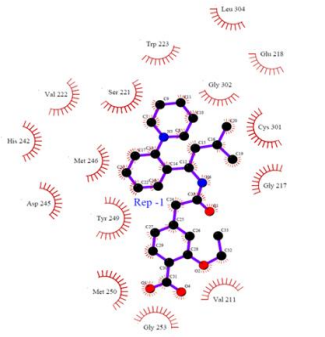
(F) Glimepiride



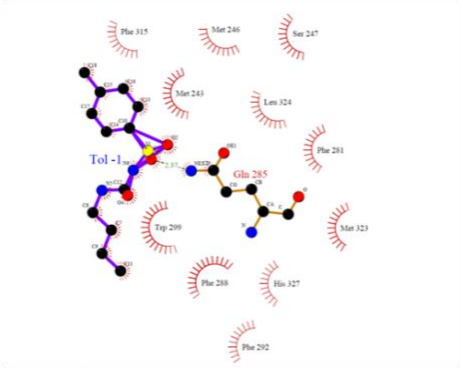
(G) Chlorpropamide



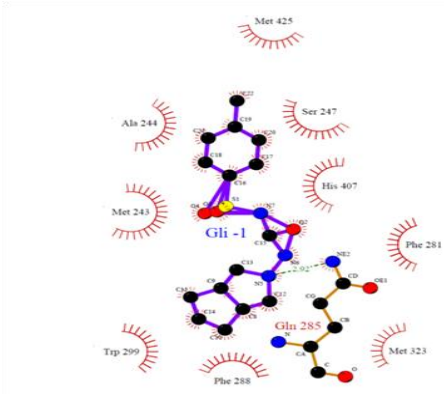
(H) Repaglinide



(I) Tolbutamide



(J) Gliclazide



(K) Dapagliflozin

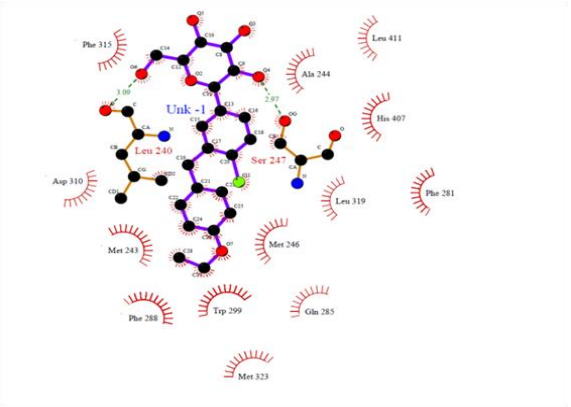


Figure 19: Molecular docking of anti-diabetic drugs with human apo-PXR-LBD. *Each of the anti-diabetic drugs in 3D mol2 format were docked into the protein structure of apo-PXR-LBD with PDB code: 1ILG (without any ligand). The amino-acid residues of the active site of PXR interacting with drugs are shown. The purple colored bonds between the atoms represent the ligand. The hydrogen bond between the amino acid residues of the active site of PXR and drugs are shown by green dotted lines, while the spoked arc represent amino acid residues of PXR making non-bonded contacts with ligand. Contact of ligand with PXR protein was plotted by LIGPLOT software.* ***(A)*** *Rifampicin* ***(B)*** *Rosiglitazone* ***(C)*** *Pioglitazone* ***(D)*** *Troglitazone* ***(E)*** *Metformin* ***(F)*** *Glimepiride* ***(G)*** *Chlorpropamide* ***(H)*** *Repaglinide* ***(I)*** *Tolbutamide* ***(J)*** *Gliclazide* ***(K)*** *Dapagliflozin.*

Table-IV: Docking Score value of drugs after binding with PXR-LBD

S.No.	Name of Drug	Docking Score
1.	Rifampicin	30.00
2.	Rosiglitazone	52.97
3.	Pioglitazone	60.88
4.	Troglitazone	40.00
5.	Metformin	20.00
6.	Glimepiride	67.30
7.	Chlorpropamide	45.36
8.	Repaglinide	57.32
9.	Tolbutamide	49.07
10.	Gliclazide	55.06
11.	Dapagliflozin	60.00

Effect of anti-diabetic drugs on human PXR localization

From previous experiments herein rosiglitazone and pioglitazone are shown to activate PXR similar to rifampicin. Also, the docking score value of these two drugs were higher than rifampicin. The docking study is therefore indicative for ligand feature of rosiglitazone and pioglitazone. To prove the receptor-ligand interaction predicted by *in silico* approach, *ex vivo* experimentation was performed. Ligand binding causes change in the localization of Nuclear Receptor by shifting it towards the nucleus. So, to visualize this type of change, PXR was tagged with a red

fluorescence protein RFP and shift in the localization pattern of PXR has been visualized.

Activation of a nuclear receptor by its ligand or activator is known to be reflected on its translocation from cytoplasm to nucleus (Kumar et al., 2006; Chaturvedi et al., 2010; Dash et al., 2017). To further confirm the phenomenon of receptor-drug interaction, we performed nuclear translocation study using fluorescent protein tagged PXR and live cell imaging. Until recently, it remained a challenge to perform PXR translocation experiments as the unliganded receptor was reported to remain predominantly nuclear in immortalized cell lines. The difficulty to perform nuclear translocation experiments was recently resolved in our laboratory after tagging PXR with red fluorescent protein (RFP) (Dash et al., 2017). This helped us to probe and identify PXR ligands which can shift the receptor into the nucleus. To elucidate the translocation potential of TZDs for PXR, which is an indicative of ligand feature of drugs, COS-1 cells has been transfected with RFP-PXR and visualized for dynamic movement. RFP-PXR was ~65% nuclear and ~35% cytosolic in unliganded state (vehicle treated). Rifampicin, which acts as a ligand of PXR, dramatically shifted cytoplasmic RFP-PXR into the nuclear compartment of the cell. Rosiglitazone and pioglitazone also shifted ~25% of cytosolic RFP-PXR towards nucleus making ~90% RFP-PXR nuclear, compared to vehicle treated cells (**Figure 20**). Overall this suggested that rosiglitazone and pioglitazone are acting as PXR ligand.

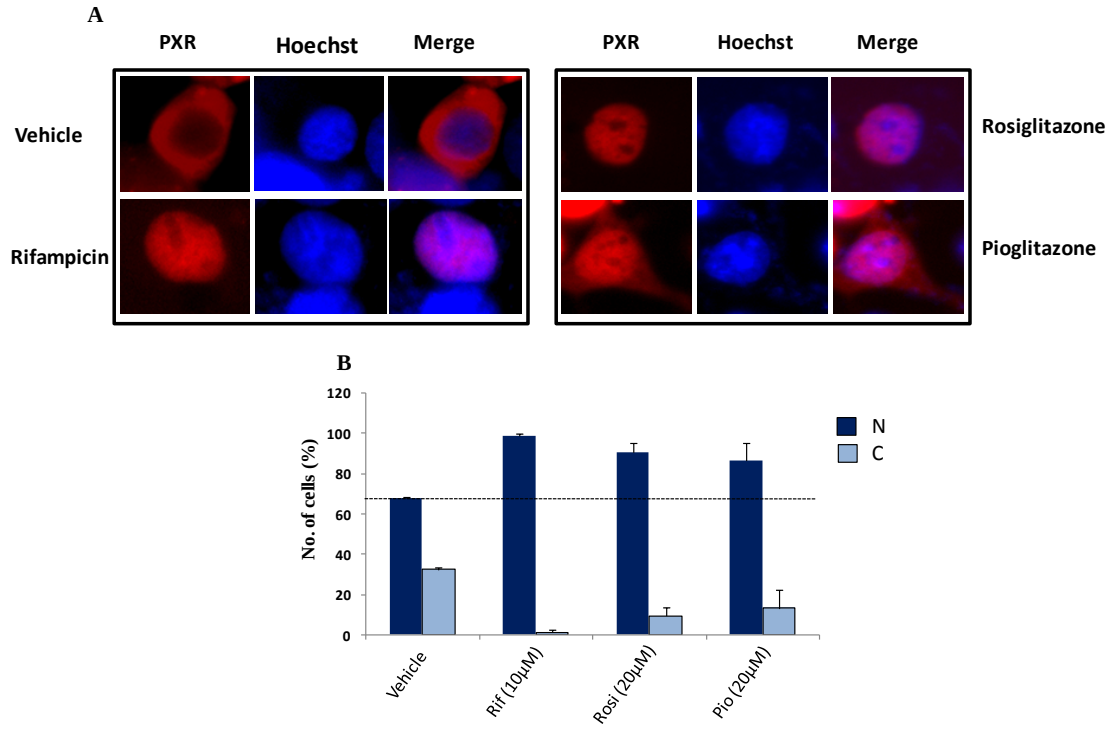


Figure 20: Effect of anti-diabetic drugs on *sub-cellular localization of RFP-hPXR.* *COS-1 cells were transfected with RFP-PXR construct. Following transfection period, cells were treated with vehicle, Rifampicin (10μM), rosiglitazone (20μM) and pioglitazone (20μM) for 24 hr and visualized by fluorescence microscope. Almost 2 hr before imaging, Hoechst (a fluorescent dye to visualize the nucleus) was added.* ***(A)*** *Live cell images were captured for PXR localization using a fluorescence microscope. In the graph, the left panel shows the sub-cellular localization of RFP-PXR (red colour) while middle panel shows the corresponding nuclei of transfected cells (blue colour) and the right panel shows merged images of both the fluorescence protein and Hoechst under the treatment of drugs* ***(B)*** *Graph represents the average number of cells of three independent experiments with ± SE for the localization of RFP-PXR under different treatments as indicated.*

Mammalian two-hybrid assay for PXR and SRC-1 interaction after rosiglitazone and pioglitazone treatment

The physiological and pharmacological actions of NRs are initiated after binding with their cognate ligands and co-regulators recruitment which co-ordinate to regulate their downstream target genes. When bound with agonist, NRs recruit co-activators to up-regulate target genes expression but down-regulate target genes when bound to antagonists, which allows co-repressors to get associated with NRs (Lonard et al., 2007). Ligand-modulated PXR forms heterodimer with RXR and binds with the response element in the promoter region of its target genes. SRC-1 family of co-activators possesses LXXLL motifs through which they interact with NRs, while co-repressors bind through their conserved LXXXIXXXL motifs, which has N-terminal

extension. Some co-regulators also possess atypical motifs along with the conserved ones as this is preferred over the latter one by some NRs. It is the AF-2 region which decides whether co-activators or co-repressors would bind (Jin and Li, 2010) and this ability to AF-2 region is provided by the nature of ligand. Selectivity of co-regulators will decide the transcriptional output of a particular NR. Agonist binding allows NRs to use their charge clamp pocket, made up of C-terminal AF-2 region of LBD, to form hydrophobic groove and accommodate LXXLL motif of co-activators (Jin and Li, 2010). Since Nuclear Receptors do not have chromatin remodeling property, they recruit co-activators in order to access the promoter regions. These co-activator proteins help in opening up the chromatin structure by unwinding DNA from nucleosome by their HAT (histone acetyl transferase) enzymatic activity. Also they work with SWI/SNF chromatin remodeler to unwind DNA in ATP-dependent manner. Along with this, they help in the recruitment of general transcription machinery to NR by bridging them (Dilworth and Chambon, 2001). On the contrary, co-repressors recruit histone-deacetylases (HDACs) to the NRs to repress their target genes. Rifampicin, St. John's Wort and hyperforin are some well-known agonists of PXR and are reported to increase the PXR transcriptional activity by tethering PXR to SRC-1 co-activator (HAT of p160 family) (Wentworth et al., 2000). Observations of computational analysis and PXR translocation study indicated that rosiglitazone and pioglitazone are behaving as PXR ligands like rifampicin. Next to the interaction of rosiglitazone and pioglitazone with PXR, we tested their ability to recruit co-activator SRC-1. To conduct this experiment, mammalian two-hybrid assay was performed.In this assay HepG2 cells were co-transfected with a construct bearing PXR-LBD, linked with activation domain of VP16 protein. Second construct has receptor interactive domain of SRC-1 ligated with DBD of GAL4 protein. Another construct contained the responsive promoter region with binding site of GAL4, known as FR-Luc. After transfecting all the constructs for 12 hr, the cells were treated with rifampicin, rosiglitazone and pioglitazone for 24 hr. Finally PXR-SRC-1 interaction was examined by luciferase assay. Rosiglitazone and pioglitazone augmented the SRC-1 recruitment to PXR in similar manner as rifampicin as shown in **Figure 21**.

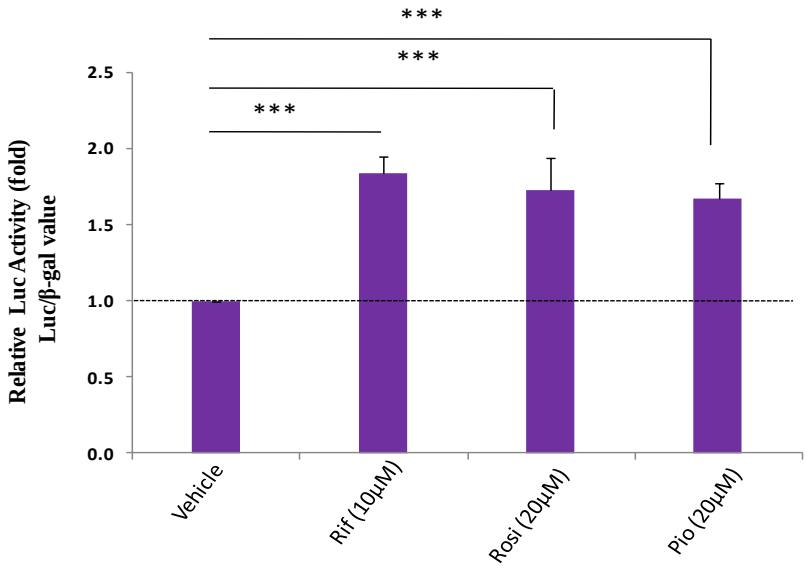


Figure 21: Mammalian two-hybrid assay for co-activator SRC-1 recruitment after treatment with anti-diabetic drugs. *HepG2 cells were co-transfected with plasmids GAL4-SRC-1 and VP16-hPXR along with construct FR-Luc in ratio of 1:1:8 in 12-well culture plate. For normalization, plasmid encoding β-gal enzyme was included at 120ng concentration/well of 12-well plate. After the transfection period, cells were treated with 20μM concentration of rosiglitazone and pioglitazone and 10μM concentration of rifampicin (a potent agonist of PXR) for 24hr followed by luciferase activity measurement and normalization with β-gal value. Data represents the mean ± SE of three independent experiments. Asterisk *** signifies luciferase values that differed significantly from the scores of control (P<0.001), in Student's t-test).*

Selective induction of SHP-promoter by anti-diabetic drugs

Another reason behind the increased PXR transcriptional activity could be the down-regulation of co-repressors expression. SHP (Short heterodimer partner) is an atypical orphan Nuclear Receptor and differs from the rest of NR member structurally, due to absence of DBD (DNA binding domain) (Zang et al., 2011).It also differs functionally from other NR members by acting as the co-repressor of its own superfamily members. It is reported to repress the activity of CAR (Constitutive androstane receptor), GR (Glucocorticoid receptor) (Krausova et al., 2011), TR (Thyroid receptor), Retenoic Acid Receptors (RAR and RXR), ER-α and ER-β (Estrogen Receptors) (Klinge et al., 2011) by either competing with co-activator binding to AF-2 domain of NRs or by introducing conformational changes in the bound NRs, which does not allows the binding of co-activators (Zhang et al., 2011). Earlier reports have shown that SHP gets induced in response to FXR ligand in

hepatic stellate cells (HSC) (Cipriani et al., 2017). PXR is also reported to be transcriptionally repressed by SHP (Krausova et al., 2011). To rule out the possibility of down-regulation of SHP gene expression by drugs rosiglitazone and pioglitazone contributing to increased transcriptional activity of PXR, SHP-promoter-reporter activity was measured after treatment with these drugs. HepG2 cells were seeded and transfected with construct SHP-Luc (harboring SHP promoter region). Subsequent to transfection period, the cells were treated with the anti-diabetic drugs included in our study along with a reference ligand rifampicin for further 24 hr, followed by luciferase assay. We observed that pioglitazone significantly induced SHP-promoter instead of down-regulating it (**Figure 22**). After literature analysis, it was found that SHP-promoter harbors PXR-RE (PXR-responsive element). So, getting induction by the ligand-activated PXR is notunexpected, as ligand-activated PXR has been shown to induce SHP to reduce its own transcription via negative feedback loop.

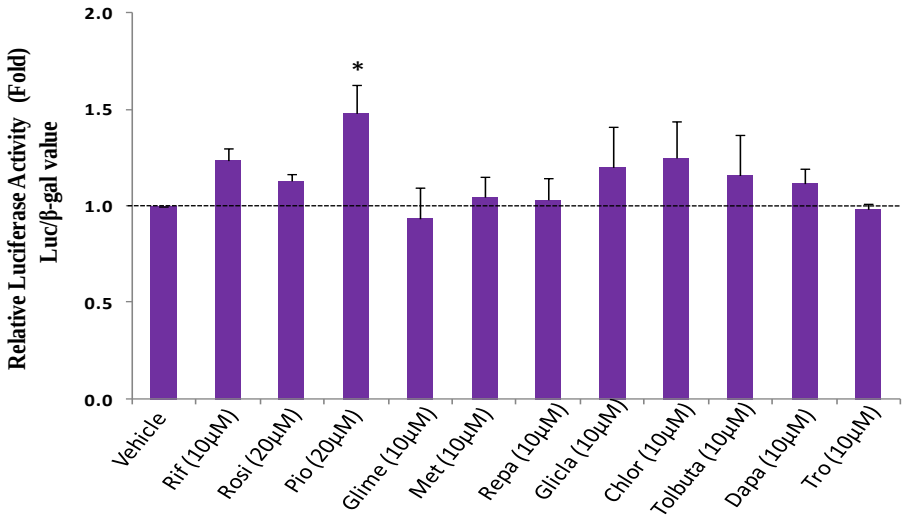


Figure 22: Effect of anti-diabetic drugs on SHP-promoter-reporter (SHP-luc). *HepG2 cells were transfected with plasmid SHP-luc in 12-well culture plate. For normalization, plasmid encoding β-gal enzyme was included at 120ng concentration/well of 12-well plate. Except rosiglitazone (20μM) and pioglitazone (20μM) all the other anti-diabetic drugs were used at concentration of 10μM after transfection period. Rifampicin, a potent agonist of PXR was also used at 10μM. After 24hrof treatment, luciferase activity were determined and normalized with β-gal value. Data represents the mean ± SE of three independent experiments. Asterisk * signifies luciferase value that differed significantly from the score of vehicle treated cells (P<0.05, in Student's t-test).*

Effect of anti-diabetic drugs on cyclic AMP (cAMP)-mediated protein kinase A (PKA) signaling pathway

Certain kinds of signaling pathways may also be modulated by these anti-diabetic drugs to strengthen the PXR transcriptional activity associated with ligand-dependent activation. This suggests both the direct activation (binding with LBD of PXR) and indirect activation (by activating certain signaling pathways) of PXR by anti-diabetic drugs. There are some reports indicating the interaction of some signaling kinases with PXR like PKA (protein kinase A), PKC (protein kinase C) and CDK 2 (cyclin dependent kinase 2) (Mackowiak and Wang, 2016). Activation of PKC and CDK 2 has been associated with decreased PXR transcriptional activity (Ding and Staudinger, 2005). Various aspects of cell differentiation, cell growth, gene regulation and release of neurotransmitters are regulated by cAMP. In general, conformational changes in G-protein coupled receptor in response to ligand binding may activate adenylate cyclase to produce cAMP. After the stimulus, cAMP may activate ion channels, guanine exchanging factors (Epac1, Epac2) and protein kinase A (Rooij et al., 1998). In mammalian cells, the primary target of cAMP is protein kinase A (Skalhegg and Tasken, 2000). PKA gets activated when cAMP binds with the regulatory subunits of the kinase (PKA-R) and causes release of catalytic subunits (PKA-C). Activated PKA-C subunits phosphorylate its several cellular target proteins on serine and threonine residues (Bockus and Humphries, 2015). There are reports showing increased CYP3A11 expression by mouse PXR after treatment with 8-Br-cAMP (a synthetic analog of cAMP) due to the SRC-1 recruitment at this promoter.

Forskolin-activated PKA has been reported to markedly increase CYP3A4 induction mediated by PXR (Ding and Staudinger, 2004). Considering all these reports, cAMP-mediated PKA activating potential of these drugs has been examined. We have predicted that indirect PKA activation by these drugs may be playing a role in activation of PXR by post-translational modification (phosphorylation). To investigate the cross-talk between PKA and PXR activation, we applied the inhibitors of adenylate cyclase (MDL-12,330A) and PKA (H-89) along with drugs. HepXREM stable cell line was treated with these inhibitors alone/with drugs, followed by luciferase reporter assay as mentioned earlier. Inhibition of adenylate cyclase by MDL-12,330A was observed to completely abolish the transcriptional activity of PXR treated with rifampicin, rosiglitazone and pioglitazone (**Figure 23A**). PKA inhibition by H-89 inhibitor also reduced the activity of PXR after the treatment with rifampicin

and pioglitazone and mildly affected rosiglitazone-activated PXR transcriptional activity (**Figure 23B**).

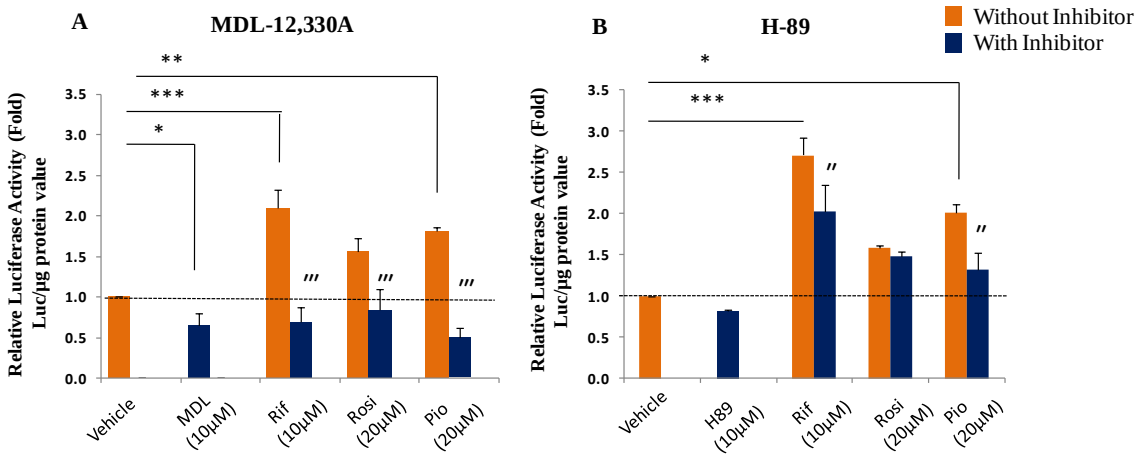


Figure 23: Effect of cAMP and PKA inhibitor on PXR transcriptional activity in HepXREM stable cells. *HepXREM cells were seeded in DMEM having 5% steroid-stripped serum without antibiotics. Subsequently, the cells were exposed to drugs for 24 hr. In the case where inhibitor is also present with drug, inhibitor was added 1hr before drug treatment.* ***(A)*** *Inhibitor of adenylate cyclase (MDL-12,330A) and* ***(B)*** *protein kinase A inhibitor (H-89) were used at 10µM concentration. Rifampicin was also used at 10µM, while rosiglitazone and pioglitazone were used at 20µM. Following treatment period, cells were harvested and processed for luciferase assay. Data represent the mean ± SE of three independent experiments. Asterisks (*, ** and ***) signify luciferase values that differed significantly from the score of vehicle (P<0.05, P<0.01 and P<0.001 respectively), while apostrophe " and ''' signify values (P<0.01, P<0.001) in the presence of inhibitor that differed significantly from values when treated with drug only (in the absence of inhibitor) in Student's t-test.*

Effect of anti-diabetic drugs on transcriptional activity of CREB

We havealready seen that the anti-diabetic drugs activate PKA signaling cascade **(Figure 23)**. To validate this result further, we performed promoter-reporter assay by co-transfecting HepG2 cells with; i) construct having CREB (cAMP response element binding protein) ligated with GAL4 DNA binding domain and;ii) construct pG5E1bLuc having the binding site of GAL4 in its promoter region ligated with Luc gene coding for luciferase enzyme. After the transfection period, cells were treated with drugs for 24 hr. PKA inhibitor H89 was used at 10µM and 8-Br-cAMP was used at 500µM concentraton. After incubation with drugs, luciferase activity was determined. A cell-permeable analog of cAMP (8-Br-cAMP) was used as activator of PKA. H89 (PKA inhibitor) decreased the basal activity of pG5E1bLuc, while cAMP derivative (8-Br-cAMP) has strongly activated GAL4-CREB and increased the

luciferase activity of pG5E1bLuc. Similar to 8-Br-cAMP, rifampicin, rosiglitazone and pioglitazone have also transactivated GAL4-CREB. In the presence of inhibitor H89, GAL4-CREB dependent activity of pG5E1bluc under the treatment of rifampicin, rosiglitazone and pioglitazone along with cAMP analog was observed to be decreased significantly (**Figure 24**). This experiment suggests the activation of PKA by these drugs.

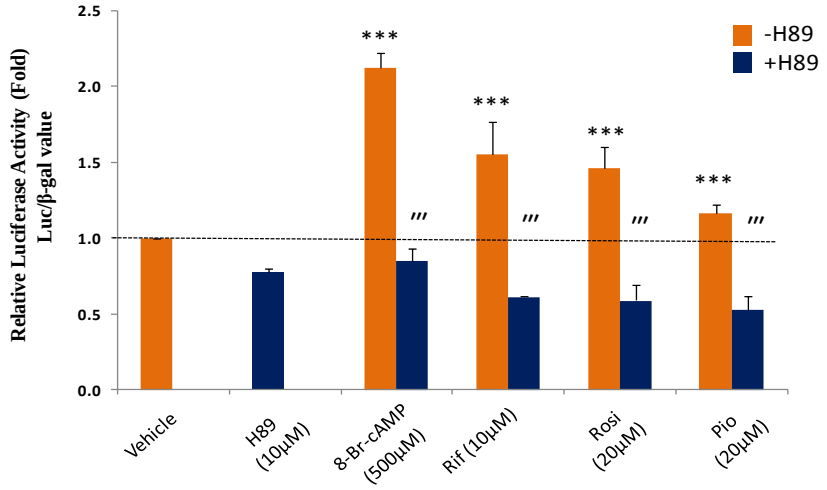


Figure 24: Effect of anti-diabetic drugs on transcriptional activity of CREB. *HepG2 cells were seeded in 12-well plates and transfected with constructs GAL4-CREB (cAMP response element binding protein) and pG5E1bLuc (having the GAL4 binding site) in 1:5 ratio. For normalization, plasmid coding for β-gal enzyme was also included at 120ng concentration/well of 12-well plate. Rifampicin and PKA inhibitor (H-89) were used at 10μM, while rosiglitazone and pioglitazone were used at 20μM for 24 hr. An analog of cAMP, 8-Br-cAMP (cell permeable) was used at 500μM concentration. After 24hr, luciferase activities were determined and normalized with β-gal value. Data represent the mean ± SE of three independent experiments. Asterisk*** signifies luciferase values that differed significantly from the score of vehicle (P<0.001), while apostrophe ''' signifies value (P<0.001) in the presence of inhibitor that differed significantly from values in the absence of inhibitor, in Student's t-test.*

Effect of anti-diabetic drugs on AMP-activated protein kinase (AMPK) signaling

Next, the possibility of AMPK-dependent PXR activation was examined, as another anti-diabetic drug metformin, was shown to activate AMPK (Krausova et al., 2011). AMP-activated protein kinase (AMPK), a serine/threonine kinase regulates the hepatic metabolism. Upon ATP depletion inside the cells due to ischemia, hypoxia or oxygen deficiency, AMPK gets activated. After activation it stops the ATP

consuming cellular processes like fatty acid/sterol synthesis, while activates ATP synthesizing processes like fatty acid catabolism. It is reported that phenobarbital-mediated activation of CAR induces AMPK to get activated (Rencurel et al., 2005). AMPK activation by metformin and thiazolidiniediones has also been reported previously (Saha et al., 2004). Nuclear Receptor HNF-α is known to regulate gluconeogenesis in liver. The transcriptional activity of this NR gets abolished due to phosphorylation introduced by AMPK, leading to degradation of HNF-α (Hong et al., 2003). On the contrary, activated AMPK induces SHP expression (Lee et al., 2010). AMPK is also shown to phosphorylate and activate p38 MAPK which then phosphorylate and reverses the transcriptional activity of GR (Nader et al., 2010). Another xenobiotic sensor CAR also acts as a substrate for AMPK, where phosphorylated CAR is unable to get translocated inside the nucleus (Kanno et al., 2010). Thus transcriptional activity of CAR gets diminished.A study by Krausova and group in 2011 has shown the PXR-dependent suppression of CYP3A4 by metformin. The report has addressed the molecular mechanism of inhibition of PXR and SRC-1 (co-activator) interaction without binding of metformin with PXR-LBD, resulting in reduction of CYP3A4 expression. They had eliminated the possibility of SHP (Small heterodimer partner) up-regulation as per the earlier reported studies. Also, metformin neither behaved as PXR antagonist nor had activated AMPK to reduce the CYP3A4 induction (Krausova et al., 2011). Considering these reports, we investigated the involvement of AMPK in PXR-dependent CYP3A4 induction by rosiglitazone and pioglitazone by applying an AMPK inhibitor dorsomorphin in HepXREM stable cells. Cells were seeded a day before treatment in complete DMEM with 5% steroid-stripped serum without antibiotics. On the following day, cells were treated with rifampicin (10µM), rosiglitazone (20µM) and pioglitazone (20µM) for 24 hr. Dorsomorphin at 10µM was added 1 hr before the treatment with experimental drugs. After 24 hr, cells were harvested for luciferase assay. Rifampicin, rosiglitazone and pioglitazone have activated PXR significantly. Dorsomorphin alone has also enhanced PXR activity, but it did not affect PXR transcriptional activity significantly, in the presence of these drugs (**Figure 25**).

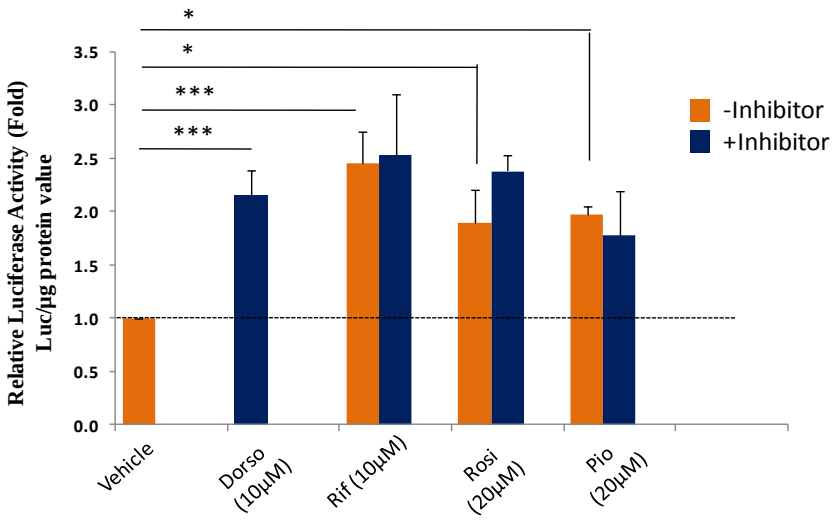


Figure 25: AMPK inhibitionenhances PXR transcriptional activity in HepXREM cell line. *HepXREM stable cells were seeded and treated with AMPK inhibitor and drugs in the same manner as mentioned before. Inhibitor of AMPK (dorsomorphin) and rifampicin were used at 10µM, rosiglitazone and pioglitazone were used at 20µM. Following treatment period, cells were harvested and processed for luciferase assay. Luciferase value of each treatment is normalized by total protein value. Data represent the mean ± SE of three independent experiments. Asterisks (* and ***) signify luciferase values that differed significantly from the score of vehicle (P<0.05 and P<0.001 respectively) in Student's t-test.*

Effect of anti-diabetic drugs on mitogen-activated protein kinase (MAPK) signaling

There are mounting evidences about post-translational modification of PXR including ubiquitination, sumoylation, acetylation and phosphorylation which regulate the PXR activity (Staudinger et al., 2011). Like aforesaid kinases initiating post translational modifications, another class of kinases is MAPKs (mitogen activated protein kinase). MAPK pathway is one of the four major signal transduction pathways in eukaryotic cells (Herlaar and Brown, 1999). There are three types of conventional MAPK pathways inside the cell: extracellular regulated protein kinase [(ERK/ (p42/44)], c-jun-NH_2-terminal kinase [JNK/(p46/54)] and p38 mitogen-activated kinase (p38 MAPK). ERK MAPK is mainly activated by growth factors; p38 MAPK is activated by stress, while JNK MAPK is activated by both stress and growth factors. These MAPKs play very important role in transducing signals from extracellular environment to the intracellular compartments (Zassadowski et al., 2012). All of these MAPKs are serine/threonine kinases which regulate the cell

growth, differentiation and survival (Garrington and Johnson, 1999) by phosphorylating their substrates.

MAPK cascade is composed of three protein kinases MAPKKK (MEKK/MAPK3), MAPKK (MEK/MAPK2) and MAPK, which activate their downstream target kinases by phosphorylation at specific serine/threonine residues. A specific recognition motif is present outside the catalytic domain of MAPKs. Catalytic domain is present at the junction of N-terminal domain and C-terminal domain. MAPKs are able to bind with both the upstream regulatory and the downstream target proteins. Both the upstream kinases MAPKKK and MAPKK are threonine/tyrosine kinases which recognize the sequence Thr-X-Tyr and phosphorylate at both Thr and Tyr residues (Cobb and Goldsmith, 1995; Pearson et al., 2001). There are 14 MAPKKKs, 7MAPKKs and 12 MAPKs reported in mammals (Zassadowski et al., 2012). It is reported that pioglitazone and 15-d-PGJ2 (both are PPAR-γ ligands) activate MEK/ERK pathway (Takeda et al., 2001). Conversely, ERK negatively regulates PPAR-γ via its NTD phosphorylation. Due to this phosphorylation event, affinity of PPAR-γ for its ligand gets reduced as a result of communication between ligand binding pocket of LDB and phosphorylated NTD (Shao et al., 1998). Few other studies have linked ERK mediated phosphorylated NTD of PPAR-γ with proteasomal degradation of this receptor (Floyd and Stephens, 2002). Phosphorylation of RXR LBD and hinge region of TR2 and ROR-α are also reported to negatively impact their transcriptional activities (Lee et al., 2000; Lechtken et al., 2007; Gupta et al., 2008; Macoritto et al., 2008). Also, recently MAPK signaling has also been reported for regulation of PXR activity (Taneja et al., 2011). In view of the above, we checked our drugs for these three MAPK activation by applying inhibitors FR180204, SP600125 and SB203580 against ERK1/2, JNK1/2/3 and p38 MAPKs respectively. HepXREM cells were seeded a day before treatment in DMEM with 5% steroid-stripped serum without antibiotics. Next day, cells were treated with specified drugs of our study for 24 hr. Inhibitors were included at least 1hr before drugs treatment. It is observed that ERK1/2 inhibitor FR180204 increased the transcriptional activity of PXR in the presence of rosiglitazone and pioglitazone (**Figure 26A**). This indicates that ERK1/2 is not involved in increasing PXR activity in the presence of TZDs. It can also be concluded that activated ERK negatively regulates PXR for the reason that inhibition of this MAPK increased rosiglitazone and pioglitazone-mediated PXR activity. Further experiments need to be carried out to confirm this proposal. Participation of

JNK and p38 MAPK has been examined by applying respective inhibitors SP600125 and SB203580 at 10µM and proceeded similarly as with ERK in HepXREM cells. It was observedthat inhibition of JNK and p38 MAPK abrogates the rosiglitazone, pioglitazone and rifampicin-mediated PXR activity (**Figure 26 B & C**). This demonstrates that rosiglitazone and pioglitazone have activated both JNK and p38 MAPK to further increase the PXR transcriptional activity, in synergy with ligand-dependent PXR activity.

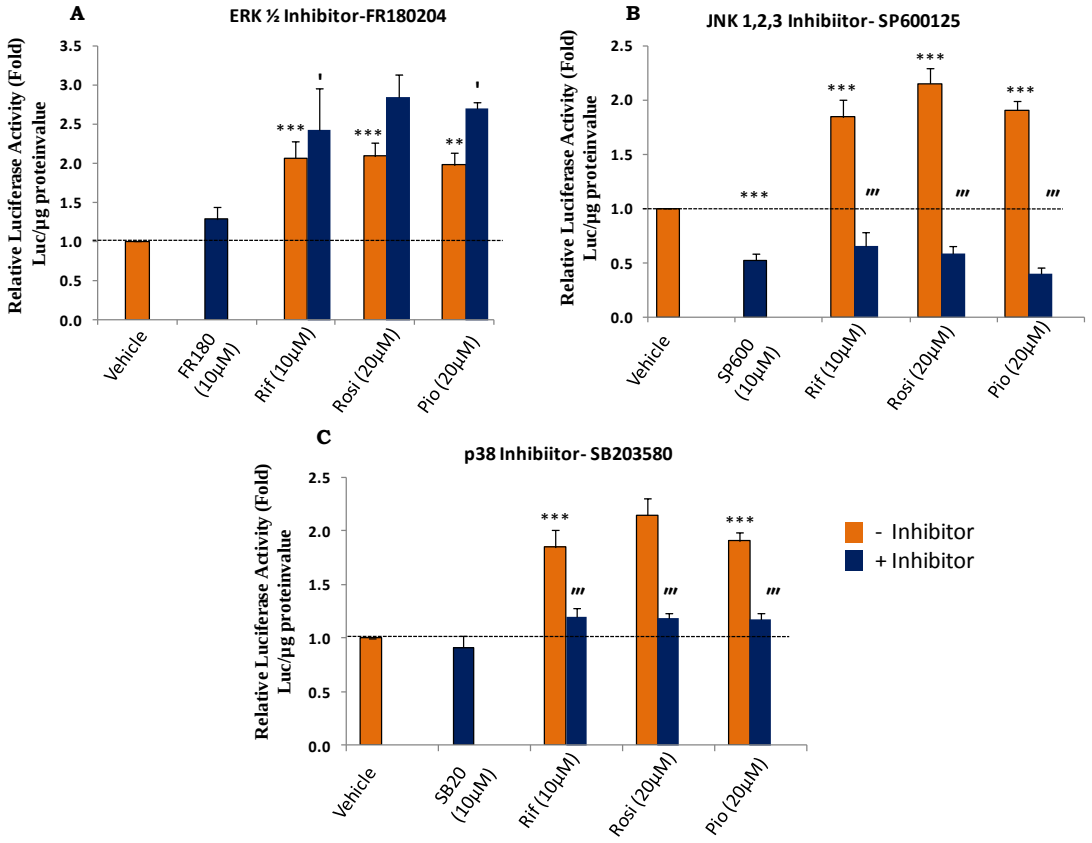


Figure 26: Effect of ERK-1/2, JNK and p38 inhibitor on PXR transcriptional activity in HepXREM cell line. *HepXREM stable cells were seeded 20-24 hr before treatment in DMEM having 5% steroid-stripped serum without antibiotics. Subsequently, the cells were treated with drugs for 24 hr. In the case where inhibitor is also present with drug, inhibitor was added 1hr before drug treatment.* ***(A)*** *Inhibitor of ERK-1/2 (FR180204)* ***(B)*** *JNK 1, 2, 3 inhibitor (SP600125) and* ***(C)*** *p38 inhibitor (SB203580) were used at 10µM concentration. Rifampicin was used at 10µM, while rosiglitazone and pioglitazone were used at 20µM. Following treatment period, cells were harvested and processed for luciferase assay. Data represent the mean ± SE of three independent experiments. Asterisks (** and ***) signify luciferase values that differed significantly from the score of vehicle (P<0.01, P<0.001) while apostrophes ' and ''' signify values (P<0.05, P<0.001) in the presence of inhibitor that*

differed significantly from values when only drug was present (in the absence of inhibitor) in Student's t-test.

Cloning and characterization of pcDNA3.1/HisC-JNK1-α1 construct

From LS180 cells, cDNA was prepared and JNK1-α1 MAPK was amplified from this template.Amplified JNK1-MAPK (1155bp) was digested with BamHI and NotI and inserted into pcDNA3.1/HisC vector to prepare a construct pcDNA3.1/HisC-JNK1-α1MAPK (**Figure 27**) as mentioned under 'Materials and Methods'. JNK1-α1 MAPK was successfully cloned in frame with N-terminal His-tag verified by sequencing. Further, experimental validation for the expression of JNK1-α1 MAPK was done. To examine the expression of JNK1-α1 at mRNA level, semi-quantitative PCR was done. COS-1 cells were transiently transfected with vector alone (pcDNA3.1/HisC) and with construct pcDNA3.1/HisC-JNK1-α1, coding for JNK1 MAPK for 12 hr. After transfection, cells were replenished with complete DMEM having 10% FBS and antibiotics and incubated further for 24 hr (**Figure 28**). After incubation, cells were processed for total RNA isolation and cDNA were prepared as mentioned under 'Materials and Methods'. Using 250ng of cDNA template from each sample, JNK1 MAPK was PCR amplified using specific primers as below;

Forward- 5'-CAGTCAGGCAAGGGATTTGTTAT-3'

Reverse- 5'-TCATCTAACTGCTTGTCAGGGA-3'

After initial denaturation at 95°C for 5 min, amplification was conducted for 28 cycles of denaturation at 95°C for 30s, annealing at 60°C for 45s, extension at 72°C for 30s and final extension was carried out at 72°C for 10 min. Amplified fragment was confirmed by running 2% agarose gel. In pcDNA3.1/HisC-JNK1 transfected cells, the intensity of 159 bp amplicon for JNK1 MAPK was more than in vector (pcDNA3.1/HisC) transfected cells (**Figure 28A**). The 129 bp region of β-actin was amplified, which acted as an endogenous control. This experiment confirmed that JNK1 MAPK is present in the construct and working after over-expression. Next level of experiment was done to see if the mRNA of JNK1 MAPK is getting translated. Again, COS-1 cells were transfected with vector alone (pcDNA3.1/HisC) and with construct pcDNA3.1/HisC-JNK1-α1. After completion of transfection period, cells

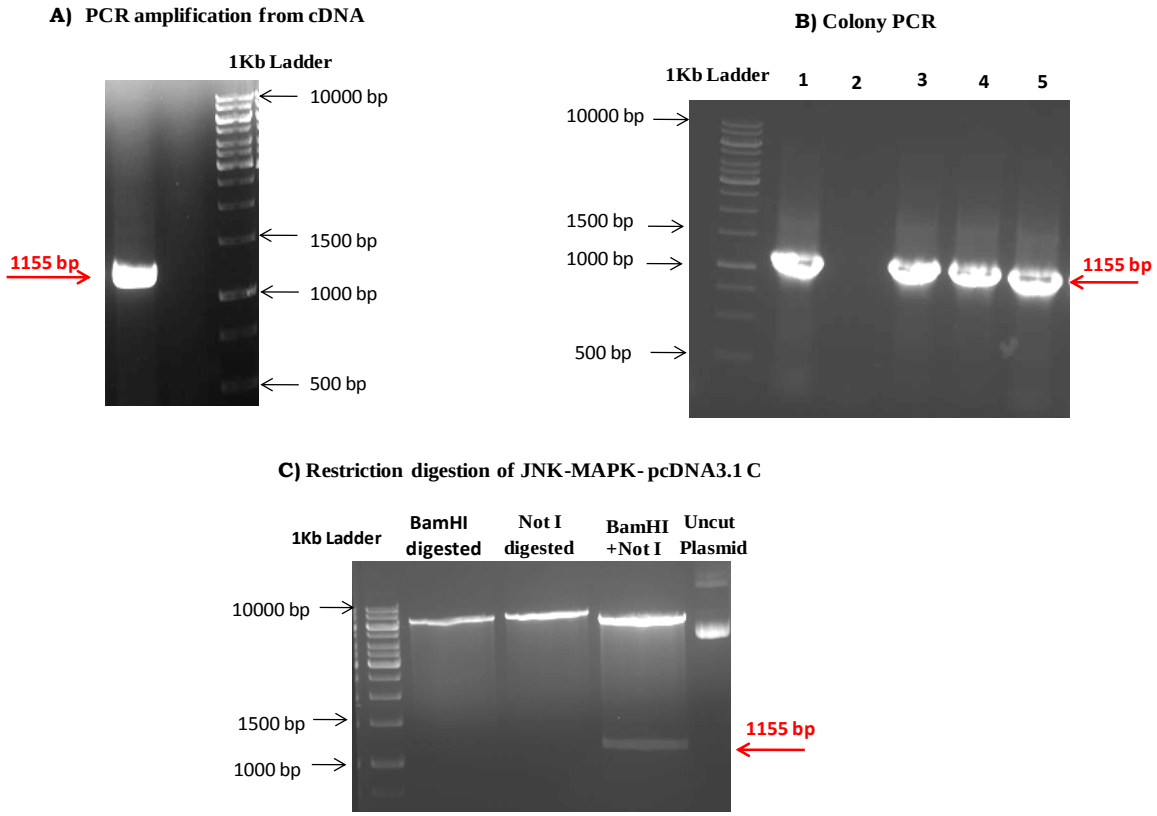


Figure 27: **Cloning of JNK1-α1 MAPK from cDNA and generation of pcDNA3.1/HisC-JNK1-α1 construct** ***(A)*** *RNA was isolated from intestinal LS180 cells and used to prepare cDNA. Gene of JNK1-α1 MAPK was PCR amplified from cDNA template using specific primers harboring BamHI and NotI restriction sites in forward and reverse primers respectively. The amplified fragment of 1155 bp and vector pcDNA3.1/His C were digested with restriction enzymes BamHI and NotI and proceeded for ligation after gel elution. Subsequently, competent bacterial cells DH10β were transformed with ligation product.* ***(B)*** *Several colonies appeared and some of them were screened using specific primers. Colonies were found positive in colony PCR.* ***(C)****Presence of insert in pcDNA3.1/His C vector was verified by restriction digestion using BamHI and NotI restriction enzymes. Fallout of 1155 bp fragment confirmed the presence of insert within the plasmid.*

were incubated for 24 hr. After the incubation, cells were washed with PBS and proceeded for western blotting. Equal amount of each protein samples (30µg) were loaded and separated on 10% SDS-PAGE, transferred on PVDF membrane and detected by primary antibody against His-tag, raised in rabbit (cell signaling technology, Massachusetts, USA). His-tagged JNK1 MAPK was detected at 43 kDa by polyclonal antibody while pcDNA3.1/HisC transfected lane remained undetected. Protein for β-actin was used to show the equal loading (**Figure 28B**). This

characterization part suggests that the construct pcDNA3.1/HisC-JNK1-α1 is getting transcribed and translated also.

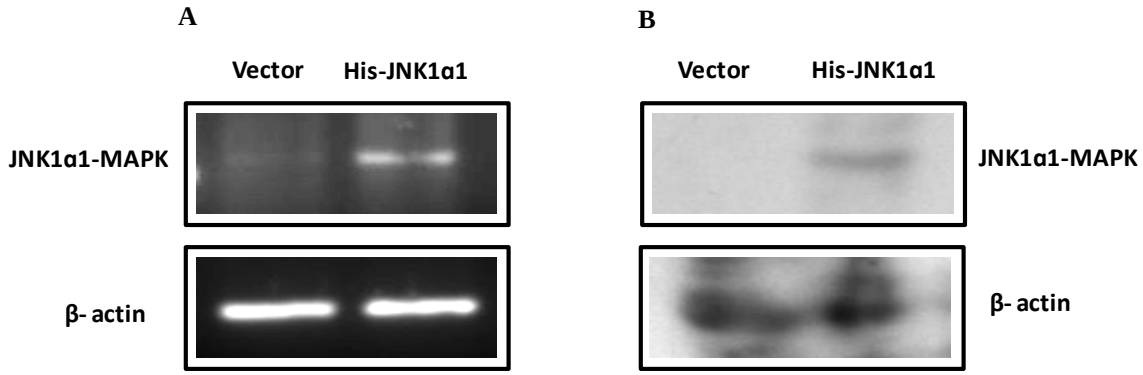


Figure 28: **Characterization of pcDNA3.1/HisC-JNK1-α1 construct.** *COS-1 cells were transfected with vector pcDNA3.1/HisC and with pcDNA3.1/HisC-JNK1-α1 construct (where coding region of JNK1-α1 has been cloned in this vector).* ***(A)*** *Total RNA was isolated from COS-1 cells and used to prepare cDNA. Expression level of JNK1-α1 MAPK was examined by semi-quantitative PCR from cDNA template using specific primers for JNK1-α1 MAPK. This set of primers amplified 159bp amplicon of JNK1α1 MAPK. β-Actin was also amplified as an endogenous control. Amplicon size of 159bp in pcDNA3.1/HisC-JNK1-α1 transfected cells suggests that JNK1-α1 MAPK is transcribed.* ***(B)*** *Protein samples were prepared from both the transfected COS-1 cells and run on 10% SDS-PAGE. Protein corresponds for His-tagged JNK1-α1 MAPK at 43 kDa. Protein for β-actin used as loading control.*

Influence of p38 MAPK and JNK MAPK expression on anti-diabetic drugs-mediated effect on PXR transcriptional activity

As is evident from **Figure 26B, 26C** transcriptional activity of PXR is reduced after introduction of specific inhibitors of JNK MAPK (**Figure 26B**) and p38 MAPK (**Figure 26C**). This suggested the involvement of p38 MAPK and JNK MAPK in regulation of PXR transcriptional activity, possibly by post-translational modification like phosphorylation. To further validate their involvement and rule out the possibility of off-target effects of their inhibitors, p38 MAPK and JNK MAPK were expressed exogenously in HepG2 cells. The cells were transiently transfected with constructs Flag-p38 MAPK and pcDNA3.1/HisC-JNK1 MAPK, PXR expression plasmid and PXR-responsive element of CYP3A4-promoter-reporter.The plasmid construct pcDNA3.1/His C-JNK1 MAPK was constructed and characterized as described in **Figure 27 & 28**. Following transfection for 12 hr, media was replaced with complete DMEM having 5% steroid-stripped serum without antibiotics. Drug treatments were given in the same medium. In this experiment rifampicin was used at 1μM while

rosiglitazone and pioglitazone were used at their 5µM concentration for 12 hr. Since rifampicin, rosiglitazone and pioglitazone were shown to give maximum PXR activity at their 10µM and 20µM concentrations during our preliminary experiments. We suspected to get no change in the activity of fully activated PXR by these drugs at their optimum concentrations. Therefore, those concentrations of drugs were selected at which drugs were associated with less PXR activity, where phosphorylation-dependent increment could be visualized compared to optimal concentrations. At these sub-optimal concentrations drugs have shown certain degree of luciferase activity (**Figure 6**). After drugs treatment, cells were harvested for promoter-reporter luciferase activity measurement. Expressions of both the kinases JNK and p38 MAPKs have been found to enhance the activity of PXR albeit moderately (**Figure 29**). This functional output explains that the phosphorylation-dependent activation may be occurring after ligand-dependent PXR activity.

Reduced PXR transcriptional activity by silencing of p38 MAPKs and JNK1 MAPKs

Next experiment was performed to validate the involvement of p38 and JNK MAPKs in PXR transcriptional activity.

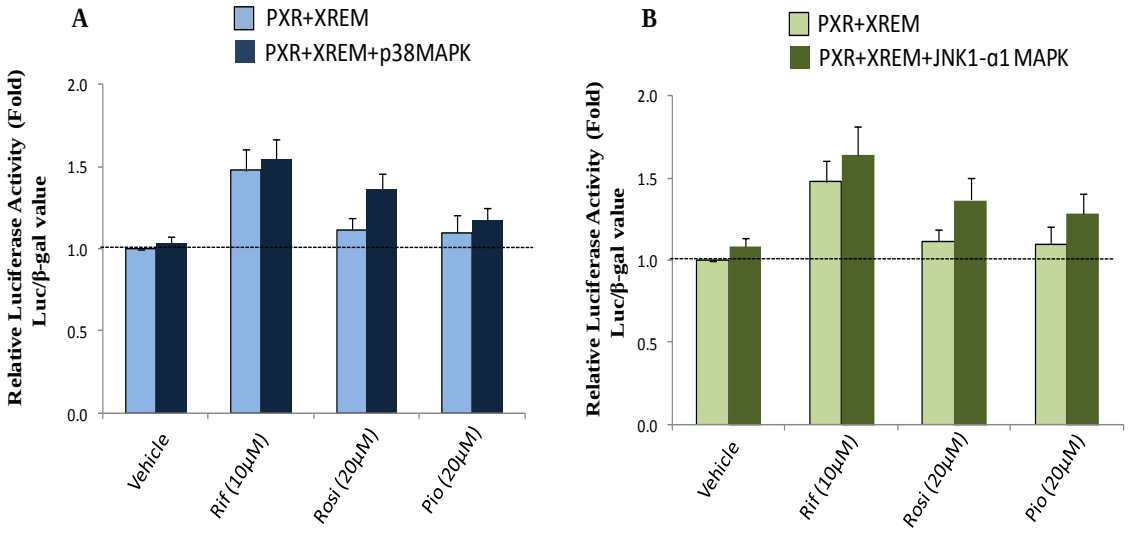


Figure 29: Effect of exogenously expressed p38 and JNK MAPKs on the anti-diabetic drug-mediated transcriptional activity of PXR. *HepG2 cells were co-transfected with pSG5-PXR, CYP3A4-promoter-reporter construct XREM-Luc in 1:4 ratio.The constructs for Flag-p38 MAPK and pcDNA3.1/HisC-JNK 1-α1MAPK were used at 10ng along with β-gal plasmid (for normalization) at 120ng/well of 12-well plate. Standard ligand of PXR, rifampicin was used at 1µM, while rosiglitazone and pioglitazone were used at their sub-optimal 5µM concentrations for 12 hr.*

Subsequently, luciferase activity were determined and normalized with β-gal value. Data represents the mean ± SE of three independent experiments.

JNK and p38 MAPKs (SAPKs) were found as intermediate signaling cascades getting affected, in the presence of rosiglitazone and pioglitazone to activate PXR indirectly (**Figure 26 B, C & Figure 29**). To confirm the involvement of SAPKs, shRNA-mediated silencing of these MAPK was done. HepG2 cells were co-transfected with constructs pSG5-PXR, XREM-Luc in 1:6 ratio. To silence p38 and JNK MAPKs, shRNA constructs (shRNA-p38 and shRNA JNK ½ MAPKs) were also co-transfected at 140ng/well of 12-well culture plate. Cells were treated with 10µM rifampicin and 20µM of rosiglitazone and pioglitazone, after 12 hr of transfection. Thereafter, cells were harvested and luciferase activity was measured. Rifampicin, rosiglitazone and pioglitazone have transactivated PXR by 4.4, 2.5 and 2.4-folds respectively. This activity got reduced in the presence of shRNA against p38 and JNK MAPK. In the presence of shRNA against p38 MAPK, PXR activity reduced from 4.4 to 2.0, 2.5 to 1.3 and 2.4 to 1.2-fold with rifampicin, rosiglitazone and pioglitazone (**Figure 30 A**). After silencing JNK1/2 MAPK, the PXR activity associated with rifampicin, rosiglitazone and pioglitazone were reduced up to 2.5, 1.68 and 1.6-folds respectively (**Figure 30 B**). This experiment exemplified the role of p38 and JNK MAPK in enhancing PXR activity probably via phosphorylation.

Examination of shRNA construct against human PXR

Before proceeding for PXR silencing experiment and to rule out the possibility of modulation of other cellular factors by selected anti-diabetic drugs, we examined whether the shRNA construct is working efficiently.To examine the silencing effects of shRNA on PXR, LS180 cells were seeded in 35 mm cell culture plate and transfected with shRNA-PXR (1µg) with Escort IV reagent for 12 hr. Cells were further incubated for 24 hr in DMEM supplemented with 5% steroid-stripped serum without antibiotics, after transfection period and thereafter processed for RNA isolation as per the procedure mentioned in ‘Materials and Methods’. Expression level of PXR mRNA was evaluated by semi-quantitative PCR using the following PXR specific primers:

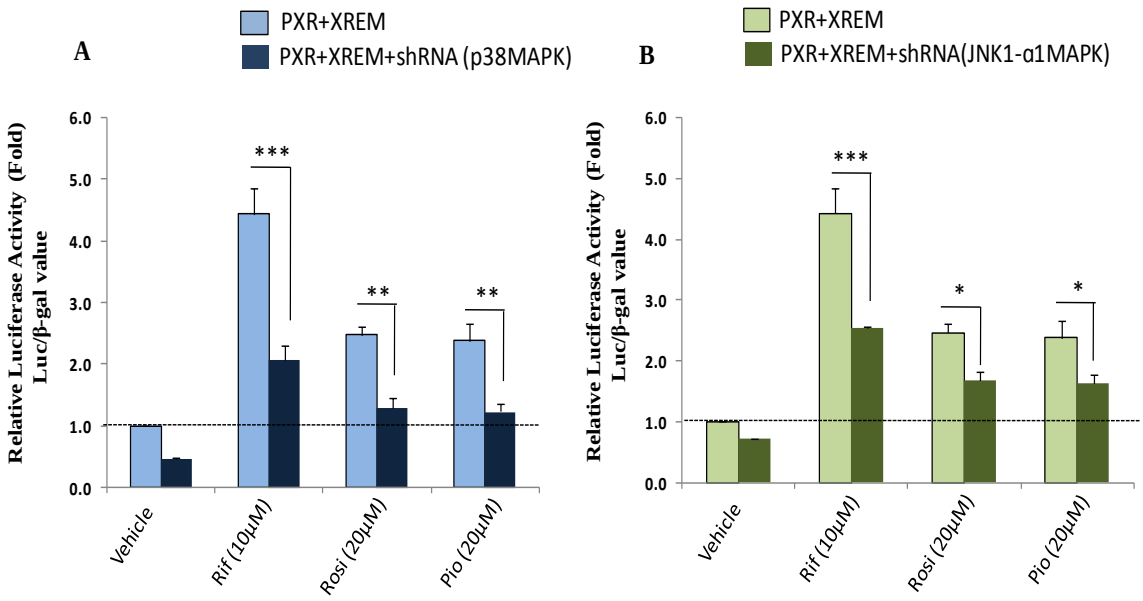


Figure 30: Effect of shRNA silenced p38 and JNK MAPK on PXR transcriptional activity in the presence of anti-diabetic drugs. *HepG2 cells were seeded in 12-well culture plate and co-transfected with pSG5-PXR, XREM-Luc constructs in 1:6 ratio. The shRNA constructs for p38 and JNK MAPK were used at 140ng along with 120ng β-gal plasmid (for normalization). Rifampicin was used at 10μM, while rosiglitazone and pioglitazone were used at their 20μM concentrations for 24 hr. After drug treatment period, luciferase activity was determined and normalized with β-gal value. Data represent the mean ± SE of three independent experiments. Asterisks (*, ** and ***) signify luciferase values of shRNA transfected cells that differed significantly from the score of their respective untransfested shRNA controls (P<0.05 and P<0.001 respectively), in Student's t-test.*

Forward- 5'-GTGAACGGACAGGGACTC-3'

Reverse-5'-ATGGGAGAAGGTAGTGTCAA-3'

The following set of primers were also used to amplify endogenous control β-actin

Forward- 5'-CCACACTGTGCCCATCTACG-3'

Reverse- 5'-GTGGTGGTGAAGCTGTAGCC-3'

The amplicon size of PXR and β-actin, amplified by the respective sets of primers were 116bp and 129bp. Amplification conditions for PXR and β-actin both were, initial denaturation at 95°C for 5 min, 25 cycle of amplification including denaturation at 95°C for 30s, annealing at 60°C for 45s, extension at 72°C for 30s and final extension was carried out at 72°C for 10 min. In shPXR transfected cells, PXR mRNA level were reduced as compared to the untransfected LS180 cells (**Figure 31A**).

A similar set of experiments were done to assess the shRNA-mediated down-regulation of PXR protein. Cell lysates were prepared from LS180 cells (untransfected and transfected ones) and 100µg of each protein samples were resolved on 10% SDS-PAGE. Transferred PXR protein (50 kDa) on PVDF membrane was detected by using polyclonal anti-PXR antibody raised in rabbit. Protein level of β-actin (42 kDa) served as loading control. Similar to the decreased mRNA level of PXR by shPXR, protein level of PXR gets diminished after introduction of shRNA designed against PXR (**Figure 31B**). These experiments established that shRNA-PXR is working on the target mRNA of PXR.

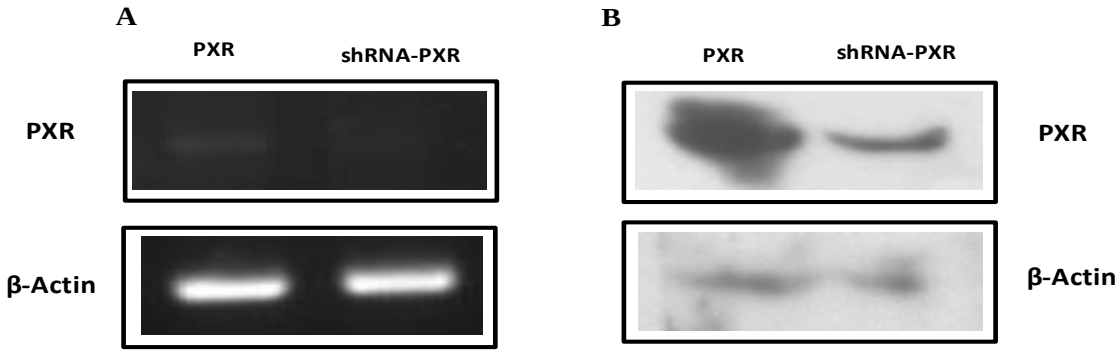


Figure 31: **Validation of the functioning of shRNA-PXR construct.** *LS180 cells wereseeded in 35 mm culture plate and transfected with 1µg of construct shRNA-PXR (shPXR).* ***(A)*** *RNA was isolated and used for preparing cDNA. Effect of shRNA on PXR mRNA level was analyzed by semi-quantitative PCR using PXR specific primers and cDNA template. β-Actin was also amplified to show the endogenous control.* ***(B)*** *Both the sets of LS180 cells were lysed and processed for protein isolation. Equal amounts (100µg) of total proteins from both sets of cells were run on 10% SDS gel followed by detection of PXR (50 kDa) by polyclonal antibody. β-Actin (42 kDa) was used as a loading control.*

PXR knockdown by shRNA abolishes CYP3A4 induction by rosiglitazone and pioglitazone

As evident from the present study, only two of the selected classes of anti-diabetic drugs, i.e. rosiglitazone and pioglitazone induced CYP3A4 (Phase I), UGT1A1 (Phase II) and MDR1 (Phase III) by activating PXR. To confirm that these drugs are actually activating PXR thereby up-regulating its target genes of 'drug metabolism and disposition' machinery, PXR was knockeddown by shRNA. To silence PXR, HepG2 cells were co-transfected with shRNA, PXR and XREM-Luc for

12-16 hr. After the incubation period, cells were treated with the experimental drugs for 24hr. After treatment period, cells were harvested and proceeded for luciferase activity.

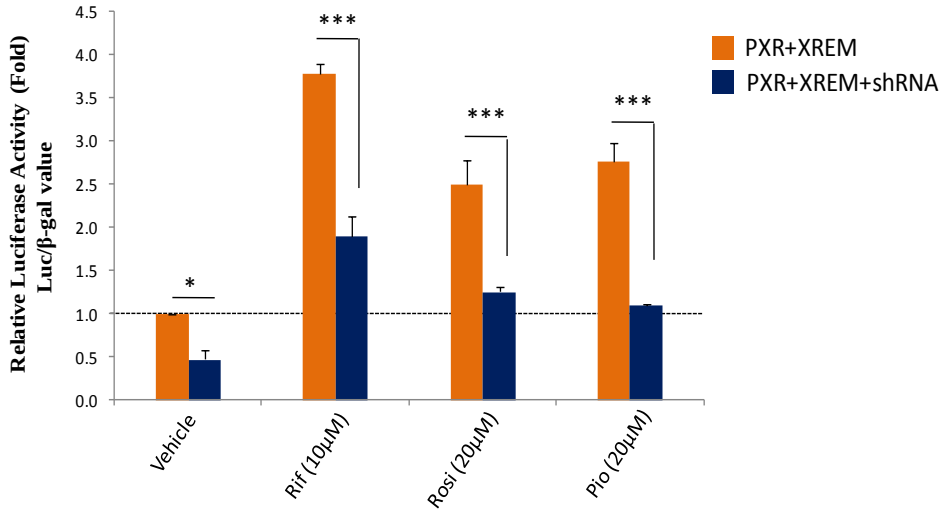


Figure 32: shRNA-mediated knockdown of PXR and the influence of anti-diabetic drugs on CYP3A4 induction. *HepG2 cells were co-transfected with PXR and XREM-Luc in 1:4 ratio along with shRNA for PXR (200ng). For normalization, plasmid coding for β-gal enzyme was included at 120ng concentration/well of 12-well plate. Rifampicin was used at 10μM; rosiglitazone and pioglitazone were used at 20μM for 24 hr. After incubation, luciferase activity was determined and normalized with respective β-gal value. Data represent the mean ± SE of three independent experiments. Asterisks (* and ***) signify luciferase values that differed significantly from the score of vehicle (P<0.05 and P<0.001 respectively), in Student's t-test.*

Similar to the effects of these drugs in stable cells HepXREM, PXR transcriptional activity was enhanced by rifampicin, followed by rosiglitazone and pioglitazone in transient transfections. After silencing of PXR by shRNA, PXR activities in the presence of rifampicin, rosiglitazone and pioglitazone were reduced significantly (**Figure 32**). This observation suggests that, activation of PXR is responsible for induction of CYP3A4 (and possibly UGT1A1 and MDR1) in the presence of these drugs, and not due to involvement of other factors.

Discussion

In our study we found that rosiglitazone and pioglitazone among the selected classes of anti-diabetic drugs (novel, established and withdrawn) at their optimum concentrations have potential to activate PXR and components of 'DMD' machinery

[phase I (CYP3A4), phase II (UGT1A1), phase III (MDR1)] (shown in previous section). In this chapter, we have tried to uncover the mechanisms behind increased transcriptional activity of PXR and associated components. To observe the mechanistic insight of PXR transactivation, firstly we looked if there were any cellular factors which get affected after the introduction of rosiglitazone and pioglitazone. As is already known that, both of these drugs belong to the TZD class of anti-diabetic drugs, which act as insulin sensitizer by increasing the glucose uptake in skeletal muscles, inhibit hepatic glucose output and also exhibit anti-inflammatory activity in metabolic tissues (Kaiser et al., 2014). Therefore, we suspected about modulation of PXR activity by TZD-activated PPAR-γ. From our study, we observed from our study that PXR activation by these drugs is not PPAR-γ dependent event.

After eliminating the involvement of PPAR-γ, we tried to hypothesize that either these drugs are directly activating PXR by behaving as PXR ligands or/and indirectly affecting PXR via signaling cascades. First thing we did was, to see the existence of direct activation mechanism. In this direction, we followed an *in silico* approach with docking of all the anti-diabetic drugs undertaken, with PXR-LBD in un-liganded state (apo-PXR-LBD). Docking score is directly proportional to the receptor-drug affinity. Scores of rosiglitazone and pioglitazone were good enough to show their receptor-ligand interaction. Experimental validation of *in silico* observations were also examined by translocation studies with our experimental drugs. There are several reports including the ones from our laboratory, about translocation of liganded NRs from cytosol towards the nucleus (Mulholland et al., 2002; Kumar et al., 2006; Chaturvedi et al., 2010; Dash et al., 2017). The localization study suggested that rosiglitazone and pioglitazone have shown similar ligand behavior as rifampicin.

Ligand-PXR interaction changes the affinity of receptor for co-regulators, followed by the replacement of co-repressors with co-activators and then PXR performs its transactivation functions. It is well-known that rifampicin binding to PXR triggers the recruitment of SRC-1 co-activator, which will help in chromatin modifications and make the promoter of its target gene accessible to bind with basal TFs machinery and RNA polymerase. Similar to rifampicin, both the anti-diabetic drugs were found to augment the SRC-1 recruitment to PXR.

Further, we suspected the down-regulation of SHP induction by rosiglitazone and pioglitazone, as these drugs have increased the PXR activity and SHP is known to inhibit PXR. SHP (Small heterodimer partner) is an orphan member of NR superfamily and represses the transcriptional activity of many of NRs (AR, ER, GR, HNF4, FXR, LXR, PXR, CAR, RARs, RXR, Nurr77, ERRs and THR) either by competing with co-activators to bind with AF-2 domain of NRs or by introducing conformational changes in the NRs, which does not allows the binding of co-activators (Zhang et al., 2011; Cipriani et al., 2017). Surprisingly, we observed a reverse relationship with PXR and SHP-promoter, where rosiglitazone and pioglitazone have induced the SHP-promoter. Pioglitazone has induced SHP more, followed by rifampicin and rosiglitazone. This observation is in accordance with the reported ones, where SHP has been shown to harbor 17 PXRRE (PXR response element) within its promoter (Zhang et al., 2011). This group has also found rifampicin-activated PXR in inducing SHP-promoter in HepG2 cells.

Ligand-dependent activation of PXR is the primary event; however mounting evidences also suggest activation of signaling pathways in post-translational regulation of PXR (Lichti-Kaiser et al., 2009; Pondugula et al., 2009; Mackowiak and Wang, 2016). Existence of such an interface between PXR and signaling event affects localization, DNA binding affinity, interaction between PXR and co-regulators and transcriptional activity of PXR (Lichti-Kaiser et al., 2009). Activation of PKA has shown species specific effect on PXR activity. In mouse hepatocyes, PXR activity gets increased; on the contrary, it gets reduced in rat and human hepatocytes (Lichti-Kaiser et al., 2009). Forskolin, an extract of *C. forskohlii* was shown to activate PXR by behaving as its ligand and also via activating PKA pathway to phosphorylate and activate PXR (Ding and Staudinger, 2004). These studies prompted us to examine the PKA activation potential of rosiglitazone and pioglitazone, which were also shown above to behave as PXR ligands. Applying the inhibitors of adenylate cyclase and PKA, enhanced PXR activity by experimental drugs got reduced, indicating the involvement of cAMP-dependent protein kinase in increasing the ligand-dependent activation of PXR. To further confirm the involvement of PKA, constructs GAL4-CREB and pG5E1bLuc (harboring the GAL4 binding element in its promoter fused with lusiferase enzyme encoding gene) were used.As expected, treatment of 8-Br-cAMP showedincrease in PKA-dependent CREB transcriptional activity and

rifampicin, rosiglitazone and pioglitazone also activated CREB with efficacy comparable to that of 8-Br-cAMP. The CREB transcriptional activity of cells treated with drugs get reduced by PKA inhibitor (H89), emphasizing the involvement of PKA in activating PXR.

In response to various stress stimuli like oxidative stress, hypoxia and hypoglycemia etc. energy-sensing kinase [(AMP)-activated protein kinase] AMPK gets activated (Sozio et al., 2011). When ATP levels decreases or AMP and ADP level goes up, AMPK gets activated. There are published data reporting the interplay between energy and xenobiotic metabolism via AMPK. Another xenobiotic sensor CAR also acts as a substrate for AMPK, where phosphorylated CAR is found to be unable to get translocated inside the nucleus (Kanno et al., 2010). Thus transcriptional activity of CAR gets diminished. AMPK-activator metformin has been shown to inhibit the transcriptional activities of PPAR-α and PPAR-γ (Sozio et al., 2011). Another matter of consideration for AMPK activation was anti-diabetic drugs taken in our study, so investigated the consequence of rosiglitazone and pioglitazone on AMPK activation. Inhibition of AMPK did not influence the transcriptional activity of PXR activated by rosiglitazone and pioglitazone, suggesting that there was no participation of AMPK on PXR transactivation under the influence of selected drugs.

MAPKs pathways get activated and initiate intracellular response against environmental stimulants like growth factors, different kinds of cellular stresses, cytokines and hormones etc. As a result, cell proliferation, growth, differentiation and survival processes get co-ordinately regulated (Yang et al., 2013). MAPKs are serine/ threonine kinases and there are different cellular proteins known as their substrates (Cargnello and Roux, 2011). Though there are diverse types of MAPKs known among which extracellular signal regulated kinase 1, 2 (ERK1/2), p38 (α, β, γ and δ) and JNK (c-jun amino terminal kinase 1/2/3) are conventional (Cargnello and Roux, 2011). These conventional MAPKs are known to phosphorylate different NRs and regulate their transcriptional activities. AR, PR, ER-α, ER-β, RARs and PPARs are reported to get phosphorylated by ERKs. On the basis of these reports we also examined the involvement of ERK MAPK in rosiglitazone and pioglitazone-mediated PXR activation. We found that increased PXR activities by the TZDs are not due to activation of ERK1/2 MAPK. In other way it can also be concluded that activated

ERK1/2 might be negatively regulating PXR activity, therefore down-regulation of ERK is increasing the transcriptional activity of PXR in reciprocal manner. After examining the participation of ERK MAPK, next we moved forward to investigate the involvement of remaining two conventional MAPKs JNK and p38. PPAR-α is reported to get phosphorylated by ERK2 and p38 MAPK in ligand-dependent manner (Barger et al., 2001). Troglitazone was withdrawn due to hepatic dysfunction leading to hepatic injury in few patients (Watkins and Whitcomb, 1998). Later, to explain the molecular mechanism, it was found that troglitazone caused oxidative stress in mitochondria by inhibiting complex I of mitochondrial electron transport chain. Overproduction of superoxide anion in mitochondria caused oxidation of thioredoxin 2 (Trx2). Activated-Trx2 triggered activation of downstream ASK1 (apoptosis signal-regulating kinase 1). Further, ASK1 activates JNK MAPK which acts as a downstream signaling pathway for Trx2/Ask1-dependent cell death (Limet al., 2008). Similarly, rosiglitazone was also shown to generate mitochondrial complex I and III-dependent oxidative stress at supratherapeutic concentration. Rosiglitazone executes its effect in PPAR-γ dependent manner at its therapeutic concentration, while it had showed cardiotoxicity at supratherapeutic concentration in PPAR-γ independent fashion (He et al., 2014). Such a supratherapeutic concentration was prescribed in patients who had become tolerant for therapeutic dose of rosiglitazone. It is well-established fact that JNK and p38 MAPKs become active in response to variety of stress stimuli like oxidative stress, hyperosmolarity, ionizing radiation, heat shock, UV irradiations, cytokines etc. (Cargnello and Roux, 2012). So, the possibility of cross-talk between TZD induced activation of stress-activated protein kinases JNK and p38 MAPK and enhancement in PXR transcriptional activity was examined.We concluded that rosiglitazone and pioglitazone-activated JNK and p38 MAPKs are increasing PXR activity probably via phosphorylation. To validate the possibility of interplay between SAPKs (JNK and p38 MAPKs) and PXR, expression plasmids for JNK1 MAPK and p38 MAPK were transfected in HepG2 cells. Cells were treated with 1µM of rifampicin while rosiglitazone and pioglitazone were used at 5µM for 12 hr. Expression of these MAPKs have slightly increased the activity of PXR. To confirm the involvement of these SAPKs, shRNA-mediated silencing of these pathways was done. PXR transcriptional activity was found reduced after silencing. This demonstrates the activation of p38 and JNK MAPK by anti-diabetic drugs to further enhance PXR transactivation.

Finally, to exclude the possibility that all the observed changes in PXR transcriptional activity and expression levels of PXR-associated components [Phase I (CYP3A4), Phase II (UGT1A1), Phase III transporter (MDR1)] were due to cellular factors other than PXR, we performed shRNA-mediated silencing of PXR. Similar to the effects of these drugs in stable cells HepXREM, PXR transcriptional activities got increased. However, the PXR activity was reduced after introducing shRNA against PXR. Therefore, knockdown of PXR reflects that, all the modulatory effect mediated by selected anti-diabetic drugs on other components/pathways are PXR-dependent events.

SUMMARY AND CONCLUSIONS

PXR acts as a 'xenosensor' to protect our body against chemical insults by detoxifying and eliminating myriads of endogenous/exogenous harmful chemicals from the cellular milieu, which otherwise may jeopardize the body's homeostasis (Dussault and Forman, 2002; Wang et al, 2012; Chai et al., 2016). The name PXR is derived from endogenous ligand 5β-pregnan-3, 20-dione with which it was found to bind initially. Ligand-activated PXR heterodimerizes with partner, Retinoid X Receptor (RXR) and together they bind to the xenobiotic responsive elements present in the promoter regions of its target genes encoding the components of 'drug metabolism and disposition (DMD)' machinery. This is followed by recruitment of co-activators like SRC-1, SRC-2, PGC-1α and PBP etc. Besides regulating the target genes of DMD machinery, which is being extensively studied over past 20 years, its anomalous expression and functions are also being unraveled in patho-physiological conditions of inflammatory bowel diseas, cancer, diabetes etc. (Wang et al., 2012; Pondugula and Mani, 2013; Mackowiak et al., 2018).

Induction of associated factors of 'DMD' machinery is responsible for drug-drug interactions (DDIs). In DDIs, the altered metabolism could decrease the efficacy or increase the toxicity of co-administered drugs (Wang et al., 2010). There are plentiful of examples in literature reporting PXR as a key mediator of drug-drug, herb-drug and food-drug interactions (Negi et al., 2008; Prakash et al., 2015). One among the reported example is St John's wort (herbal drug), showing DDIs with wide ranges of drug molecules (Moore et al., 2000; Ernst, 2002). It has shown drug-drug interactions with i) immune-suppressant cyclosporine and sirolimus; ii) with cytotoxic drugs doxorubicin, etoposide, paclitaxel, vinblastin; iii) with cardiovascular drugs digoxin, amiodarone and with; iv) indinavir, ritonavir, saquinavir etc. (anti-HIV drugs) decreasing the efficacy of these co-administered drugs (Tirona et al., 2006; Negi et al., 2008). Due to severe consequences of drug-drug interactions with ketoconazole, drug terfenadine has been withdrawn from clinical practices. Similarly, mibefradil showing DDIs was also withdrawn (Eddershaw et al., 1999). Probability of failure of any medicine in co-medication therapy is high if one of the used agents influences the ADME (absorption, distribution, metabolism and elimination) profiles of other medications, leading to drug-drug interactions (Pal et al., 2006).

In view of the possible instances of drug-drug interactions it is reasonable to formulate an early-stage screening of small molecules, and must be conducted well-before their clinical trial to avoid failures at later stages due to 'DMD' machinery activation and drug-drug interactions. In case of sulfonylurea and nateglinide class of anti-diabetic drugs, CYP2C9 (among the Phase I drug metabolizing enzymes) has been shown to affect their metabolism. Similarly, up-regulation of CYP2C8 has been associated with altered metabolism of repaglinide and TZD class of oral anti-diabetic agents (Tornio et al., 2012). In T2DM condition, multidrug therapy is a common practice, for example TZD are prescribed in addition to sulfonylurea and with metformin.

Therefore, it is desirable that during the early stages of drug development, the small drug molecules must be screened on PXR-platform. To address this issue, in the present study we have selected representatives of classical withdrawn (redundant), established and novel anti-diabetic drug molecules to examine if the success and failure of small molecule modulators can be pre-assessed on PXR platform. We have elaborated the study with rosiglitazone and pioglitazone (thiazolidinediones, TZDs), which are oral anti-diabetic formulations and have been withdrawn from the several countries owing to their association with cardiotoxicity and bladder cancer respectively (Nissen and Wolski, 2007; 2010; Zhu et al., 2012). A retrospective cohort study was conducted on T2DM patients of Indian origin of different age group, multiple prescribed doses of pioglitazone and duration of pioglitazone therapy. On the basis of this study, it was found that pioglitazone was not associated with the risk of bladder cancer across diabetic patients (Newmann et al., 2012), if prescribed for less than 12 months or if, its cumulative dose remained below 10500mg. The risk increases slightly, if the cumulative dose falls within the range of 10501-28000 mg and increases significantly, once the cumulative exposure dose goes beyond 28000mg (Zhu et al., 2012). However, the mechanistic approach for tumour initiating potential of pioglitazone has not been concluded so far. In India, earlier the average consumption of pioglitazone used to be 30mg/day which now-a-days has been mostly limited to 7.5mg/day, based on BMI. This dose is highly efficacious with reduced side effects and would take longer time to reach the cumulative dose of 28000mg (Gupta et al., 2015).

Induction and involvement of CYP3A4 is primarily responsible for oxidative metabolism of ~60% of clinical drugs, which could lead to DDIs with co-medicated drugs and therefore result in therapeutic failure of the latter (Zhou et al., 2005). There are several examples of drugs like St John's wort, carbamazepine, phenytoin, topiramate and rifampicin that induce CYP3A4 in PXR-dependent manner (Johannessen et al., 2010). A DDI changes the drug's pharmacokinetic behaviour leading to undesirable failures at some stages of clinical trials (Mizuno et al., 2003). PXR has now been proposed as the main transcriptional regulator of CYP3A4. Any small molecule that activates PXR is likely to fail in clinical settings. Thus, screening of drugs for their potential to activate PXR appears to be important in early stages of drug discovery processes, to assess their pre-clinical metabolism. In our study, we tested whether anti-diabetic drugs (novel, established and withdrawn) had potential to transactivate PXR and PXR-dependent induction of CYP3A4, UGT1A1 and MDR1. To evaluate the success of small molecules on PXR platform, we have ulitized the HepG2-derived stable cellular models as tools. To assess the efficacy of drug molecules on PXR protein level, HepXREM stable cells have been generated. HepG2 cells were stably transfected with constructs coding for PXR protein along with CYP3A4-promoter-reporter (XREM-Luc) to generate HepXREM cells. However, to evaluate the PXR induction potential of small drug molecules, Hepx-497/+43 cells has been generated, by stable transfection of PXR-promoter region (-497/+43) in HepG2 cells. In this context, using HepXREM stable cell line, we have directly demonstrated that PXR-dependent CYP3A4 induction by the withdrawn TZD drugs (rosiglitazone and pioglitazone) is the plausible reason of their clinical failure. Interestingly, when CYP3A4 mRNA expression after the treatment of the same drugs was quantified by real-time PCR, it also supported the claims. Other anti-diabetic drugs metformin, repaglinide, glimepiride, chlorpropamide, tolbutamide, gliclazide and dapagliflozin did not activate/induce PXR in preliminary transactivation assays in HepXREM and Hepx-497/+43 cells, and were therefore, excluded from subsequent downstream study. In the LS180 intestinal cells, rifampicin has shown maximum PXR-dependent induction of CYP3A4 mRNA, followed by rosiglitazone and pioglitazone. Like CYP3A4 mRNA level, similar pattern for induction of CYP3A4 protein level have also been observed. Endogenous PXR protein level remained unaffected by these drugs. LS180 acts as a model cell line for such studies as it is

considered to express significantly higher PXR levels (Gupta et al., 2008; Harmsen et al., 2008).

Among the phase II conjugating enzymes, the UGT1A1, a member of UGTs family, plays a major role in increasing the hydrophilicity of drugs. We also evaluated its induction by the same anti-diabetic drugs. We observed that there was a significant increase in the activity of UGT1A1 promoter-reporter construct along with induction of its mRNA by rosiglitazone and pioglitazone.

MDR1 (Phase III transporter) causes efflux of a broad range of structurally diverse and low affinity xenobiotics/endobiotics (Mizuno et al., 2003). This broad range substrate specificity appears to contribute further to drug-drug interactions during co-medication. Induction of MDR1-promoter via PXR is shown by both TZD treatments which were also validated by the up-regulation in the MDR1 mRNA level. Overall, our findings suggest that rosiglitazone and pioglitazone are highly potent activators of PXR and its target genes of 'DMD' machinery thereby, mediating undesirable clinical consequences and withdrawal.

Further, we attempted to gain mechanistic insights as to how these drugs are activating PXR. To elucidate the possibility of ligand feature of these drugs, molecular docking of the experimental drugs with apo-PXR-LBD was performed. Docking score values of rosiglitazone and pioglitazone were observed to be higher than rifampicin, reflecting interactions of these TZD as ligands with PXR-LBD. From the *in vitro* live cell imaging study, we observed that these TZDs are capable of driving RFP-PXR from cytosol into the nucleus, giving another indication of interaction of these drugs with PXR. Subsequent to nuclear import study, mammalian two-hybrid experiments were also performed to further investigate if the recruitment of co-activator SRC-1 with ligand bound PXR is imminent. We observed augmentation in the interaction of co-activator SRC-1 with PXR-LBD after the treatment of cells with rosiglitazone and pioglitazone.

Although the primary and direct event to activate PXR is ligand binding, increasing amount of evidences suggest that cell signaling pathways and modulation of PXR/co-regulators phosphorylation status also determines overall responsiveness to environmental stimuli (Lichti-Kaiser, 2008). PXR is also reported to cross-talk with

other NRs or signaling pathways (Zhou et al., 2006; Pascussi et al., 2008; Kumar et al., 2010). Activation of these signaling pathways introduce post-translational modifications like acetylation, deacetylation, phosphorylation, dephosphorylation and sumoylation which are also implicated to modulate other NRs functions including PXR (Pondugula et al., 2009; Smutny T et al., 2013; Priyanka et al., 2016). These reported studies suggest that a drug molecule can transactivate PXR by behaving like its ligand and/or by modulating signaling pathways which can synergistically enhance ligand-activated PXR activity. We found that the two TZDs rosiglitazone and pioglitazone are activating PXR by modulating cAMP-dependent PKA signaling, JNK and p38 MAPKs. However, ERK/MEK-2 MAPK and AMPK signaling pathways did not influence receptor functioning.

Finally, to resolve the controversy associated with the TZDs, modulating some other cellular factors/transcription factors, which may also alter target gene expression in PXR-independent manner, shRNA-mediated silencing of PXR was performed. We observed induction of the CYP3A4-promoter by TZDs in transient transcription assays, in the absence of shRNA, which correlated well with stable cell line HepXREM reporter activity. After the introduction of shRNA, the luciferase activity associated with CYP3A4-promoter (XREM) was decreased subsequent to drug treatment. Based on this shRNA silencing experiment, it was concluded that the observed PXR activity imparted by rosiglitazone and pioglitazone is actually mediated via ligand-receptor relationship.

Perturbations in the co-ordinated action of drug metabolizing enzymes and efflux transporter would affect the bioavailability/toxicity of co-administered drugs and potentially cause drug-drug interactions (Wacher et al., 1995; Prakash et al., 2015). Thus, a more in-depth pre-assessment of the pharmacokinetic properties of any small molecules or drugs for PXR activity at cellular level, before proceeding to clinical trials on humans, will not only extend better health benefits but also reduce the financial losses. In this context, the present study suggests to evaluate small molecules during preclinical stages by examining the PXR activation/induction potential using stable cellular models projected herein. Such an exercise is expected to prevent any deleterious clinical consequences, loss of time and resources in

developing a superior therapeutic molecule. Based on our observations, the major findings from present study are highlighted below:

- Anti-diabetic drugs rosiglitazone and pioglitazone at 20µM concentration transactivated PXR as potently as rifampicin. While, troglitazone showed a gradual decrease in PXR activity starting from 20µM concentration.

- There was no effect on cell viability at 20µM of rosiglitazone, pioglitazone and at 10µM of other anti-diabetic drugs. Among all the drugs, only rosiglitazone and pioglitazone have shown significant enhancement of PXR activity at 20µM. Therefore, 20µM for both of these TZDs and 10µM for the rest of the selected anti-diabetic drugs were considered optimal for further downstream experiments.

- Rosiglitazone at 20µM and dapagliflozin at 100µM concentration increased the PXR-promoter activity. Like HepXREM stable cells, troglitazone has shown gradual decrease in PXR-promoter activity. The decreased luciferase activity in both the stable cells, shown by troglitazone is attributed to cytotoxicity and not the antagonistic nature of the experimental molecule.

- Following PXR-promoter activity, endogenous protein level of PXR was examined with 10µM rifampicin, 20µM and 50µM of rosiglitazone, 20µM pioglitazone and 100µM of dapagliflozin in LS180 cells. Unlike the PXR-promoter activity in stable cell Hepx-497/+43, PXR protein level remained unchanged by these drugs. This difference may be due to the promoter lengths integrated in these two cell lines. In Hepx-497/+43 cells, a small portion of PXR-promoter (promoter region -497/+43) has been stably integrated, while in LS180 cells, we have examined the effect of drugs on endogenous PXR-promoter. Rosiglitazone and pioglitazone increased the CYP3A4 mRNA level by 3.4-fold and 1.77-fold respectively, which is comparable to the induction with standard PXR ligand rifampicin (4-fold). Similarly, rosiglitazone and rifampicin enhanced the CYP3A4 protein (57 kDa) level by 2-fold, followed by pioglitazone (1.5-fold).

- Like Phase I metabolising enzyme CYP3A4, Phase II conjugating enzyme UGT1A1-pomoter-reporter activity was observed to be increased by rosiglitazone and pioglitazone, which was significantly higher than the positive reference ligand rifampicin. To confirm the induction of UGT1A1, mRNA expression levels were analyzed and found to be increased up to 2.5-fold, by rifampicin and rosiglitazone, while for pioglitazone it was up to 2.0-fold.

- Rosiglitazone and pioglitazone increased PXR-mediated transactivation of MDR1-promoter more than that of rifampicin. Likewise in promoter-reporter assay, MDR1 mRNA level was increased up to 2.0-fold and 1.4-fold after the treatment of rosiglitazone and pioglitazone respectively. Rifampicin exhibited maximum induction of 4.0-fold.

- Transcriptional activity of CAR was also modulated by these two TZDs. Promoter of CYP2B6 (a prototypical target gene of CAR), linked with luciferase enzyme encoding gene was co-transfected with CAR. Pioglitazone induced maximum promoter activity followed by rifampicin and rosiglitazone.

- PXR-mediated cross-transactivation of CYP2B6-promoter was found to be increased by rosiglitazone and pioglitazone. On the contrary, there was no effect of any of these drugs on transactivation of CYP3A4-promoter mediated by CAR.

- To examine the cross-talk between PXR and PPAR-γ (TZDs are well-known ligands of PPAR-γ), a selective PPAR-γ inhibitor GW9662 was applied. In the absence of inhibitor, rosiglitazone and pioglitazone enhanced PXR transcriptional activity in HepXREM stable cells. After applying GW9662, PXR transcriptional activity remained uninfluenced in the presence of both of these TZDs, which has nullified the possibility of this cross-talk.

- To examine if the activation of PXR by the selected anti-diabetic drugs is direct, (direct activation), computational approach was followed by performing the docking of drugs with apo-PXR-LBD. The value of docking score reflects the binding affinity of drugs with unliganded PXR. Docking

score value of rosiglitazone and pioglitazone was considerably higher than the rifampicin. These *in silico* outputs indicated the ligand behavior of these drugs for PXR.

- To further approve the ligand characteristics of the two TZDs, ligand-dependent translocation study of RFP-PXR under the influence of these drugs was performed. In the absence of any ligand, RFP-PXR is ~65% nuclear and ~35% cytosolic. Rifampicin, a standard ligand of PXR completely shifted cytosolic fractions of PXR into the nucleus. A considerable portion of cytosolic PXR was also observed to be shifted by rosiglitazone and pioglitazone. Like *in silico* predictions, this experimental study also denoted the ligand nature of these TZDs towards PXR.

- Rifampicin and other ligands of PXR are known to recruit the co-activators to increase the PXR transcriptional activity. So, to find the interaction between PXR and SRC-1 (co-activator), mammalian two-hybrid assay was performed. Rosiglitazone and pioglitazone were found to recruit SRC-1 to similar extent as rifampicin, implying that rosiglitazone and pioglitazone may actlike PXR ligands.

- SHP (Small heterodimer partner), one of the member of NR superfamily, is reported to act as a co-repressor protein for different NRs including PXR. We suspected that SHP down-regulation by two TZDs may be one of the possible mechanisms to enhance PXR activity. Therefore, we transfected HepG2 with SHP-Luc (promoter of SHP) and treated with drugs. Instead of getting inhibition, we observed induction of SHP by pioglitazone. Rifampicin and rosiglitazone also mildly induced the SHP-promoter. Rifampicin is known to induce SHP to keep the PXR activity at desired level through a negative feedback loop.

- In addition to direct activation (binding with ligand) of PXR, indirect activation (modulation of signaling pathways) is also reported. Such an interface between direct and indirect activation influences the transcriptional function of PXR. To examine the involvement of reported signaling pathways, we applied specific inhibitors of signaling pathways. After applying the

inhibitors of i) adenylate cyclase (MDL-12,330A); ii) PKA (H-89); iii) AMPK (dorsomorphin) and iv) MAPKs; ERK1/2 (FR180204), JNK1/2/3 (SP600125), P38 (SB203580), PXR transcriptional activity was measured. We observed that rosiglitazone and pioglitazone have activated PKA, JNK and p38 MAPK signaling cascade but did not influence ERK/MEK-2 MAPK and AMPK signaling. So, it was possible that after activation of these signaling pathways by drugs, PXR activity may be further enhanced.

- Next level of experiment was done to further validate the involvement of PKA and MAPKs in PXR activation. To verify PKA activation by rosiglitazone and pioglitazone, construct GAL4-CREB (having DNA binding domain of GAL4) and pG5E1bLuc (harboring GAL4 binding sites in the promoter region, cloned in frame with luciferase coding gene) were co-transfected. An analog of cAMP (8-Br-cAMP) acted as a positive control and showed maximum CREB associated luciferase activity. Similarly, rosiglitazone and pioglitazone have also shown increased CREB activity. This demonstrated that these drugs activate PKA signaling events. Further, to examine the activation of p38 and JNK MAPK by two of the TZD, expression plasmids for the respective MAPKs were ectopically expressed in HepG2, along with PXR and XREM constructs. Expression of these MAPKs showenhancement in the PXR transcriptional activity. To confirm the role of these MAPKs, p38 and JNK MAPKs were silenced by their respective shRNAs. The decrease in PXR activity after silencing, confirmed the modulation of p38 and JNK MAPKs by anti-diabetic drugs in enhancing PXR activity.

- In order to avoid the possibility of modulation of other cellular factors by anti-diabetic drugs in regulating PXR activity, silencing of PXR was done with shRNA. HepG2 cells were co-transfected with shRNA against PXR along with constructs XREM and PXR, followed by drug treatments. Introduction of shRNA-PXR significantly decreased rifampicin, rosiglitazone and pioglitazone-mediated PXR activity. This observation ruled out the involvement of any cellular factors in PXR activation. This suggests that rosiglitazone and pioglitazone are acting on PXR and regulating the associated components of ‘drug metabolism and disposition’ machinery.

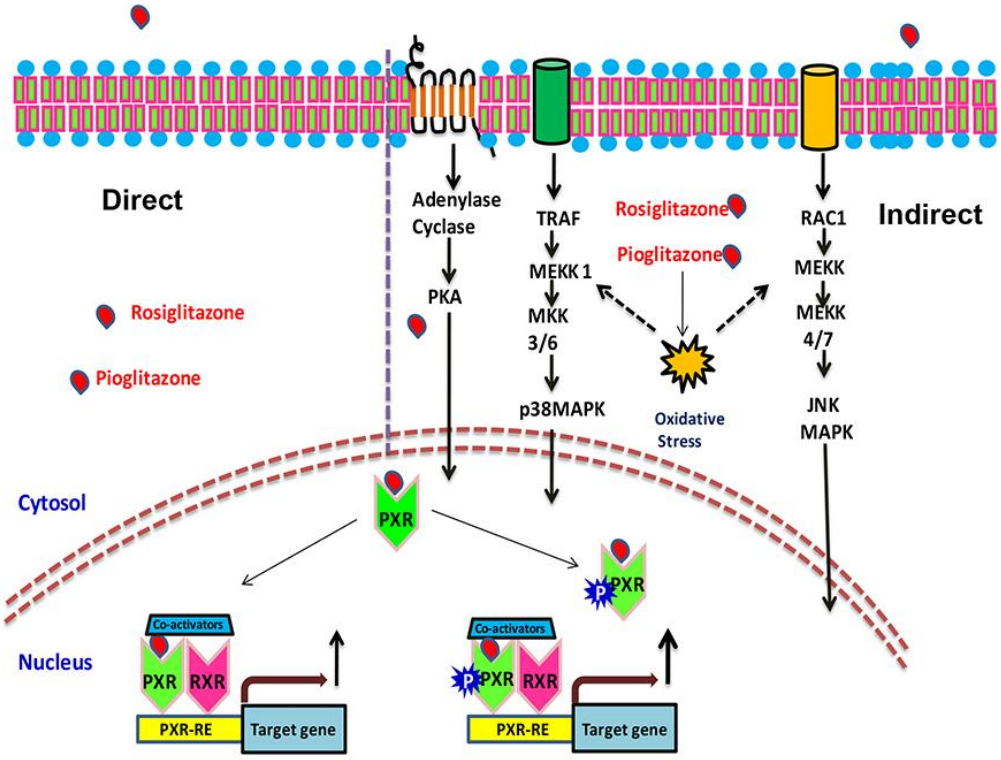


Figure 33: Schematic illustrations of i) direct and ii) indirect activation of PXR by rosiglitazone and pioglitazone resulting in augmentation of ligand-dependent activation of PXR. *In direct activation mechanism, rosiglitazone and pioglitazone bind with the LBD of PXR, augment the recruitment of co-activators, which leads to enhanced PXR transcriptional activity. Additionally, rosiglitazone and pioglitazone activate SAPKs (JNK and p38 MAPKs) via indirect activation, which further strengthen the PXR transactivation by phosphorylating the receptor and engaging the assembly of co-activators. To sum up, we tempted to speculate that, rosiglitazone and pioglitazone activate JNK and p38 MAPKs to phosphorylate and then increase the PXR transcriptional activity in addition to ligand-dependent (TZD-PXR interaction) activation.*

In conclusion, we observed that, though the TZDs may have been withdrawn from clinical practices by projecting other issues but the data in this study reveals that, had PXR activation and induction of its associated components were done prior to their launch in the market, these failures would have been predicted well in advance by the using stable cellular models as screening tools. These stable cells served as high-throughput screening tools to identify PXR modulators. Emergence of idiosyncratic toxicity behavior is generally unpredictable and comes to limelight only because of the genetic variations among individuals or distinct population, which cannot be always evaluated during clinical trials or before the launch of drugs. Unlike prediction of idiosyncratic behavior, it is possible to predict the induction of PXR-

mediated components of 'DMD' machinery by the potential therapeutic molecules. Both transiently and stably transfected cell lines were used and compared to assess and evaluate selected anti-diabetic drugs. Both of the TZD, rosiglitazone and pioglitazone exhibited direct activation of PXR by interacting with ligand binding domain of PXR, thus behaving like PXR ligand, as well as, indirectly by modulating some key signaling pathways which post-translationally activate ligand-bound PXR. Furthermore, up-regulation of genes of 'DMD' machinery CYP3A4, UGT1A1 and MDR1 is a down-stream consequence of PXR activation by these TZD. Rosiglitazone and pioglitazone are activating both the MAPK pathways (JNK and p38), which responds to certain kind of stresses. Based on the conclusions from our study, we proposed the speculative model of PXR activation by anti-diabetic drugs rosiglitazone and pioglitazone (**Figure 33**).

A studyhas proposed themechanism behind troglitazone-induced hepatic cellular injury is oxidative stress. Because of this oxidative stress thioredoxin-2/ASK1 signaling are activated, which leads to the further activation of JNK-MAPK, leading to mitochondrial permeability transition and causing cell injury (Priscilla et al., 2007). Troglitazone is also reported to induce CYP3A4 via activation of PXR (Chiarelli and Marzio, 2008). It is also reported that MAPKs phosphorylate NRs/co-regulators, leading to activation of NRs and induction of target genes. To further investigate whether there is any oxidative stress associated with the treatment of rosiglitazone and pioglitazone, detection of free radical would be required. Also, to validate the involvement of PKA, p38 and JNK MAPKs, expression levels of CYP3A4, UGT1A1 and MDR1 may be measured by applying their respective inhibitors. CHIP experiment would be required to be carried out, to establishwhether there is enhanced binding of PXR with CYP3A4, UGT1A1 and MDR1 promoter region after the treatment of rosiglitazone and pioglitazone.

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PUBLICATIONS

1) **Shashi Kala Singh** and Rakesh K. Tyagi (2017) Thiazolidinedione class of anti-diabetic drugs modulate nuclear receptor CAR function. *Journal of Endocrinology and Reproduction*, 21: 117-124.

2) Seema Negi, **Shashi Kala Singh**, Sanjay Kumar, Subodh Kumar and Rakesh K. Tyagi (2018) Stable cellular models of nuclear receptor PXR for high-throughput evaluation of small molecules. *Toxicology In Vitro.,* 52: 222-234.

3) Amit K. Dash, Ashutosh S. Yende, Sudhir Kumar, **Shashi Kala Singh**, Deepak Kotiya, Manjul Rana and Rakesh K. Tyagi (2016) The Constitutive Androstane Receptor (CAR): a nuclear receptor in health and disease. *Journal of Endocrinology and Reproduction,* 20: 1-27.

4) Sam P. Mathew, Keshav Thakur, Sudhir Kumar, Ashutosh S. Yende, **Shashi Kala Singh**, Amit K. Dash, and Rakesh K. Tyagi (2018) A Comprehensive Analysis and Prediction of Sub-Cellular Localization of Human Nuclear Receptors. *Nuclear Receptor Research*, Vol. 5, Article ID 101324.

5) **Shashi Kala Singh** and Rakesh K. Tyagi. A comprehensive evaluation of anti-diabetic drugs on nuclear receptor PXR (manuscript under preparation).

Conference Presentations/Abstracts

1**) Shashi Kala Singh**, Seema Negi, Mallampati Saradhi and Rakesh K. Tyagi. Evaluation of efficacy of anti-diabetic drugs by xenosensing nuclear receptors at the international symposium on "integrative physiology and comparative endocrinology (ISIPCE-2016)" & 34th annual meeting of the "society for reproduction biology and comparative endocrinology", held on 12th-14th feb 2016 in Varanasi at BHU.

2) **Shashi Kala Singh**, Seema Negi, Mallampati Saradhi and Rakesh K. Tyagi. Evaluation of efficacy of anti-diabetic drugs by xenosensing nuclear receptors at Society of Biological Chemists'' held on 21th-24th Nov 2016 in Mysuru, India.

3) **Shashi Kala Singh**, Seema Negi, Mallampati Saradhi and Rakesh K. Tyagi. Evaluation of efficacy of anti-diabetic drugs by xenosensing nuclear receptors at the National Science Day Celebration at Jawaharlal Nehru University Convention Centre, 28th February, 2016.

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Thiazolidinedione Class of Anti-Diabetic Drugs Modulate Nuclear Receptor CAR Function

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Abstract

The human body has developed a defence system to prevent the accumulation of endogenous (bile acids, steroids, cholesterol metabolites, neurotransmitters, etc.) as well as exogenous (xenobiotics, clinical drugs, etc.) small molecules at toxic levels. This task is accomplished by 'drug metabolism and disposition (DMD) machinery' which entails phase I and phase II enzymes, and phase III transporter proteins. The components of this machinery act in a coordinated manner to biotransform and facilitate the elimination of small toxic molecules from the cellular milieu. Constitutive androstane receptor (CAR), a member of the nuclear receptor superfamily, acts as one of the major transcriptional regulators of the DMD machinery. Prescription of combination therapy is a common regimen during the treatment of diverse metabolic disorders and infectious diseases. In such combination therapies one drug may modulate the expression of genes of DMD, influencing the metabolism of another co-administered drug. This leads to decreased bioavailability or increased toxicity of the latter. Evaluation of drug-drug interactions (DDIs) has now become a major safety concern during drug discovery and development processes. Pre-assessment of the small molecules for modulatory effects on CAR and induction of the components of DMD can resolve the safety concerns, treatment failures and drug withdrawals due to the harmful DDIs. In the present study, we have followed a 'reverse approach' to assess CAR activation by drugs previously withdrawn from clinical practices. We selected three redundant members of thiazolidinedione family of anti-diabetic drugs and examined their potential in regulation of CAR and its target gene CYP2B6. These drugs showed differential transcriptional activation of CAR. Two of the TZD i.e., rosiglitazone and pioglitazone enhanced CAR activity by behaving as receptor ligands while the other (troglitazone) did not influence the receptor function and was justly withdrawn since it inflicted cytotoxicity.

Keywords: Anti-Diabetic Drugs, Constitutive Androstane Receptor, Drug Metabolism and Disposition Machinery, Nuclear Receptor, Nuclear Translocation

1. Introduction

Nuclear Receptors (NRs) constitute a superfamily of ligand-modulated transcription factors with 48 members identified in the human genome [10, 23, 5]. Members of the NR superfamily are involved in almost all the major aspects of biological processes such as growth, development, metabolism, homeostasis, etc. They execute their transcriptional functions in response to small lipophilic ligands like hormones, xenobiotics, fatty acids, vitamins, all-trans retinoic acid (RA), 9-cis-retinoic acid (9-cis-RA) and diverse endogenous metabolites[5]. Constitutive androstane receptor (CAR: NR1I3) is one of the key members of the human NR superfamily. It was isolated for the first time in 1994 from human liver using degenerate oligonucleotide probes[1,4]. It is predominantly expressed in liver and intestine, the primary site of drug detoxification [21]. CAR acts as a promiscuous receptor as it gets activated by a broad range of structurally dissimilar xenobiotics[21]. CAR exhibits differential subcellular localization and transcription function behaviour depending on cell and tissue type[26]. Recently, unliganded red fluorescent protein-tagged CAR (RFP-CAR) was observed to shift preferentially to the cytoplasmic compartment making it amenable for nuclear translocation studies [6]. CAR has a high basal activity without the involvement of binding to

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Stable cellular models of nuclear receptor PXR for high-throughput evaluation of small molecules

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ABSTRACT

Pregnane & Xenobiotic Receptor (PXR) is one of the 48 members of the ligand-modulated transcription factors belonging to nuclear receptor superfamily. Though PXR is now well-established as a 'xenosensor', regulating the central detoxification and drug metabolizing machinery, it has also emerged as a key player in several metabolic disorders. This makes PXR attractive to both, researchers and pharmaceutical industry since clinical success of small drug molecules can be pre-evaluated on PXR platform. At the early stages of drug discovery, cell-based assays are used for high-throughput screening of small molecules. The future success or failure of a drug can be predicted by this approach saving expensive resources and time. In view of this, we have developed human liver cell line-based, dual-level screening and validation protocol on PXR platform having application to assess small molecules. We have generated two different stably transfected cell lines, (i) a stable promoter-reporter cell line (HepXREM) expressing PXR and a commonly used CYP3A4 promoter-reporter i.e. XREM-luciferase; and (ii) two stable cell lines integrated with proximal PXR-promoter-reporter (Hepx − 1096/ + 43 and Hepx − 497/ + 43). Employing HepXREM, Hepx-1096/ + 43 and Hepx-497/ + 43 stable cell lines > 25 anti-cancer herbal drug ingredients were screened for examining their modulatory effects on a) PXR transcriptional activity and, b) PXR-promoter activity. In conclusion, the present report provides a convenient and economical, dual-level screening system to facilitate the identification of superior therapeutic small molecules.

1. Introduction

Presently, cell-based assays are used in more than half of all high throughput drug screenings performed for target validation and ADMET (absorption, distribution, metabolism, elimination and toxicity) analyses in the early stages of drug discovery (Michelini et al., 2010; Zang et al., 2012; Nierode et al., 2016). Although several drug screening methods are available to assess the pharmacological properties of small molecules, but due to the high cost, lengthy experimental duration these approaches offer only limited use. In this context, alternative methods which provide faster, easier and more reproducible results are desirable. Experimental processes in drug discovery often involve screening a large number of new or modified compounds using defined biochemical assays in an ultra high-throughput format (Westby et al., 2005; Korn and Krausz, 2007; Michelini et al., 2010; Macarron et al., 2011; Nierode et al., 2016). However, the pharmacodynamic processes are complex and involve interactions at multiple levels that cannot be predicted using biochemical assays alone. This complexity may be resolved by judicious use of cell-based screening assays. Cell-based assays are biologically more relevant to predict the response of the organism towards the experimental drugs. In addition, at some point in the drug discovery process, predicting cellular toxicity is also important. In general, to meet these needs various approaches are followed to screen or evaluate novel molecules or compounds. Some of these are cell-free ligand binding assays (like fluorescence polarization) which do not mimic the cellular environment and are physiologically less relevant. Unlike cell-free systems, cell-based two hybrid assays may give convincing observations about the therapeutic behaviour of drugs inside the cells but may not represent same structure and functionality of target protein of interest. So, due to the involvement of only a portion (s) of transcription factors there is no distinction between agonists and antagonists during receptor binding assays. Today, for high throughput screening of drugs, cell-based transactivation assays may be used to supersede the drawback of assays mentioned above (Pinne and Raucy, 2014). Furthermore, cell-based assays are relatively more convenient and physiologically relevant during primary screens. Now-a-days stable cell lines of various nuclear receptors are in common use for such purposes (Sonneveld et al., 2005; Gijsbers et al., 2011; Novotna et al., 2012; Campana et al., 2016). In order to screen small molecules, including endocrine or metabolic disruptors, stable cell lines of estrogen/

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The Constitutive Androstane Receptor (CAR): a nuclear receptor in health and disease

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Summary

Constitutive Androstane Receptor (CAR, NR1I3), a member of the nuclear receptor superfamily of transcription factors, has emerged as one of the key regulators of the drug and xenobiotic metabolism. The unique feature that separates CAR from other members of the superfamily is that it remains active in the absence of ligand and is further regulated by activators. From its first isolation in 1994, a number of studies related to its distribution, characteristics, functions, and relation to other members of the superfamily have been conducted that place it centrally, governing many key events of the body. Human CAR is expressed relatively higher in liver and epithelial cells of the small intestine villi and less in heart, muscle, kidney, brain and lung. Though there are some controversies regarding its subcellular localization in different cell lines, in general, the subcellular localization of CAR is reported to be predominantly cytoplasmic, in complex with co-chaperone partners HSP90 and CCRP (cytoplasmic CAR retention protein). To execute transcription functions, nuclear translocation is a prerequisite event for a NR, including CAR. In this context, existence of two pathways is suggested, i) direct mechanism of action; and ii) indirect mechanism of action that is governed via nuclear translocation of CAR. Additionally, existence of species-specific differences in its modulation with ligands acting either as an agonist, antagonist or inverse agonist is also apparent. Like the other xenobiotic receptor PXR, CAR also functions as an alternative 'xenosensor' to defend the body against persistent chemical insults. It responds to diverse array of chemically distinct compounds, including endobiotics and xenobiotics, to regulate the clearance of noxious chemicals and toxic metabolites in liver and intestine via induction of genes involved in their metabolism. The usefulness of targeting CAR in metabolic diseases including bilirubinemia, obesity, type 2 diabetes mellitus, atherosclerosis, preeclampsia, hypertension, cholestasis and also in liver cancer is being extensively studied in animal models. However, to determine the human relevance it requires further investigation. Though a large number of natural and synthetic compounds act as modulators of CAR, designing new derivatives with defined therapeutic benefit need to be investigated. The purpose of this review is to highlight the general aspects of nuclear receptor CAR, its mechanism of action and importance in human health and disease.

Key Words: Nuclear receptors, Transcription factors, Constitutive Androstane Receptor, Xenosensor, Drug metabolism, Metabolic diseases, Sub-cellular localization, Cancer.

Introduction

Nuclear Receptors (NRs) belong to a superfamily of phylogenetically-related proteins comprised of 48 members in humans. They act as transcriptional switches by responding to their cognate ligands including various hormones, vitamins, lipids, steroids, etc., and share a general modular structure (Mangelsdorf et al., 1995; Nuclear Receptors Nomenclature Committee, 1999; Burris et al., 2012). The members of this superfamily have a central DNA binding domain (DBD), also termed 'C region' which is highly conserved in sequence. There is a highly variable region on the amino-terminal to the C region called region A/B which contains the activation function 1 (AF-1) whose function (transcriptional activity) is independent of the presence of ligand. On the carboxy-terminal to the DBD, another conserved region is found, which is termed as the ligand binding domain (LBD) or E region and contains the activation function 2 (AF-2) whose action (transcriptional activity) is ligand-dependant. This region is responsible for recognition and binding of the specific ligands. There is a comparatively shorter region which connects C and E regions, called the hinge region or region D. On the extreme carboxy terminal to the LBD, some receptors may contain a region of unknown function called F region (Burris et al., 2012) (Fig. 1).

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Research Article

A Comprehensive Analysis and Prediction of Sub-Cellular Localization of Human Nuclear Receptors

Sam P. Mathew, Keshav Thakur, Sudhir Kumar, Ashutosh S. Yende, Shashi Kala Singh, Amit K. Dash, and Rakesh K. Tyagi

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Abstract. The Nuclear Receptor (NR) superfamily comprises of conserved ligand-modulated intracellular transcription factors which in the presence of their cognate ligands activate a plethora of signaling networks, thereby commencing their respective transcription functions. All NRs are nuclear when liganded or active. However, their localization may differ between nucleus and cytoplasm when unliganded or inactive. NRs control a majority of physiological processes in body ranging from metabolism to reproduction and development. Hitherto, in case of humans, 48 NRs have been identified which are localized either in cytosolic, nuclear or both compartments of the cell. Sub-cellular localization of proteins has great relevance in relation to their function. However, specific sub-cellular localization patterns of human NRs are clouded with ambiguity and are mostly ridden with controversy, with only a few of them being well-studied and established under specific physiological conditions. In the present study, we attempted to bridge the gap and attempted to draw conclusions in relation to sub-cellular localization of human NRs based on published experimental data and by *in-silico* prediction methods. This comprehensive analysis may not only be useful to draw conclusions on their control of physiological processes but may also open new avenues towards understanding of the molecular basis of NR-mediated diseases attributed to their mislocalization and malfunctioning.

Keywords: Nuclear Receptors, sub-cellular localization, *in-silico,* ngLOC, Hum-mPLoc 3.0

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1. Introduction

Nuclear receptors (NRs) are transcription factors which on activation by physiological stimuli, bind to the specific DNA sequences and bring about regulation of complex biological pathways [1]. These receptors function alongside other proteins to regulate the expression of specific target genes, thereby effectively controlling vital cellular functions such as development, homeostasis and metabolism in an organism.

NRs constitute a large superfamily of evolutionarily-conserved proteins. The NR superfamily can be broadly categorized into four subfamilies based on their DNA-binding properties and dimerization preferences. Class I receptors include steroid hormone receptors, such as GR, MR, PR, AR, ER etc., which act as ligand-induced homodimers and bind to the half-sites of target DNA oriented as inverted repeats. Class II consists of receptors which heterodimerize with RXR such as VDR, RAR, TR etc., and bind to the direct repeat half-sites. Class III and class IV receptors are orphan receptors where class III receptors bind to the direct repeat as homodimers while class IV receptors typically bind to extended core sites as monomers [1, 2].

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