Modulation of Pregnane & Xenobiotic Receptor functions by prospective anti-cancer herbal drugs

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CERTIFICATE

The research work embodied in this doctoral thesis entitled **'Modulation of Pregnane & Xenobiotic Receptor functions by prospective anti-cancer herbal drugs'** 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 or Institution elsewhere.

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TO MY LOVING PARENTS

Who have always been the constant sources of inspiration and encouragement.....

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Acknowledgement

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(Seema Negi)

ABBREVIATIONS

µg	Microgram
µl	Microlitre
µM	Micromolar
^{o}C	Degree centigrade
ABCA1	ATP Binding Cassette Transporter A1
ACs	Adenylyl Cyclases
AF	Activation function
AICAR	5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside
AMP	5' adenosine monophosphate
AMPK	AMP-activated Protein Kinase
APS	Ammonium persulphate
ATP	Adenosine tri phosphate
AR	Androgen Receptor
bp	Base pair
CaMKKβ	Calcium/calmodulin-dependent kinase kinase β
cAMP	Cyclic AMP
CAR	Constitutive Androstane Receptor
CARM1	Coactivator-associated arginine methyltransferase 1
cDNA	Complementary DNA
CYP	Cytochrome P450
CBP	CREB-binding protein
CDK	Cyclin dependent kinase
CREB	cAMP-response element binding protein
DAX1	Dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1
DBD	DNA-binding domain
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetic acid
ER	Estrogen Receptor
ERK	Extracellular Regulated Kinase
FBS	Fetal Bovine Serum
FXR	Farnesoid X Receptor
g	Gram
GR	Glucocorticoid Receptor

GCNF1	Germ cell nuclear factor 1
GRIP1	Glucocorticoid Receptor Interacting Protein 1
h	Hour
HAT	Histone acetyl transferase
HDAC	Histone deacetylase
HEPES	4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid
HMT	Histone methyl transferase
HNF-4	Hepatocyte Nuclear Factor-4
kDa	Kilodalton
LBD	Ligand-binding domain
LKB1	Liver Kinase B1
LSD1	Lysine specific demethylase
LXR	Liver X Receptor
M	Molar
MAPK	Mitogen-activated protein kinase
MDR1	Multi-drug resistance 1
min	Minute
ml	Millilitre
mM	Millimolar
mRNA	Messenger RNA
mW	Molecular weight
MR	Mineralocorticoid Receptor
N-CoR	Nuclear Receptor corepressor
ng	Nanogram
NGFI-B	Nerve growth factor-induced factor B
NLS	Nuclear localization signal
NTD	N-terminal domain
NR	Nuclear Receptor
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBP	PPAR Binding Protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGC-1α	Peroxisome Proliferator Activated Receptor Gamma Coactivator 1 alpha
pH	Power of hydrogen
PI3K	Phosphatidylinositol 3-OH Kinase
PKA	Cyclic-AMP-dependent Protein Kinase A
PKC	Protein Kinase C
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenyl methyl sulphonyl fluoride
PPAR	Peroxisome Proliferator-activated Receptor

PSA	Prostate specific antigen
PR	Progesterone Receptor
PVDF	Poly-Vinyl-di-Fluoride
PXR	Pregnane & Xenobiotic Receptor
RAR	Retinoic Acid Receptor
ROR	Retinoic acid receptor-related orphan receptor
RT	Room temperature
RXR	Retinoid X Receptor
SDS	Sodium dodecyl sulphate
s	Second
SF1	Steroidogenic factor 1
SHP	Small heterodimer partner
SIRT1	NAD-dependent deacetylase sirtuin 1
SRC-1	Steroid Receptor Coactivator-1
SRC-3	Steroid Receptor Coactivator-3
SREBP-1c	Sterol regulatory element-binding protein 1c
SMRT	Silencing mediator for retinoid and thyroid-hormone receptors
Sp1	Specificity protein 1
STAT	Signal transducer and activator of transcription
TAE	Tris acetate, EDTA buffer
TBS	Tris buffer Saline
TBP	TATA-binding protein
TEMED	N, N, N', N'-tetra methyl ethylene diamine
TR	Thyroid Receptor
Tris	Tris-(hydroxyl methyl)-amino methane
VDR	Vitamin D Receptor
XREM	Xenobiotic responsive enhancer module

INTRODUCTION

Nuclear receptors (NRs) represent potential therapeutic targets because they play a vital role in various biological processes of fundamental importance. Thus, considerable efforts are spent in drug discovery programs to identify nuclear receptor agonists and antagonists that may possess the desired pharmacological activity. In drug discovery and development not only pharmacological properties of potential new drugs come into account but also much emphasis is laid on the compound's toxicology, ADME (absorption, distribution, metabolism, excretion) properties, and safety profile. Extensive preclinical studies address these qualities of a drug candidate before it can be administered to humans. In recent years, herbal drugs have gained much importance in the therapeutic and treatment process. Herbal drugs are readily used by millions of people without prescription under the belief that anything natural is safe. Like allopathic (prescription) drugs, herbal medicines also have different pharmacokinetic and pharmacodynamic properties ultimately leading to different therapeutic responses, but also have adverse actions due to drug-herbal interactions. The concurrent use of herbal medicines and conventional (prescription) drugs by patients suffering from different diseases has progressively increased. Co-administration of herbal medicines with conventional drugs increases the risk of undesirable interactions between the two. Interactions between drugs can affect the pharmacokinetics of concomitantly administered chemotherapeutic agents. An important mechanism that underlies these interactions is the induction of drug metabolizing enzymes and efflux transporters (CYP3A4 and MDR1). The transcription of many of these functional proteins involved in pharmacokinetics is regulated by a NR called Pregnane & Xenobiotic Receptor (PXR) (Willson and Kliewer, 2002; Meijerman et al, 2006; Harmsen et al, 2007). For example, if one drug activates PXR, it can be predicted that administration of this drug will promote the elimination of other co-administered drugs that are also metabolized and eliminated by PXR-target gene products, thereby reducing the efficacy of multi-drug therapies in patients on combination therapy. In this prospective, PXR antagonist(s) may have a role by impeding the induction of drug metabolism through inhibition of PXR activity (Wang et al, 2008a; Mani et al, 2013). Studies on PXR interactions with herbal drugs in

changing physiological environment both under normal and pathophysiological conditions may give important clues in evaluating the herbal drugs.

The PXR, a member of the nuclear receptor super-family is a well-known xenobiotic sensing receptor. The highly promiscuous nature of PXR allows it to interact with a wide variety of structurally distinct ligands such as xenobiotics (including pharmaceutical drugs, herbal drugs, endocrine disruptors, pesticides and environmental contaminants etc.) and endobiotics (including pregnanes, bile acids, hormones and dietary vitamins, etc.) (Kliewer et al, 1998; Kliewer, 2003; Orans et al, 2005; Saradhi et al, 2006, Chang, 2009). The ability of PXR to interact with such a diverse range of compounds makes it promiscuous in nature and this promiscuity lies in its LBD region. The PXR LBD contains additional five strands of ß-sheets unlike other nuclear receptors that normally contain only two-three strands. The insertion of these extra sheets (of ~60 residues) add up to a larger volume of ligand binding pocket (>1,300A) as compared to ligand binding pocket of other NRs (Watkins et al, 2001; 2003). This insertion leads to an enlarged, flexible and hydrophobic LBD which is capable of fitting and accommodating diverse ligands. The large and conformable binding pocket probably contributes to its ability to respond to low-affinity compounds, including endobiotics (Chrencik et al, 2005; Xue et al, 2007b). PXR is highly expressed in the major organs that are important in xenobiotic biotransformation, including the liver and intestine (Kliewer et al, 1998). PXR transcriptionally regulates the expression of genes involved in all phases of drug metabolism and elimination, therefore PXR is referred as the 'master regulator' of the expression of the CYP3A4 which metabolizes more than 50% of the drugs (Orans et al, 2005; EI Sankary et al, 2000; Goodwin et al, 2001; Falkner et al, 2001; Wilson and Kliewer, 2002; Meijerman et al, 2006; Harmsen et al, 2007). Under the physiological conditions, it maintains the homeostasis of the body, primarily mediating the rapid and timely elimination of toxic endogenous metabolites and exogenous chemicals. Recently, PXR role is also emerged in hepatic steatosis, vitamin D homeostasis, bile acids homeostasis, steroid hormones homeostasis, inflammatory bowel diseases, cancer, etc. (Wilson

and Kliewer, 2002; Zhou et al, 2009; Ihunnah et al, 2011; Gao and Xie, 2012; Pondugula and Mani, 2013). Although the primary event leading to activation of PXR is ligand binding, increasing amounts of evidences suggest that cell signalling pathways and modulation of PXR-cofactor-phosphorylation status also determines overall responsiveness to environmental stimuli (Rochette-Egly, 2003; Staudinger and Lichti-Kaiser, 2008). Post-translational modifications like acetylation, deacetylation, phosphorylation, dephosphorylation, sumoylation have also been implicated in gene transcription regulation of many nuclear receptors incuding PXR (Pondugula et al, 2009). A few recent reports indicate that some of the metabolic signal transduction pathways interface with PXR (Lichti-Kaiser et al, 2009; Pondugula et al, 2009). Nonetheless, the regulation of PXR gene transcription remains unexplored by signalling pathways. While PXR is known to be transcriptionally activate many genes, its own transcriptional mechanisms remains inadequately explored. So an important question arises as to what regulates the master regulator PXR? A number of xenobiotics that activate PXR have been identified; however, the mechanisms controlling PXR expression are largely undetermined. Nevertheless, its plausible involvement under patho-physiological conditions like cancers, osteomalacia, drug-drug interactions, etc. is also becoming apparent. Recent findings suggest that PXR is augmented under certain physiological conditions including pregnancy and also in certain patho-physiological conditions including several malignancies (breast, endometrial, colon, ovarian cancers etc.). The significance of PXR expression in these malignancies remains ambiguous and need to be investigated. Various contradictory finding has been reported in recent years regarding the role of PXR in cancer. While few research findings delineate its involvement in cancer cell proliferation and drug resistance, in contrast, others reveal the role of PXR in apoptosis (Masuyama et al, 2003; Gupta et al, 2008).

The majority of research on transcriptional regulation of PXR has been descriptive and the role of PXR in cancer is also controversial. In the light of existing literature, the aims of the present study were to gain better insight into the regulation of PXR functions and signalling by herbal anticancer

drugs and their mechanism of actions. The major objectives covered in present study are as stated below:

- To generate and characterize stable hepatic cell lines for screening and identification of herbal drugs having modulatory effects on xenobiotic receptor PXR.
- To study the modulation of critical PXR-responsive genes by anti-cancer herbal drugs involved in xenobiotic metabolism and elimination.
- To investigate the action of herbal drugs on PXR-mediated signalling events in hepatic cancer cell lines.

The objectives of this research proposal were to screen various prospective anti-cancer herbal drugs that are plausible PXR modulators, evaluate the dynamics of PXR promoter activity and protein expression in presence of these herbal drugs, and to determine the magnitude of the changes in metabolizing enzymes and their activity. The ability of these prospective anticancer herbal drugs to trigger the PXR activity and the induction of metabolizing enzymes are studied using appropriate model cell lines. There are various methods, *in vivo* and *in vitro* to assess the pharmacological properties (therapeutic activity and safety) for screening of drugs. Due to lengthy experimental duration, high cost and intensive labour, only small number of drugs may be tested *in vivo* using animal models. In addition to this *in vivo* model offers limited predictive values because of species variations and other factors. Alternative methods such as *ex vivo* cell-based assays have gained more attention because of less time consumption, low cost, reproducibility of results and better adaptability for high-throughput screening strategies (Naylor, 1999). In this context, a cell-based screening approach for evaluating the therapeutic value and safety assessment of clinical or herbal drugs, xenobiotics and endobiotics are addressed at two main levels by engaging (i) PXR protein based or (ii) PXR-promoter based transcription assays (**Figure 1**).

Level 1. Drugs, xenobiotics and endobiotics that bind and activate PXR protein may be assayed to identify and eliminate the possibility of drug-drug, herbal-herbal, drug-herbal interactions during treatment regime.

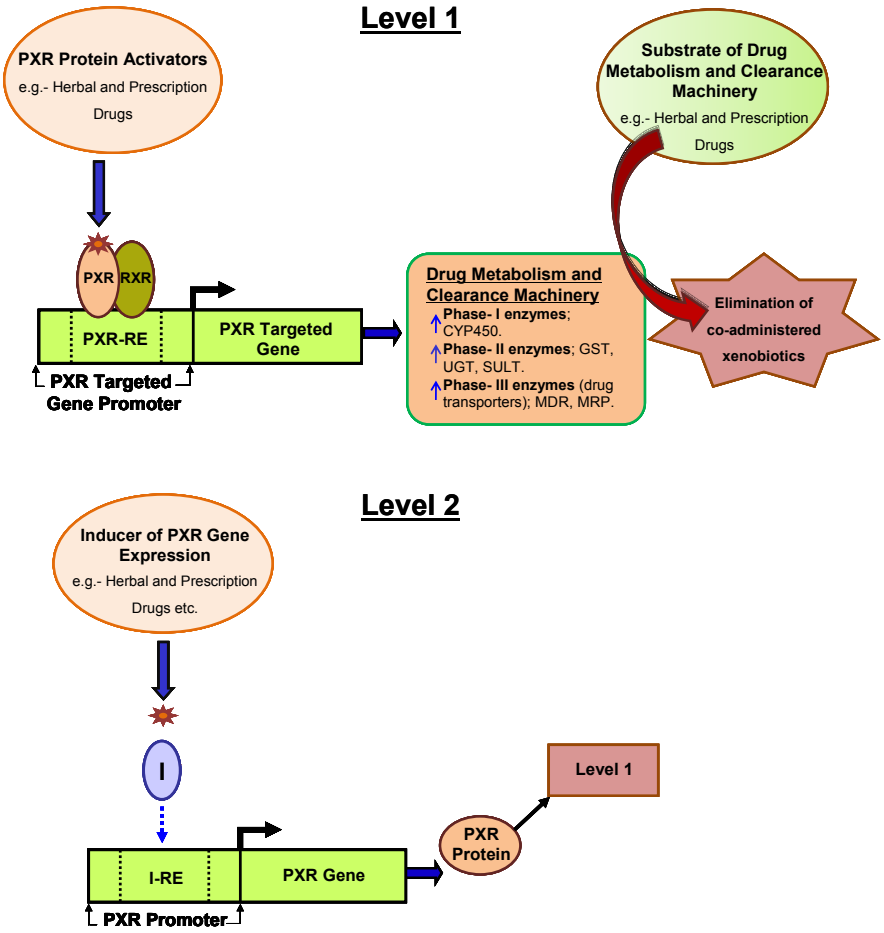

Figure 1: A schematic representation depicts the response of drugs, xenobiotics and endobiotics on PXR protein (Level 1) and PXR-promoter (Level 2). *Level 1: After entering the cell, PXR protein activator like drugs, xenobiotics and endobiotics bind to PXR protein which then heterodimerizes with its endogenous partner RXR (Retinoid X Receptor). This complex binds to PXR response element (PXR-RE) present in the promoter of PXR targeted genes. This in turn accelerates the cascade of detoxification machinery by up regulating Phase I, II, III components. As a consequence drugs, xenobiotics and endobiotics that bind to PXR protein are eliminated by the upregulated detoxification machinery. Similarly, other co-administered drug, xenobiotics and endobiotics that do not bind to PXR protein are also eliminated by the same upregulated detoxification machinery. Level 2: We hypothesize that some drugs, xenobiotics and endobiotics can act as inducers of PXR-promoter either alone or in conjunction with a hitherto unknown DNA binding protein(s). When inducer is bound to PXR-promoter through I-RE the expression of PXR protein will be increased (Level 2). Increased availability of PXR protein will lead to upregulation of detoxification machinery as depicted in Level 1. For evaluating drugs, xenobiotics and endobiotics at Level 2 we generated the human liver cell line(s) with stably integrated PXR-promoter-reporter construct that is useful for high throughput screening of above mentioned compounds at Level 2. In addition to this, the cell line will be useful in identifying unknown regulatory factors which have the capability to bind and modulate (repress or activate) PXR-promoter functions. In the figure above, RXR denotes Retinod X Receptor; PXR-*

RE: PXR response element. 'I' denotes any regulatory drug, xenobiotic or endobiotic or its complex with hitherto unknown DNA binding protein(s) that has a capability to interact with PXR-promoter region i.e. I-RE (inducer-response element).

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.

Findings from the current study may unravel the unexplored regulatory mechanisms of PXR gene and receptor functions by herbal anti-cancer drugs and expose the signalling events taking place in PXR transcription and transactivation. Subsequently, this study may provide some novel PXR agonists(s) and antagonist(s) holding therapeutic potentials. PXR agonist(s) may hold therapeutic potential for metabolic diseases and inflammatory diseases while antagonists may be advantageous to withdraw the adverse effects of PXR in drug-drug interactions and chemotherapeutic drug resistance. Finally, this study also offered reproducible cell based preclinical screening models for evaluating the therapeutic value of clinical or herbal drugs, xenobiotics and endobiotics at both PXR protein and PXR-promoter levels (**Figure1**).

REVIEW OF LITERATURE

The Nuclear Receptor Superfamily

Overview

The nuclear receptor (NR) superfamily is a class of ligand-modulated transcription factors that work in concert with co-activators and co-repressors to regulate target gene expression. These are important transcriptional regulators involved in diverse physiological functions and playing important roles in control of embryonic development, cell differentiation, homeostasis, reproduction, metabolism and immunity (Mangelsdorf et al, 1995; Laudet and Gronemeyer, 2002). Dysregulation of NR signalling has been associated with many patholological conditions including reproductive dysfunctions and metabolic disorders such as cancer, diabetes, neurological disorders and cardiovascular diseases (Laudet and Gronemeyer, 2002; Germain et al, 2006). NRs emerged in the earliest of metazoan evolution long before the divergence of vertebrates and invertebrates (Escriva et al, 1997; Owen and Zelent, 2000). Estrogen receptor was among the first NRs being identified biochemically in 1960s by Elwood Jensen (Jensen and Khan, 2004) which was later cloned by Pierre Chambon in 1980's. At the same time, two other NRs including glucocortioid receptor, estrogen receptor and thyroid receptor were cloned by Ron Evans and Bjorn Vennstrom respectively (Hollenberg et al, 1985; Green et al, 1986; Greene et al, 1986). Later, several NRs were characterized and they have become recognized as a superfamily of transcription factors. The family includes nuclear hormone receptors (NHR), orphan nuclear receptors and 'adopted' orphan nuclear receptors based on the ligand binding nature of NRs. As the name suggest, NHR utilize classical hormones as their ligands for signalling. Orphan nuclear receptors are those that share similar structure to the identified receptors but whose physiological ligands were not known at the time of their identification and are awaiting ligand identification. Lastly, 'adopted' orphan nuclear receptors are those for which ligands have only recently been identified. The search for ligands for orphan receptors and the identification of novel signalling pathways has become a very active research field in recent past few years (Gustafsson, 1999; Kliewer et al, 1999). The NR research field has undergone very rapid development and covers areas ranging from structural

and functional analyses to the molecular mechanisms of transcription regulation. The ability of NRs for binding ligands makes them potential pharmaceutical targets. Their successes as drug targets are highlighted by the common use of retinoic acid for retinoid acid receptor (RAR) (targeted in acute promyelocytic leukemia), casodex for androgen receptor (AR) (targeted in prostate cancer), tamoxifen for estrogen receptor (ER) (targeted in breast cancer), thiazolidinediones for peroxisome proliferator-activated receptor-γ (PPARγ) (targeted in type II diabetes) or dexamethasone for the glucocorticoid receptor (GR) (targeted in inflammatory diseases), spironolactone for the mineralocorticoid receptor (MR) (targeted in congestive heart failure), progestins for progesterone receptor (PR) (targeted in menstrual cycle disorders and endometriosis), and dovonex for the vitamin D receptor (VDR) (target in osteoporosis) (Laudet and Gronemeyer, 2002; Gronemeyer et al, 2004; Chambon, 2005; Evans, 2005; Chen, 2008; Huang et al, 2010).

Successful identification of selective NR modulators has revolutionized the NR drug discovery strategy from the designing of synthetic compounds that mimic the function of cognate ligands to development of selective nuclear receptor modulators (SNuRMs) that specifically modulate the functional activity of an NR. As nuclear receptors bind small molecules that can easily be modified by drug design, and control functions associated with major diseases (e.g. cancer, osteoporosis and diabetes), they are promising pharmacological targets. Since all body tissues express a subset of NRs, overall NRs have pivotal control on the whole organism homeostasis and particularly on the patho-physiological status of an organism. Hence NRs are feasible therapeutic targets for dozens of human diseases and the discovery and development of compounds that finely modulate the activity of NRs may result in potential drugs only if a deeper knowledge of each ligand-NR interaction can be achieved. In the past decade, a number of molecular, genetic, structural and pharmacological studies have contributed to our understanding towards the molecular mechanisms involved in NR action. Moreover, these studies provided novel molecular assays for the rapid identification and screening of potential drugs with the desired pharmacological profile and have facilitated the rational designing of the next generation of pharmaceuticals.

Canonical structure

Nuclear receptors share a common structural organization (**Figure 2**). The N-terminal region (A/B domain) is highly variable, and contains at least one constitutionally active transactivation region (AF-1) and several autonomous transactivation domains (AD); A/B domains are variable in length, from less than 50 to more than 500 amino acids, and their 3D structure is not known. The most conserved region is the DNA-binding domain (DBD, C domain), which notably contains the P-box, a short motif responsible for DNA-binding specificity on sequences typically containing the AGGTCA motif, and is involved in dimerization of nuclear receptors. This dimerization includes homodimers as well as heterodimers. The 3D structure of DBD has been resolved for a number of nuclear receptors and contains two highly conserved zinc-fingers – C-X2-C-X13-C-X2-C and CX5-C-X9-C-X2-C – the four cysteines of each finger chelating one Zn^{2+} ion that are responsible for DNA binding and dimerization (Bourguet et al, 2000). Between the DNA-binding and ligand binding domains is a less conserved region (D domain) that behaves as a flexible hinge between the C and E domains, and contains the nuclear localization signal (NLS), which may overlap on the C domain (Robinson-Rechavi et al, 2003). The largest domain is the moderately conserved ligand-binding domain (LBD, E domain), whose secondary structure of 12 α-helixes is better conserved than the primary sequence. The 3D structure has been determined for several nuclear receptors (Moras and Gronemeyer, 1998), unliganded (apo) or liganded (holo), allowing much better understanding of the mechanisms involved in ligand binding. Lastly, it contains an activation function helix, termed AF-2, in the extreme C-terminal of the LBD which is required for ligand-dependent activation and co-activator recruitment. Domain F is a C-terminal domain which is highly variable among all nuclear receptors and is supposed to mediate repression effect but its functional relevance is still unknown.

Nomenclature and classification of nuclear receptors

Nuclear receptors form a superfamily of phylogenetically related proteins, with the 21 genes in the complete genome of the fly *Drosophila melanogaster*

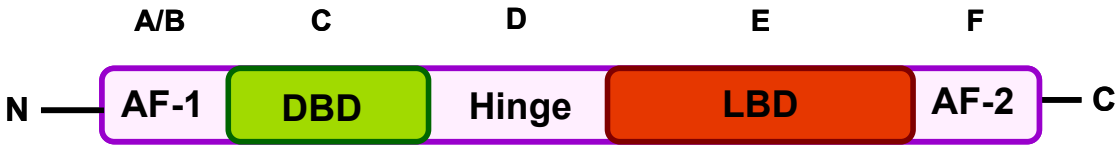

Figure 2: Structure of nuclear receptors. *Members of the nuclear receptor superfamily consist of four modular domains: a highly variable N-terminal region that harbors an activation function (AF-1), a DNA binding domain (DBD) consisting of two zinc-finger motifs, a flexible hinge domain, and the ligand binding domain (LBD) that also contains an activation function (AF-2).*

(Adams et al, 2000), 48 in humans (Robinson-Rechavi et al, 2001), 49 in mice (FXRβ is extra in mice) (Robinson-Rechavi and Laudet, 2003) and unexpectedly more than 270 genes in the nematode worm *Caenorhabditis elegans* (Sluder et al, 1999). This diversity has been organized in a phylogeny-based nomenclature (Nuclear Receptors Nomenclature Committee, 1999) of the form NRxyz, where x is the sub-family, y is the group and z the gene. In addition to nuclear receptors that have both DNA-binding and ligand-binding domains, sub-family NR0 contains those nuclear receptors that lack either of these domains, and are not represented in the phylogenetic tree. They include notably Knirps, KNRL and EGON (NR0A1, 2, 3) in Drosophila, and DAX1 and SHP (NR0B1, 2) in vertebrates.

Based on sequence alignment and phylogenetic tree construction, human NR family has been classified into six subfamilies (NR1-NR6) (Nuclear Receptor Nomenclature Committee, 1999; Escriva et al, 2000; Thornton and De Salle, 2000):

1. **The subfamily I** also known as Thyroid Hormone Receptor like proteins which includes TRs, RARs, VDR, and PPARs, as well as orphan receptors such as RORs, Rev-erbs, CAR (NR1I3), PXR (NR1I2), LXRs, and others.

2. The subfamily II also known as Retinoid Receptor-like protein and includes RXRs, COUP-TF, and HNF-4.

3. The subfamily III also known as Estrogen Receptor-like protein includes the steroid receptors with AR, ER, GR, PR, and as well as the ERR.

4. The subfamily IV also known as Nerve Growth Factor IB-like and contains NGFI-B (NR4A1), NURR1 (NR4A2), and NOR1 (NR4A3)].

5. The subfamily V also known as Steriodogenic Factor-like protein is another small group that includes the steroidogenic factor 1 (NR5A1) and the receptors related to the Drosophila FTZ-F1.

6. The subfamily VI also known as Germ Cell Nuclear Factor-like and this subfamily contain only the GCNF1 receptor (NR6A1), which does not fit well into any other subfamilies.

A detailed outline of classification and nomenclature of human nuclear receptor is described in **Table I**.

Table I: Nomenclature and Classification for Nuclear Receptors.

Subfamily and Group	NR/Gene	Trivial Names	Accession Number
1A	NR1A1	TRα, c-erbA-1, THRA	M24748
	NR1A2	TRβ, c-erbA-2, THRB	X04707
1B	NR1B1	RARα	X06538
	NR1B2	RARβ, HAP	Y00291
	NR1B3	RARγ, RARD	M57707
1C	NR1C1	PPARα	L02932
	NR1C2	PPARβ, NUC1, PPARδ, FAAR	L07592
	NR1C3	PPARγ	L40904
1D	NR1D1	REVERBα, EAR1, EAR1A	M24898
	NR1D2	REVERBβ, EAR1β, BD73, RVR, HZF2	L31785
	NR1D3	E75	X51548
1E	NR1E1	E78, DR-78	U01087
1F	NR1F1	RORα, RZRα	U04897
	NR1F2	RORβ, RZRβ	Y08639
	NR1F3	RORγ, TOR	U16997
	NR1F4	HR3, DHR3, MHR3, GHR3, CNR3,	M90806
		CHR3	U13075
1G	NR1G1	CNR14	U13074
1H	NR1H1	ECR	M74078
	NR1H2	UR, OR-1, NER1, RIP15, LXRβ	U07132
	NR1H3	RLD1, LXR, LXRα	U22662
	NR1H4	FXR, RIP14, HRR1	U09416
1I	NR1I1	VDR	J03258

	NR1I2	ONR1, PXR, SXR, BXR	X75163
	NR1I3	MB67, CAR1, CARα	Z30425
	NR1I4	CAR2, CARβ	AF00932
1J	NR1J1	DHR96	U36792
1K	NR1K1	NHR1	U19360
2A	NR2A1	HNF4	X76930
	NR2A2	HNF4G	Z49826
	NR2A3	HNF4B	Z49827
	NR2A4	DHNF4, HNF4D	U70874
2B	NR2B1	RXRA	X52773
	NR2B2	RXRB, H-2RIIBP, RCoR-1	M84820
	NR2B3	RXRG	X66225
	NR2B4	USP, Ultraspiracle, 2C1, CF1	X52591
2C	NR2C1	TR2, TR2-11	M29960
	NR2C2	TR4, TAK1	L27586
2D	NR2D1	DHR78	U36791
2E	NR2E1	TLL, TLX, XTLL	S72373
	NR2E2	TLL, Tailless	M34639
2F	NR2F1	COUP-TFI, COUPTFA, EAR3, SVP44	X12795
	NR2F2	COUP-TFII, COUPTFB, ARP1, SVP40	M64497
	NR2F3	SVP, COUP-TF	M28863
	NR2F4	COUP-TFIII, COUPTFG	X63092
	NR2F5	SVP46	X70300
	NR2F6	EAR2	X12794
3A	NR3A1	ERα	X03635
	NR3A2	ERβ	U57439
3B	NR3B1	ERR1, ERRα	X51416
	NR3B2	ERR2, ERRβ	X51417
3C	NR3C1	GR	X03225
	NR3C2	MR	M16801
	NR3C3	PR	M15716
	NR3C4	AR	M20132
4A	NR4A1	NGFIB, TR3, N10, NUR77, NAK1	L13740
	NR4A2	NURR1, NOT, RNR1, HZF-3, TINOR	X75918
	NR4A3	NOR1, MINOR	D38530
	NR4A4	DHR38, NGFIB	U36762
		CNR8, C48D5	U13076
5A	NR5A1	SF1, ELP, FTZ-F1,	D88155

		AD4BP	
	NR5A2	LRH1, xFF1rA, xFF1rB, FFLR, PHR, FTF	U93553
	NR5A3	FTZ-F1	M63711
5B	NR5B1	DHR39, FTZF1B	L06423
6A	NR6A1	GCNF1, RTR	U14666
0A	NR0A1	KNI, Knirps	X13331
	NR0A2	KNRL, Knirps related	X14153
	NR0A3	EGON, Embryonic gonad, EAGLE	X16631
	NR0A4	ODR7	U16708
	NR0A5	Trithorax	M31617
0B	NR0B1	DAX1, AHCH	S74720
	NR0B2	SHP	L76571

(Adapted and modified from Nuclear Receptors Nomenclature Committee, 1999)

Mode of action

Nuclear receptors classically act in three steps (Laudet and Gronemeyer, 2002): repression, derepression and transcription activation (**Figure 3**). Repression is a characteristic feature of apo-nuclear receptor, which recruits a corepressor complex with histone deacetylase activity (HDAC; represented in the lower half of the bottom-right inset). Derepression occurs following ligand binding, which dissociates this complex and recruits a first coactivator complex, with histone acetyltransferase (HAT) activity, resulting in chromatin decondensation, which is believed to be necessary but not sufficient for activation of the target gene. In the third step, the HAT complex dissociates and a second coactivator complex is assembled (TRAP/DRIP/ARC), making a contact with the basal transcription machinery, and thereby resulting in transcription activation of the target gene. Though simplified, this mechanism is not general for all NRs, since some NRs may act as activators without a ligand, whereas others are unable to interact with the target gene promoter in the absence of ligand (the 'repression' step).

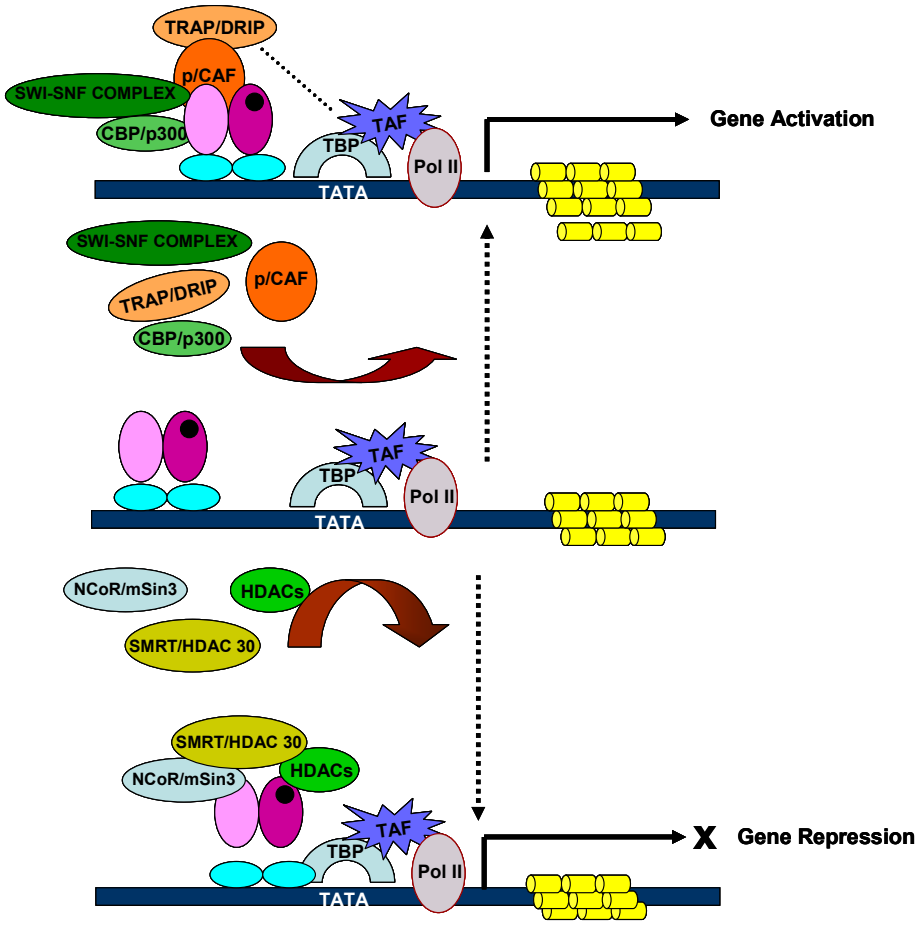

Figure 3: A simplified model of nuclear receptor signalling. *This represents a hypothetical schematic of the exchange of coregulators involved in activation of a gene by nuclear receptors (adapted from Thakur and Paramanik, 2009).*

Pregnane & Xenobiotic Receptor (PXR): the xenobiotic-sensing receptor

It was originally noted in the 1970s that certain pharmaceutical compounds regulated the expression of a number of enzymes capable of protecting against toxic effects of xenobiotics (Selye, 1971). The receptor capable of sensing the presence of such exogenous compounds and, ultimately, the upregulation of various metabolizing enzymes was later identified as PXR (Blumberg et al, 1998; Kliewer et al, 1998). The pregnane & Xenobiotic receptor [PXR, NR1I2, also known as steroid X receptor (SXR)] is a novel ligand-activated intracellular transcription factor belonging to the nuclear receptor super-family. It has been independently discovered in mice and humans by three groups in 1998. These investigators used either homology cloning or database mining

techniques (Bertilsson et al, 1998; Blumberg et al, 1998; Kliewer et al, 1998; Lehmann et al, 1998). PXR is structurally characterized by its four distinct domains i.e., an amino-terminal transactivation domain, a central DNA-binding domain (DBD), the hinge region, and a carboxy-terminal ligand-binding domain (LBD). Unlike other nuclear receptor orthologs, PXR ortholog shares less amino acid homology in the LBD, providing the possibility for marked variation in its activation profile across species. Activation of PXR was shown to be induced by natural steroids as well as synthetic corticoids. The human ortholog, originally named PXR as well as SXR (steroid and xenobiotic receptor) and PAR (pregnane-activated receptor), was found to be highly expressed in the liver, intestine and kidney the tissues where maximum detoxification of noxious compounds occur. But low levels of PXR have also been found in the peripheral blood monocytes, blood brain barrier, uterus, ovary, placenta, breast, osteoclasts, heart, adrenal glands, bone marrow, and specific brain regions of various species (Zhang et al, 1999; Bauer et al, 2004; Lamba et al, 2004; Lamba et al, 2005; Miki et al, 2005; Pollock et al, 2007). PXR is activated by a pleothera of xenobiotics including pharmaceutical agents like rifampicin, rifaximin (Ma et al, 2007), dexamethasone (Lehman et al, 1998), troglitazone (Jones et al, 2000), tamoxifen (Ma et al, 2008), ritonavir (Dussault et al, 2001); environmental pollutants like pesticides; endocrine disrupters (Zhou et al, 2009; Chaturvedi et al, 2010) and medicinal herbal drugs (Negi et al, 2008; Chang 2009) etc. After activation by endogenous or exogenous ligands, PXR heterodimerizes with the 9-cis retinoic acid receptor (RXR), binds to the xenobiotic response elements of the target genes and modulates their expression, leading to detoxification and elimination of the xenobiotics. To date, a large number of ligands act in an agonistic manner to upregulate its gene expression but so far, few antagonists have been described including ketoconazole (Wang et al, 2007; Mani et al, 2013), sulphoraphane (Zhou et al, 2007), ecteinascidin-743 (ET-743) (Synold et al, 2001) and coumestrol (Wang et al, 2008a) that have been subsequently shown to disrupt the binding of coregulators to the surface of PXR in an agonist-dependent fashion (Huang et al, 2007).

PXR acts as master regulator of detoxification defence machinery, i.e., phase-I and phase-II enzymes, as well as several drug transporters (Kliewer, 2003). Other than its function as a vital xenosensor, PXR also plays a key role in the metabolism of endobiotics (steroids, bile acids and their derivatives, vitamins, etc.) and xenobiotics (synthetic drugs, herbal medicines, endocrine disruptors, etc.). Ligand-mediated transcriptional activation of PXR is one of the principal mechanisms underlying the induction of drug metabolizing enzymes and drug transporters that ultimately leads to interactions of co-administered drugs. Irrespective of its ligand-bound or ligand-free status, PXR is predominantly present in the nuclear compartment and associates with condensed chromosomes during all the stages of mitosis (Saradhi et al, 2005b). In addition to the role of PXR in detoxification, bile homeostasis (Saini et al, 2005) and bone metabolism, its role in cancer is also becoming apparent. Therefore, PXR appears to be an important and promiscuous xenosensor in human health and disease (Saradhi et al, 2006). After completing a decade, the research outcome of several new findings on PXR reveal the diverse role of PXR in normal physiological control and patho- physiological situations. Additionally, other studies not only expound the involvement of PXR in drug-drug/herbal interactions via modulating detoxification defence machinery but also in designing safer therapeutic molecules (Staudinger et al, 2006; Pal and Mitra, 2006).

Structure of Pregnane and Xenobiotic Receptor

Gene organization

Human PXR is located on chromosome 3, locus 3q12–q13.3 and spans approximately 20 kbs (Hustert et al, 2001; Kliewer et al, 2002). It is composed of 10 exons separated by 9 intronic regions (**Figure 4**) (Hustert et al, 2001; Kliewer et al, 2002). Multiple transcript isoforms of PXR are generated by alternative splicing and alternate promoter usage. Three alternatively spliced transcripts that encode different isoforms of PXR have been described (Bertilsson et al, 1998; Blumberg et al, 1998; Kliewer et al, 1998). The first two exons are used as alternative 5' ends of human PXR transcripts. The human PXR isoform-1 (mRNA (NM_003889) and the corresponding isoform-1 protein

(NP_003880)) originate from exon 1. Isoform 1 is generally accepted as the wild-type form of the receptor. The human PXR isoform 2 originates from exon 2 and characterized by the extension of 39 amino acid residues at the N-terminus (Bertilsson et al, 1998). The human PXR isoform-3 originates from an in-frame deletion of 111 bps of the 5' part of exon 5. This hPXR variant has lacking of 37 amino acids in the LBD and considered shortest among three (Dotzlaw et al, 1999). In **Figure 4**, the human PXR isoform-1 mRNA (NM_003889) and the corresponding isoform-1 protein (NP_003880) are represented. The 434 amino acid long human PXR isoform-1 (PXR.1) is composed of the DBD; encoded by exons 3 and 4, the H region; encoded by exon 5 and the LBD; encoded by exons 5–10 (Hustert et al, 2001).

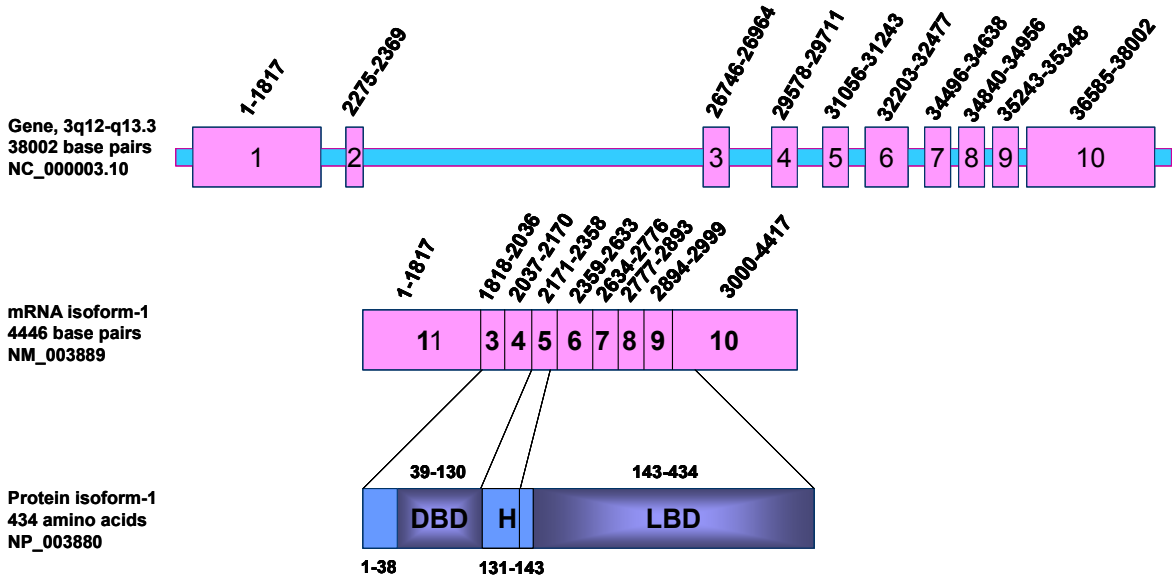

Figure 4: PXR gene, mRNA and protein organization. *The hPXR gene, localized on chromosome 3, is composed of 10 exons and 9 introns. Three hPXR isoforms, containing different combinations of splicing, have been so far identified. The isoform-1 is considered the wild-type one. The isoform-1 encodes a 434 amino acid long xenosensor. The DBD is encoded by exons 3 and 4, the H region is encoded by part of exon 5, and the LBD is encoded by exons 5–10 (Adapted and modified from di-Masi et al, 2009)*

Protein Structure

PXR, like all the members of the NR super-family, is modular protein sharing common regions, including the N-terminal DBD, the H region, and the

C-terminal LBD (**Figure 5**). These regions participate in the formation of independent but allosterically interacting functional domains (Ribeiro et al, 1995; Kumar and Thompson, 1999; Olefsky, 2001; Mohan and Heyman, 2003; McEwan, 2004; Ascenzi et al, 2006). It contains the highly conserved DNA binding domain (DBD), a characteristic structure of NRs. The far N-terminus is a short activation function-1 (AF-1) region which permits the regulation of receptor action in a ligand-independent manner. The structure of the PXR DBD is highly similar to that of the retinoid X receptor α (RXRα) DBD, which is a double zinc-finger motif that contacts DNA in a sequence-specific fashion. The response elements include direct repeats with 3 to 5 bases separating the DBD binding sites (DR-3, DR-4 and DR-5) and inverted repeats (with the beginning of each sequence in proximity) separated by 6 or 8 bases (ER-6 and ER-8, respectively) (Lehmann et al, 1998, Blumberg et al, 1998, Kast et al, 2002). PXR target genes, multidrug resistance protein 1 (MDR1) and cytochrome P450 3A4 (CYP3A4), contain DR-4/ER-6 (Geick et al, 2001) and DR-3/ER-6 (Blumberg et al, 1998, Goodwin et al, 1999) in their promoter regions, respectively. The PXR DBD also contains a bipartite nuclear localization sequence (Kawana et al, 2003). The DBD is linked to the ligand-binding domain (LBD) in PXR by a hinge region which is considerably shorter than that observed in the majority of NRs. The LBD

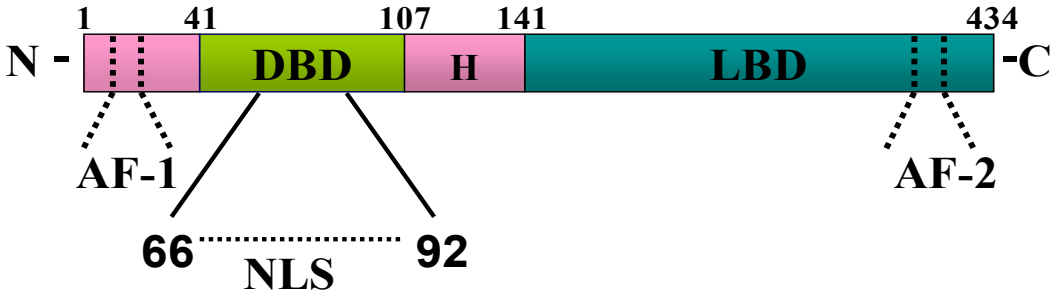

Figure 5: Schematic presentation of functional domains in human PXR. *The domain structure of human PXR is presented, including the N-terminal ligand independent activation function 1 (AF-1), the DNA binding domain (DBD), the relatively short hinge region, and the ligand binding domain (LBD), which contains the ligand-dependent activation function 2 (AF-2).*

contains the ligand‐dependent activation function 2 (AF‐2) region and the ligand-binding pocket. The LBD is the most prominent feature in PXR. It is possible for the LBD of PXR to heterodimerize with the LBD of RXRα, similar to the known structures of other NR LBDs with the RXRα LBDs (Zhang et al, 2001). Conformational changes upon ligand binding in AF‐2, which are responsible for the recruitment of coregulator proteins, lead to changes in the transcription of target genes (Renaud et al 1995, Nolte et al, 1998; Xu et al, 2002).

Crystal structures of PXR-LBD: Promiscuity and species specific variation of PXR

To function as a xenobiotic sensor, PXR has evolved the ability to recognize a wide variety of toxic substances. Despite this promiscuity, striking species differences are seen in the activation profiles between PXR orthologs. Crystal structures of the human PXR-LBD shed light on the molecular basis of these properties (Watkins et al, 2001). LBD is largely hydrophobic with five polar residues capable of both donating and accepting hydrogen bonds. According to the 3-D structure, the LBD comprises three sets of α-helices: α 1/ α 3, α 4/ α 5/ α 8, and α 7/ α 10. In addition, a layer of five stranded anti-parallel β-sheets includes two novel β-strands not observed in other NRs: β 1 and β 1' (**Figure 6**). In contrast to other NRs of known structures, PXR contains an insert of approximately 60 amino acids between helices α 1 and α 3, which contribute to the formation of the novel helix α 2, β 1, and β 1'. In human PXR, a flexible loop encompassing residues 309-321 replaces helix α 6 (Orans et al, 2005). These features combine to give PXR a spherical ligand binding pocket that has a volume of more than 1150Å.

The crystal structure of the LBD of PXR suggested which amino acid differences in the LBD of human PXR (human and mouse PXR LBD exhibit only 77% amino acid identity) contributed to known species differences in ligand activation of mouse and human PXR (Lehmann et al, 1998; Xie et al, 2000) and induction of CYP3A (Kocarek et al, 1995). Watkins and colleagues humanized the mouse PXR by converting four of the polar amino acids in the ligand binding pocket from the mouse PXR sequence to the corresponding residues

found in human PXR (Watkins et al, 2001; 2003a). This group generated humanized mouse model so that it recognized ligands that typically only bind and activate human PXR. The results of these studies suggest that these residues are either involved in direct binding with the ligand and/or altering the shape of the binding site significantly. The structural models also provide insights into the marked differences in the activation of PXR across species.

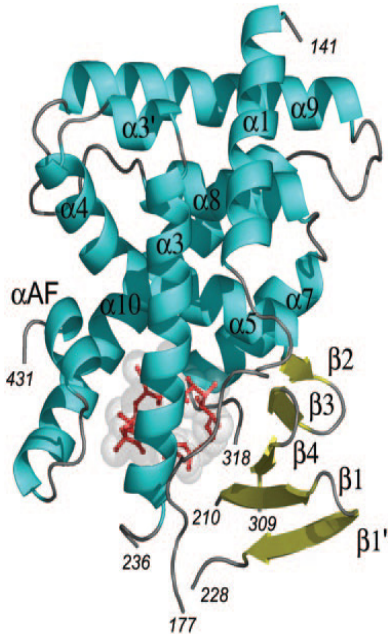

Figure 6: Crystal structure of the LBD of human PXR in complex with rifampicin (red) at 2.8 Å resolution. *The three-dimensional structure of the human PXR-LBD is presented as a ribbon diagram. The PXR-LBD consists of a three-layered a-helical sandwich with a five-stranded anti-parallel β-sheet. The a helices are in cyan and β strands are in yellow, including the two novel β-strands, β-1 and β-1' that complete the five-stranded anti-parallel β-sheet observed in this structure. The large solvent-accessible ligand-binding pocket is outlined in white. The following loop regions of PXR are disordered in this complex: residues 178–209, 229–235, and 310–317. (Adapted from Chrencik et al, 2005).*

Several x-ray structures of the PXR LBD in complex with agonists have been reported. The cholesterol-lowering drug SR12813 forms complex in three distinct positions within the cavity (Watkins et al, 2001) and PXR-SR12813 in complex with an SRC-1 coactivator peptide revealed a single agonist binding mode (Watkins et al, 2003a). Thus, Redinbo and coworkers demonstrated that

the PXR ligand-binding pocket can accommodate ligands in multiple positions, and upon coactivator binding, the ligand is stabilized into a single active orientation (Watkins et al, 2001; Watkins et al, 2003a). In active state, SR12813 interacts with Ser247 and His407 through hydrogen bonding involving 11 hydrophobic amino acid side chains. The active component of the herbal antidepressant St. John's wort, hyperforin, forms hydrogen bonds with Ser247, Gln285, and His407 (Watkins et al, 2003b). The antibiotic rifampicin, one of the largest known PXR ligands, also forms hydrogen bonds with Ser247, Gln285, and His407 (Chrencik et al, 2005). The liver X receptor (LXR) agonist T0901317 was shown to interact with the hPXR LBD through polar interactions with the Gln285 and His407 (Xue et al, 2007). The hops constituent colupulone forms hydrogen bonds with His407 and bonds to Gln285 through a water molecule (Teotico et al, 2008).

The PXR-ligand interaction appears to be a dynamic process, leading to structural changes that quite possibly alter the interaction between PXR and its coactivator or corepressor. The growing number of crystal structures will prove to be invaluable in uncovering the complex relationship among ligand, receptor, coregulators, and target DNA. Structural information from all the complexes obtained to date reveals a large and expandable ligand binding pocket that can harbor ligands of varying sizes, with different chemical and structural properties, thus explaining the promiscuity of PXR in contrast to other NRs.

Molecular mechanism of PXR action

After binding with its cognate ligand, PXR heterodimerizes with RXRα to bind DNA response elements in the promoter regions of their target genes and modulates their transcription (Ihunnah et al, 2011; Gao and Xie, 2012) (**Figure 7**). PXR can bind to a variety of DNA response elements containing two copies of the half site consensus sequence AG(G/T)TCA with various spacing, which includes direct repeats DR-3, DR-4, and DR-5, and everted repeats ER-6 and ER-8 (Orans et al, 2005). The two most important PXR target genes, multidrug resistance protein 1 (MDR1) and cytochrome P450 3A4 (CYP3A4), contain DR-4/ER-6 (Geick et al, 2001) and DR-3/ER-6

(Blumberg et al, 1998, Goodwin et al, 1999) in their promoter regions, respectively. PXR is also capable of recruiting a host of coactivators which includes members of the p160 family of coactivators such as steroid receptor coactivators 1 (SRC-1), TIF/GRIP (SRC-2), and peroxisome proliferator activated receptor gamma coactivator 1a (PGC-1a) (Li et al, 2005; Mangelsdorf and Evans, 1995; McKenna et al, 1999). The DBD of PXR facilitates DNA binding specificity via two highly conserved zinc finger motifs as well as a P-Box motif and D-Box motif which allow the receptor to target and bind its xenobiotic response elements (XREs) located in the 5′ promoter region of PXR target genes (Umesono et al, 1989).

Physiologic functions of PXR

Many xenobiotics and endobiotics, as well as their metabolites can activate PXR. Subsequently, the activated PXR regulates the transcription of key enzymes involved in the metabolism of xenobiotics and endobiotics. Although roles of PXR in xenobiotic and endobiotic gene regulation has been descriptive, it remains to be determined whether the regulatory functions of PXR can be taken advantage of in preventing and treating human diseases. Several emerging avenues of research have revealed novel and mostly unanticipated roles for PXR in inflammation, bone homeostasis, vitamin D metabolism, energy homeostasis, endocrine disruption, T lymphocyte function and cancer (Staudinger et al, 2001; Stedman et al, 2005; Saradhi et al, 2006; Tabb and Blumberg, 2006; Zhou et al, 2009; Dubrac et al, 2010; Konno et al, 2008; Lichti-Kaiser et al, 2009; Gao and Xie, 2012) (**Figure 8**).

- **PXR function in xenobiotic metabolism**

PXR plays an important role in the transcriptional regulation of various genes involved in xenobiotic detoxification (Synold et al, 2001; Maglich et al, 2002). PXR can be activated by a diverse set of xenobiotic chemicals, leading to activation of Phase I (cytochrome 450), Phase II (glutathione S-transferase, GST; sulfotransferases, SULT and uridine 5'-diphosphate glucuronosyltransferases, UGT) drug metabolizing enzymes and Phase III (MDR1 and OATP2) (Goodwin et al, 2001; Dussault et al, 2001) drug

transporters (**Figure 7**). Further, gene knockout studies carried out in mice have also confirmed a role for PXR in regulating the metabolism of endogenous steroids, dietary and xenobiotic compounds (Staudinger et al, 2001; Xie et al, 2001). Thus, these studies define the role of PXR as a key mediator of an elaborate network of genes involved in the detoxification and clearance of xenobiotics. Nonetheless, in the era of poly-pharmacy, the regulation of drug metabolizing enzymes by PXR poses a severe problem of drug-drug interactions where the PXR inducer mediates the induction of CYP3A4 machinery and hence accelerates the metabolism of co-administered drug(s) and raises the serious health concerns (Kliewer et al, 1999; Kliewer, 2005).

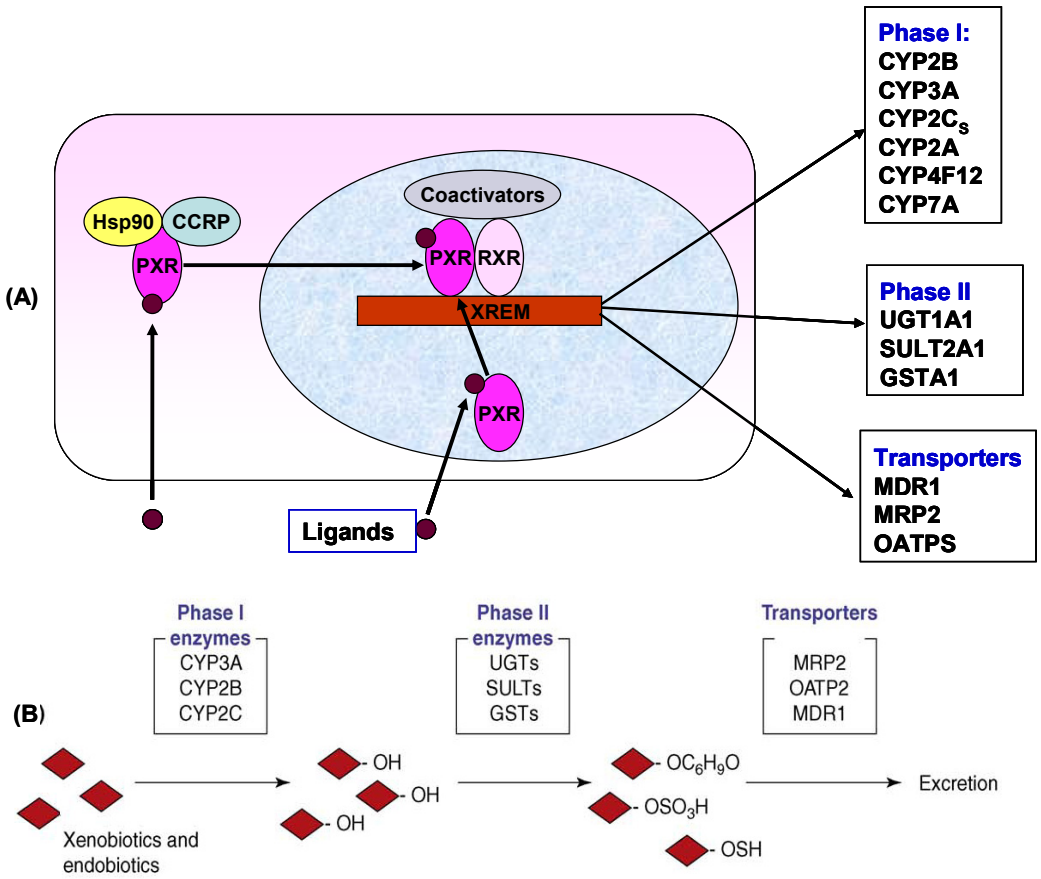

Figure 7: Schematic illustration of the activation mechanisms and target genes of PXR. ***(A)*** *Activation of PXR is purely ligand dependent. PXR target genes i.e Phase I, Phase II drug metabolizing enzymes and Phase III drug transpoter are grouped in separate boxes,* ***(B)*** *Transcription of genes encoding Phase I and Phase II metabolizing enzymes and drug transporters. Abbreviations: CYP, cytochrome P450; GST, glutathione S-transferase; SULT, sulfotransferase; UGT, UDP-glucuronosyltransferase. MDR, multidrug resistance*

protein; MRP, multidrug-resistance-associated protein; OATP, organic anion transporter polypeptide; XREM, xenobiotic responsive enhancer module; RXR, retinoid X receptor (Adapted and modified from Wada et al, 2009; Tolson and Wang, 2010).

Regulation of Phase I enzymes

PXR is capable of modulating the metabolism process through induction of the major Phase I cytochromes P450 enzymes (CYPs). CYPs are a superfamily of heme-dependent monooxygenases, which catalyze the first step of detoxification of aliphatic or lipophillic compounds (Poulos, 1988; Poulos, 2005). Like PXR, CYP450 enzymes are also highly expressed in the liver and intestine (Poulos, 2005) and convert target compounds into more soluble derivatives that are easier to excrete from the body use hydroxylation and/or oxidation reactions (Poulos, 1988). PXR has been shown to be activated by broad range of compounds and its activation leads to the transcription of CYP genes including CYP3A4, CYP3A23, CYP2B6, CYP2C8, CYP2C9, CYP2C19, and CYP1A (Lehmann et al, 1998; Drocourt et al, 2001; Sonoda et al, 2002; Ferguson et al, 2005; Miki et al, 2005; Kishida et al, 2008; Mueller et al, 2010).

Regulation of Phase II enzymes

PXR also can regulate the expression of Phase II drug metabolizing enzymes, including UDP-glucuronosyl transferase (UGT), sulfotransferase (SULT) and glutathione Stransferase (GST) enzymes [Xu et al, 2005]. Phase II metabolic transformations add sites that are ideal for Phase II conjugates and also add polar molecules onto xenobiotics and endobiotics, producing water soluble, non-toxic metabolites amenable to biliary and/or urinary excretion (Wang et al, 2002). Indeed, a major consequence of PXR mediated Phase II metabolic enzyme regulation is the metabolism and detoxification of bile acids, estrogens, thyroxin, xenobiotics, and carcinogens (Xie et al, 2003).

UGTs, SULTs and GSTs contribute extensively to metabolism by catalyzing the addition of a UDP-glucuronic acid, sulphate conjugates, glutathione (GSH) respectively to endo- and xenobiotics, enhancing their water solubility and elimination (Bian et al, 2007; Knight et al, 2008; Buckley and Klaassen, 2009; Bock, 2010; Cui et al, 2010). PXR activation by

carbamazepin, rifampicin, dexamethasone and phenytoin has been shown to transcription activator of several UGTs, including UGT1A1, UGT1A6, UGT1A3 and UGT1A4 (Xie et al, 2003; Gardner-Stephen et al, 2004; Trottier, 2006; Bock, 2010). Dexamethasone treatment has been shown to up-regulate SULT2A1 in human liver cells, but rifampicin treatment has been shown to have both inductive and suppressive effects (Duanmu et al, 2002; Fang et al, 2007; Fang et al, 2005). PXR activation by benzo[a]pyrene leads to subsequent up-regulation of several GSTs including GSTA1, GSTA2 and GSTM1 (Naspinski et al, 2008).

Regulation of drug transporter

Drug transporters work in concert with Phase I and II enzymes. The major xenobiotic transporters focus to PXR regulation include the ATP binding cassette family (ABC) proteins expressed in hepatocytes, enterocytes, kidney, and blood brain barrier that regulate cellular export of drugs (Lemmen et al, 2013). Examples of PXR target ABC transporters include the multidrug resistance 1 or P-glycoprotein (MDR1/P-gp), multidrug resistance associated proteins (MRP2, MRP3, MRP4, and MRP5), and breast cancer resistance protein (BCRP) (Schrenk et al, 2001; Assem et al, 2004; Mills et al, 2004; Jigorel et al, 2006). The organic anion transporting polypeptide family (SLC/OATP), which regulates drug and endobiotic influx/uptake into the liver, is also regulated by PXR (Hagenbuch and Meier, 2004). The known PXR target SLC/OATP genes include SLCO1A2/OATP1A2, SLCO1B1/OATP1B1, and SLCO1B3/OATP1B3. Finally, the organic ion transporter family, particularly the organic cation transporter SLC22A5/OCTN2, is proposed to have moderate PXR related regulation (Rae et al, 2001).

- **PXR function in endobiotic metabolism**

PXR was originally defined as xenobiotic receptor. Emerging evidences suggested its role in endobiotic metabolism and thus functioning as an 'endobiotic receptor' as well. Although the endobiotic functions of PXR have been appreciated, identification of physiologically relevant endogenous ligands for PXR will be beneficial in understanding the role of PXR as an endobiotic sensor. Like xenobiotics, it is activated by a diverse set of natural steroids

(pregnanes, androstanes and estranges) and endobiotic molecules (bile acids and their derivatives, oxysterols and vitamins). As an endobiotic receptor, new avenues of research have revealed the role for PXR in energy homeostasis through the regulation of glucose and lipids metabolism (Wada et al, 2009; Gao and Xie, 2010). In contrast, disruptions of energy homeostasis, such as those observed in obesity and diabetes, also have a major impact on drug metabolism (Hansen and Connolly, 2008).

- **PXR as a novel target for drug development**

PXR in glucose metabolism

Glucose is the necessary fuel source in central and peripheral tissues in human and important macromolecule to execute the gluconeogenesis, glycogenesis and glycogenolysis pathways. Glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK1) are the rate limiting enzymes in gluconeogenesis and glycogenolysis respectively (Foster and Nordlie, 2002; Quinn and Yeagley, 2005). The genes encoding for these enzymes are upregulated by glucagon and glucocorticoids. Glucagon increases the formation of intracellular cAMP, which activates protein kinase A (PKA) to stimulate cAMP-response element binding protein (CREB) that binds to and regulates the transcription of PEPCK1 and G6Pase (Herzig et al, 2001; Kurukulasuriya et al, 2003). Similarly, glucocorticoids induce PEPCK1 expression through glucocorticoid response element (Imai et al, 1990). On the other hand, insulin, suppresses gluconeogenesis by downregulating the transcription of G6Pase and PEPCK1 (Nakae et al, 2001). The forkhead transcription factor 1(FoxO1) functions as an activator of G6Pase and PEPCK1 in the absence of insulin but in the presence of insulin, FoxO1 is excluded out from the nucleus through phosphatidylinositol-3 kinase (PI3K)-Akt pathway, resulting in a repressed expression of G6Pase and PEPCK1 and decreased glucose production (Matsuzaki et al, 2003). PXR plays a role in hepatic gluconeogenesis where it reduces the expression of PEPCK1 and G6Pase in VP-hPXR transgenic mice (Zhou et al, 2006). PCN bound PXR downregulated G6Pase gene expression in wild type but not in PXR-/- mice. Studies by Kodama and colleagues suggested crosstalk between PXR, CREB and FoxO1 in

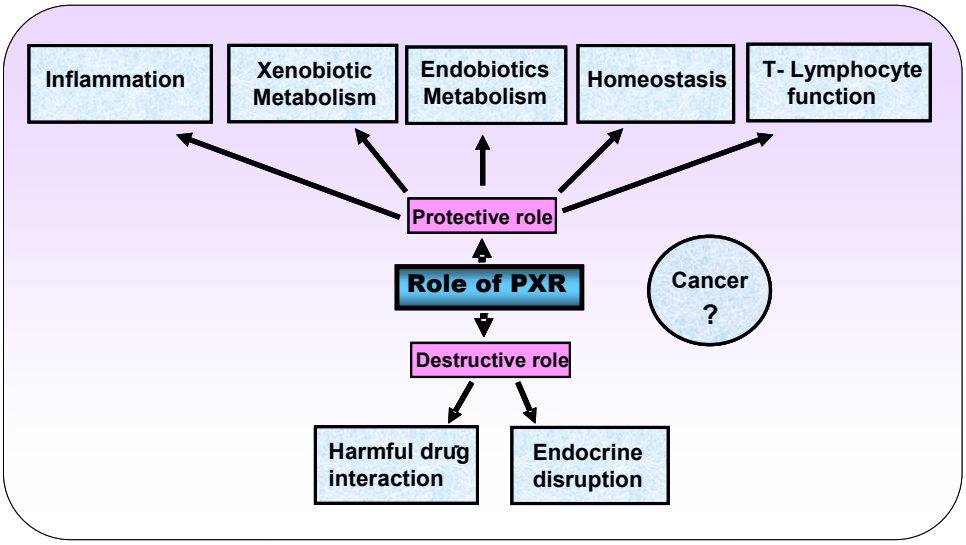

Figure 8: A simplified model depicting the role of PXR. *In addition to its established role in inducing xenobiotic metabolism and triggering drug-drug interaction, PXR has potential roles in various other biological functions including regulation of lipid and adrenal steroid homeostasis, retinoic acid metabolism, bone homeostasis, glucose metabolism, inflammation and cancer.*

regulating gluconeogenesis. It is shown that PXR directly interacted with CREB and prevented its binding to the G6Pase gene promoter. Thus, by forming a complex with phosphorylated CREB, ligand activated PXR repressed CREB mediated gene transcription. Additionally, ligand activated PXR interacted with FoxO1, which prevents FoxO1 from binding to Insulin Response Sequence (IRS), leading to suppression of G6Pase and PEPCK1 gene expression and gluconeogenesis (Konno et al, 2008). The hepatocyte nuclear factor 4α (HNF4α) also positively regulates gluconeogenesis with the nuclear receptor coactivator PGC-1α. Bhalla and colleagues showed that PXR could compete with HNF4α for PGC-1α and thus suppress gluconeogenesis (Bhalla et al, 2004).

PXR in lipid metabolism

PXR plays an important role in hepatic lipogenesis by activating sterol regulatory element binding protein (SREBP)- independent lipogenic pathway. PXR mediates its SREBP-independent pathway by activating the free fatty acid (FFA) uptake transporter CD36, PPARγ and other accessory lipogenic enzymes, such as stearoyl CoA desaturase-1 (SCD-1) and long-chain free fatty acid

elongase (FAE) (Bradbury 2006). Promoter analysis has established CD36 as a transcriptional target of LXR and PXR (Zhou et al, 2008a). The activation of PXR has been associated with the upregulation of PPARγ, a positive regulator of CD36. The cross-regulation of CD36 by LXR in SREBP dependent pathway and by PXR in SREBP-independent pathway suggests that this fatty acid transporter functions as a common target (Zhou et al, 2008a). PXR mediated lipid accumulation is also found to be associated with normal progression of liver regeneration in case of a hepatic injury (Dai et al, 2008).

PXR in bile homeostasis

Bile acids are the catabolic end products of cholesterol metabolism which are produced by the liver for the absorption of dietary lipids and fat soluble vitamins. Production of bile acid is under the stringent regulation because excessive bile acid may prove to be toxic to the body. In humans, bile acids bind to their specific nuclear receptor known as Farnesoid X Receptor (FXR, NR1H4) that upon activation regulates the expression of genes involved in bile acid biosynthesis and transport (Zhu et al, 2011). FXR exerts a feedforward-feedback mechanism on bile acid synthesis by suppressing CYP7A1 synthesis which is a key enzyme required for conversion of cholesterol to bile acids. Furthermore, data with PXR null mice also confirmed the role of PXR in CYP7A1 regulation other than the mechanism which is distinct from FXR's mechanism of activation (Sinal et al, 2000). Along with FXR, PXR has also been demonstrated as a lithocholic acid (LCA, a secondary bile acid) sensor. Studies have shown with animal model that activation of PXR protected against severe liver damage induced by LCA (Ma and Lu, 2008). Indeed, in one of the report, bile acid activated FXR can concurrently block synthesis of bile acids and activate PXR and subsequently PXR was identified as a target of FXR (Jung et al, 2006). Thus, co-ordinated activity of PXR and FXR constitutes an efficient mechanism for protection against bile acid induced liver damage and also the activation of PXR by selective activators provides a therapeutic utility in the treatment of biliary cholestasis (Kliewer and Willson, 2002).

PXR in vitamin metabolism and bone metabolism

Vitamin K2 is a critical nutrient for blood coagulation and functions as a mediator of bone formation (Tabb et al, 2003). Vitamin K2 is reported to activate PXR and stimulate its target gene expression. Studies done by Ichikawa et al, 2006, identified various PXR targeted genes (tsukushi, matrilin-2, osteopontin and CD-14 antigen) with bone related functions (Ichikawa et al, 2006). Similar studies done by Igarashi et al, 2007, showed the PXR activation by vitamin K2 also induced the expression of Msh homeobox 2, a oseteoblastogenic transcription factor (Igarashi et al, 2007). Thus, activated PXR shows an osteoprotective role and plays a novel role as a mediator of bone homeostasis (Tabb et al, 2003).

Similarly, vitamin D plays an essential role in the maintenance of calcium homeostasis and also in the maintenance of bone metabolism through its cognate receptor; Vitamin D receptor (VDR). In the target tissues, VDR upon binding to its ligand $1,25(OH)_2D$ forms a heterodimer with RXR and binds to specific vitamin D responsive element (VDREs) motifs on the 25-hydroxyvitamin D-24-hydroxylase (CYP24) gene which is responsible for the catabolism of 25(OH)D and $1,25(OH)_2D$. PXR shares almost 60% homology of the amino acids sequence in the DNA binding domain region when compared with vitamin D receptor (VDR) (Bertilsson et al, 1998). Due to homology of PXR with VDR, PXR forms a heterodimer with RXR and recognizes the same VDREs motifs present in CYP24 promoter and transactivates target gene expression resulting in various physiological effects including calcium/bone metabolism, cell growth maturation, rennin and insulin production (Pascussi et al, 2005; Holick, 2005). This crosstalk between PXR and VDR controls diverse physiological processes and has important implications in bone health.

PXR in retinoic acid metabolism

Retinoic acid (RA) is the metabolite of vitamin A that binds to and activates the retinoic acid receptor (RAR). RAR forms a heterodimer with RXR and activates the transcription of genes associated with cell differentiation (Park et al, 1999) and apoptosis (Altucci et al, 2001), leading to inhibition of cell growth. Therefore, RAs have been used or tested as anti-cancer agents in

several human cancer types (Soprano et al, 2004). Ligand activated PXR can induce expression of CYP3A and transporters such as MDR1A, MRP3 and OATP2, which accelerate RA metabolism (Wang et al, 2008b). It has been suggested that PXR antagonists might be useful in preventing RA resistance.

PXR in glucocorticoid and mineralocorticoid homeostasis

Studies by Zhai et al, 2007 showed the importance of PXR in adrenal steroid homeostasis. Both genetic and pharmacological activation of PXR increased plasma levels of corticosterone and aldosterone that subsequently activate adrenal steroidogenic enzymes, such as CYP11a1, CYP11b1, CYP11b2, and 3β-hydroxysteroid dehydrogenase (3β-HSD). Interestingly, the PXR transgenic mice exhibited normal ACTH secretion in pituitary and intact suppression of dexamethasone by corticosterone, indicating a functional hypothalamus-pituitary–adrenal axis in spite of severely disrupted adrenal steroid homeostasis. Several clinical studies reported that rifampicin increased steroid secretion in urine and may have resulted in misdiagnosis of Cushing's syndrome (Terzolo et al, 1995; Zawawi et al, 1996). Therefore, PXR has a potential to disrupt endocrine homeostasis, and it may be broadly implicated in drug-hormone interactions.

PXR in androgen metabolism

The androgen–androgen receptor signalling pathway plays an important role in the initiation and progression of prostate cancer. Accordingly, androgen deprivation has been the most effective endocrine therapy for hormone-dependent prostate cancer. PXR target genes, CYP3A and SULT2A1 are known to play a role in the metabolic deactivation of androgens. CYP3A catalyzes hydroxylation of testosterone and progesterone, leading to inactive hormones (Niwa et al, 1998). SULT2A1 is responsible for androgen sulfonation (Strott et al, 2002). Recent study showed that activation of PXR lowered androgenic activity and inhibited androgen-dependent prostate regeneration in castrated male mice that received daily injections of testosterone by inducing the expression of CYP3As and SULT2A1 (Zhang et al, 2010). In human prostate cancer cells, treatment with the PXR agonist rifampicin inhibited androgen

dependent proliferation of LAPC-4 cells but had little effect on the growth of the androgen-independent isogenic LA99 cells. Downregulation of PXR or SULT2A1 in LAPC-4 cells by shRNA or siRNA abolished the rifampicin effect, indicating that the inhibitory effect of rifampicin on androgens was PXR and SULT2A1 dependent. PXR may represent a novel therapeutic target to lower androgen activity and may aid in the treatment and prevention of hormone-dependent prostate cancer.

PXR in cancer and chemotherapy

PXR is expressed not only normal tissues but also in numerous types of cancerous tissues. Most significantly, the expression levels of PXR in these cancer tissues are usually higher than in non-neoplastic tissues (Qiao et al, 2013). PXR has been reported to be expressed in various malignant tissues like breast (Miki et al, 2006; Conde et al, 2008; Verma et al, 2009; Dotzlaw et al, 1999), osteosarcoma (Mensah-Osman et al, 2007), prostate (Chen et al, 2007), ovarian (Gupta et al, 2008; Yue et al, 2010), colon (Zhou et al, 2008b), endometrial (Masuyama et al, 2003; Masuyama et al, 2007), and esophageal (Takeyama et al, 2010) cancers where it is believed to be associated with decreased sensitivity to anticancer drugs (Chen et al, 2007; Gupta et al, 2008; Mensah-Osman et al, 2007; Masuyama et al, 2007), and drug-drug interactions (Harmsen et al, 2007). Due to its ligand promiscuity, PXR can be activated by many anticancer drugs, including cyclophosphamide, tamoxifen, taxol, vincristine, and vinblastine (Koyano et al. 2002; Poso and Honkakoski, 2006; Smith et al, 2010; Synold et al, 2001). Moreover, cancer patients are usually treated with combination therapy in addition to anticancer drugs, which also increases the possibility of drug-mediated PXR activation. Accordingly, recent studies support the idea that activation of PXR may compromise the effectiveness of anticancer drugs and contribute to acquire multi-drug resistance during anticancer chemotherapy (Chen, 2010). In PXR-expressing cancer cells such as prostate, colon, and endometrial cancer, PXR agonists can lead to increased resistance of cancer cells to chemotherapeutic agents, while the cancer cells can be sensitized to these anticancer agents by knockdown of PXR (Chen et al, 2007; Masuyama et al, 2007; Ouyang et al,

2010). On other hand, PXR mediated chemoresistance originating from inducible activity of PXR can also be blocked by pharmacologic intervention, leading to enhanced efficacy of chemotherapy (Pondugula and Mani, 2013). A recent study demonstrated that the reduced chemosensitivity of colorectal cancer cells to irinotecan was reversed by the PXR antagonist sulforaphane, while the activation of PXR decreased the effectiveness of this drug (Raynal et al, 2010). Thus the concept has been proposed to tackle resistance to anticancer drug by pharmacologically antagonizing PXR (Chen, 2010). The discovery and development of nontoxic, specific, and potent PXR antagonists will provide an effective way to improve the efficacy of anticancer drugs for the treatment of PXR-positive cancers.

Herbal drugs and PXR

The history of herbal medicines is as old as human civilization. The documents, many of which are of great antiquity, reveal that plants were used as medicines in China, India, Amazon Basin, Egypt and Greece, long before the beginning of the Christian era. India is very rich in natural resources and traditional knowledge. The use of plants as a source of herbal medicine has been an innate and vital aspect of India's healthcare system. The three Indian traditional systems of medicine (Ayurveda, Siddha and Unani) have identified more than 1,500 medicinal plants, of which nearly 700 are commonly used (Agarwal and Raju, 2006). According to an estimate by the World Health Organization (WHO), 70-80% of the world population, especially in developing countries, relies on traditional medicines, mostly plant drugs, for their primary healthcare needs (WHO, 2002; Agarwal and Raju, 2006). Recent reports reveal that the worldwide market of herbal medicines is estimated to be around US $80 to 100 billion, and it is projected to reach up to US $2,500 billion by the year 2010 (Mathur, 2003; Agarwal and Raju, 2006).

The ability of PXR to interact with such a diverse range of compounds makes it promiscuous in nature and this promiscuity lies in its LBD region. An enlarged, flexible and hydrophobic LBD is capable of fitting and accommodating diverse ligands. To date, a large number of ligands act in an agonistic manner to up regulate its gene expression but so far, few

antagonists have been described including ketoconazole (Wang et al, 2007; Mani et al, 2013), sulphoraphane (Zhou et al, 2007), ecteinascidin-743 (ET-743) (Synold et al, 2001) and coumestrol (Wang et al, 2008a) that have been reported. Because of the enormous diversity of PXR agonists and antagonists, it is well suited to accommodate its metabolic role as part of a protective system towards endobiotic and xenobiotic insult. Thus its ability to respond to a diverse array of chemically-distinct ligands including many endogenous compounds and xenobiotics makes PXR, a master regulator of the body. Some of the constituents of herbal medicines activates PXR are shown in **Figure 9**.

Activation of PXR by herbal medicines

Considerably less is known about the effect of complex chemical mixtures, such as herbal medicines on PXR activity. St. John's wort was the first herbal medicine shown to activate PXR (Moore et al, 2000; Wentworth et al, 2000). Since then, various other herbal medicines have also been identified as activators of PXR (**Figure 9**) (Negi et al, 2008). The following is an overview of our current knowledge on the effect of specific herbal medicines on PXR activity.

Hypericum perforatum (St. John's wort)

St. John's wort (*Hypericum perforatum*) is a herbal remedy widely used for the treatment of depression. The crude extract of St. John's wort contains a complex mixture of several active chemical constituents such as hypericin, quercetin, isoquercitin, biflavonoids, hyperforin, naphthodanthrones, procyanidines, Catechin, tannins, chlorogenic acid, etc. The principal compound responsible for antidepressant action of St. John's wort is hyperforin, the response of which is mediated primarily via inhibition of synaptic reuptake of neurotransmitters (serotonin, norepinephrine and dopamine) (Moore et al, 2000).

St. John's wort is an efficacious activator of PXR in cell-based reporter assays (Moore et al, 2000; Wentworth et al., 2000) and its activation induces hepatic drug metabolism (Moore et al, 2000; Gödtel-Armbrust et al, 2007). PXR

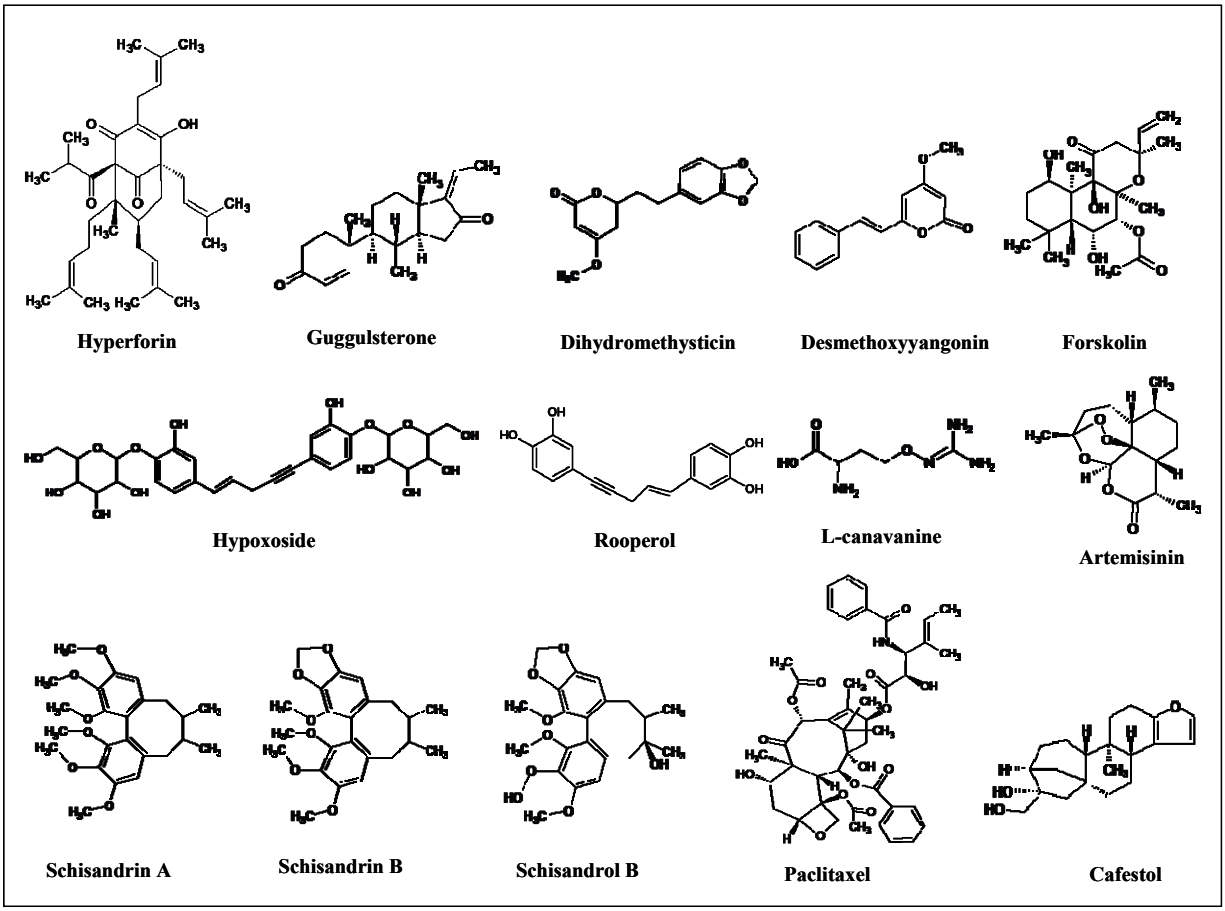

Figure 9: Chemical structure of some of the active constituents of herbal medicines that activate xenobiotic receptor, PXR. *This figure includes active constituents such as hyperforin (in St. John's wort), guggulsterone (in Mukul myrth), dihydromethysticin and desmethoxyyangonin (in Kava kava), forskolin (in Coleus forskohlii); hypoxoside (an inactive constituent in Hypoxis sp, which is converted into active metabolite rooperol in the gut), L-canvanine (in Sutherlandia sp), artemisinin (in Qing hao), schisandrin A, schisandrin B and schisandrol B (in Wu wei zi), Paclitaxel, also called taxol (in Pacific yew), and cafestol (in coffee).*

activation leads to up-regulation of CYP3A4 expression and an increase in metabolism of CYP3A4 substrates (e.g., cyclosporin). St. John's wort has been shown to alter the expression and function of P-glycoprotein in animal and human subjects, resulting in decreased concentrations of drugs in plasma (e.g., digoxin) (Durr et al, 2000; Fugh-Berman and Ernst, 2001; Gutmann et al, 2006). St. John's wort is responsible for a number of clinically relevant drug interactions that reduce the efficacy of several therapies, such as in transplantation, AIDS, cancer, etc. It has also been shown that St. John's wort enhances the metabolism of a variety of prescription drugs. These include oral

contraceptives, cyclosporine, digoxin, warfarin, indinavir and theophylline (Johne et al, 1999; Nebel et al, 1999; Breidenbach et al, 2000; Durr et al, 2000; Karliova et al, 2000; Mai et al, 2000; Piscitelli et al, 2000; Ruschitzka et al, 2000; Hennessy et al, 2002; Brazier and Levine, 2003). Several reports disclose around 80-85 drug-herbal interaction cases with St. John's wort, of which 54 cases were with the drug cyclosporine. Other drug categories interacting with St. John's wort are oral contraceptives (12 cases), antidepressants (09 cases), warfarin (07 cases) and one case each with theophylline, phenprocoumon and loperamide (Kast et al, 2002). A recent study identified 37 cases of interactions for St. John's wort with digoxin (13 cases), clopidogrel (06 cases), indinavir (08 cases), irinotecan (05 cases), antipsychotics (03 cases), tacrolimus (01 case) and with an anesthetic (01 case) (Gokhil and Patel, 2007). Induction of drug metabolizing enzymes by St. John's wort lowers the plasma concentration of co-administered prescription drug. Similarly, prolonged intake of herbal supplement (inducer) may result in sub-therapeutic concentrations of coadministered drug (Pal and Mitra, 2006). All these reports indicate that St. John's wort is somewhat a risky proposition when combined with drugs in the categories mentioned above. Most of these drugs are metabolized by phase-I enzymes, especially CYP3A, and these interactions are mediated by the involvement of PXR. St. John's wort compounds bind to PXR, and strengthen the interaction between PXR and the steroid receptor co-activator 1 (SRC-1) (Wentworth et al, 2000). The findings suggest that structurally modified drugs that do not bind and activate PXR will be safer anti-depressants since unfavourable drug interactions can be prevented during co-administration of other drugs.

***Commiphora mukul* (Mukul myrth)**

Mukul myrth (*Commiphora mukul*) is an Ayurvedic medicine used to treat hyperlipidemia (Beg et al., 1996). The stereoisomers E- and Z-guggulsterone are the active constituents of gugulipid that diminish hepatic cholesterol levels (Singh et al., 1990; Urizar et al., 2002). The therapeutic effect of guggulsterone is believed to be mediated through the antagonism of the nuclear receptor Farnesoid X Receptor (FXR) (Urizar et al, 2002).

Recently, by using promoter-reporter assays it was shown that guggulsterone activates PXR. More over, gugulipid and guggulsterone treatments stimulate CYP3A4 gene expression through PXR in hepatocytes. Although this herbal drug produces desirable therapeutic effects in lipid disorders, it may cause adverse drug-herbal interactions on combination therapy through activation of PXR. Protein interaction assays show that guggulsterone activates PXR by recruiting the co-activator SRC-1 (Brobst et al, 2004). The results of a well-controlled human study revealed that gugulipid interacts with prescription drugs such as diltiazem and propranolol and reduces their peak plasma concentrations (Dalvi et al, 1994). Studies have shown that guggulsterone not only modulate FXR and PXR but also modulate the activity of multiple nuclear receptors, including CAR, glucocorticoid receptor, progesterone receptor, mineralocorticoid receptor, androgen receptor and estrogen receptor (Dalvi et al, 1994; Burris et al, 2005; Ding and Staudinger, 2005c).

***Piper methysticum* (Kava kava)**

Kava kava (*Piper methysticum*) is a herbal remedy widely used as an anti-anxiety drug. It is also used to treat a wide variety of disorders including insomnia, stress, restlessness, muscle fatigue, gonorrhoea and vaginitis. The therapeutically important compounds in Kava kava are a group of structurally related lactones, collectively termed as kavalactones. The effects of kavalactones are believed to be mediated by g-aminobutyric acid (GABA) receptors in the central nervous system (CNS) (Jussofie et al, 1994). In addition to their effect on the CNS, kavalactones have been shown to modulate the activities of hepatic CYP enzymes. The activities of CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 are inhibited by kavalactones through a competitive mechanism (Mathews et al, 2002). Kavalactones: desmethoxyyangonin and dihydromethysticin, activate PXR in reporter gene assays but with lesser efficacy as compared to the classical PXR agonist rifampicin. These two kavalactones are responsible for the induction of CYP3A23 gene expression mediated via PXR activation. Kava kava exerts dual effects on CYP enzyme: (i) competitive inhibition, and (ii) induction of CYP3A

gene expression. Both these kava lactones affect the therapeutic efficacy of co-administered drugs such as levodopa, hydrochlorthiazide, promethazine, fluspirilen and biperiden (Ma et al, 2004). All these reports suggest that Kava kava would affect the metabolism of co-administrated drugs in a manner similar to St. John's wort.

Coleus forskohlii

The extract of plant *Coleus forskohlii* has been used as an Ayurvedic medicine to treat various disorders including hypothyroidism, hypertension, congestive heart failure, eczema, respiratory disorders and convulsions (Ammon and Muller, 1985). It is also known to be used as an anti-obesity agent, in view of its 'fat burning' property. The two diterpene compounds forskolin and 1, 9-dideoxyforskolin are the active constituents of *C. forskohlii*. Forskolin has both cAMP-dependent and cAMP-independent activities. Forskolin is widely used as a biochemical tool to activate adenyl cyclase and increase intracellular concentration of cAMP with subsequent activation of protein kinase A (PKA) signal transduction pathway (Seamon et al, 1981). It was shown that both forskolin and 1, 9 dideoxyforskolin (non-adenylcyclase activating analog) induce CYP3A gene expression in primary cultures of rodent hepatocytes (Sidhu and Omiecinski, 1996). Recent studies reveal that both these compounds are potent PXR activators (Ding and Staudinger, 2005a; Dowless et al, 2005), which work by displacing the co-repressor N-CoR and recruiting coactivators such as SRC-1. Forskolin, 1, 9-dideoxyforskolin and *C. forskohlii* extract produce PXR-mediated induction of CYP3A. In addition to this, activation of PKA signal transduction pathway potentiates PXR-mediated xenobiotic response as well as interaction between PXR and known co-activator proteins in cell-based assays. Interestingly, PKA and PXR signalling pathways have a synergistic effect on the induction of CYP3A gene expression in primary mouse hepatocyte cultures (Ding and Staudinger, 2005b). Consumption of *C. forskohlii* extract is not advised with anti-hypertensives and anti-coagulants due to the high potential for drug-herbal interactions.

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Hypoxis- and Sutherlandia

Hypoxis hemerocallidea and *Sutherlandia frutescens* are African herbal and traditional remedies used for HIV treatment (Mills et al, 2005a). Both the herbs are recommended by the South African Ministry of Health for HIV therapy but the molecular mechanisms underlying their therapeutic effects are not yet known. An important biologically active compound in *H. hemerocallidea*, hypoxoside, is responsible for the medicinal property, which readily converts to aglycone rooperol in the human gut (Albrecht et al, 1995). The principal constituents of *S. frutescens* include L-canavanine, GABA and D-pinitol. L-canavanine is responsible for the anti-viral effects.

Recent *in vitro* experiments indicate that crude extracts of both the herbs inhibit CYP3A4 activity and P-glycoprotein expression while activate PXR. The findings suggest that co-administration of these herbal drugs with other drugs results in inhibition of drug metabolism and transport during short term therapy. However, prolonged therapy results in induction of the same detoxification machinery. This observation is exemplified with an antiretroviral agent (Mills et al, 2005b). It remains to be determined if the active herbal compounds in the extracts affecting CYP3A4 and PXR are different from those having therapeutic activities and, if so, the compounds with potential therapeutic activities could be purified to treat patients and avoid unwanted side effects caused by CYP3A4 and PXR intervention.

***Artemisia annua* (Qing hao)**

Qing hao (*Artemisia annua*) is a Chinese herbal medicine used in treatment of malaria. The therapeutically active compound it contains is artemisinin (or qinghaosu). Artemisinin and some of its active synthetic derivatives (artemether, arteether and artesunate), collectively called artemisinin drugs, are used worldwide as effective and popular anti-malarial drugs because the malarial parasite has not yet developed resistance against these drugs (van Agtmael et al, 1999). But, then, allopathic drugs coadministered with artemisinin drugs will result in lowered plasma concentration and decreased therapeutic efficacy of the allopathic drugs due to induction of detoxification machinery (Hassan Alin et al, 1996; Ashton et

al, 1998). This inference is based on the fact that artemisinin activates PXR and CAR in reporter gene assays and also known to induce CYP2B6, CYP3A4 and MDR1 gene expressions in primary human hepatocytes and the human intestinal cell line LS174T (Burk et al, 2005). These findings reveal that artemisinin has a higher risk of potential drug-herbal interactions via induction of CYP3A4 and MDR1 through activation of PXR and CAR.

***Schisandra chinensis* (Wu wei zi)**

Wu wei zi (*Schisandra chinensis*), a traditional Chinese medicine, means 'five-flavor fruit' in Chinese since it has all the five basic flavors: salty, sweet, sour, pungent (spicy) and bitter. It has been already reported that Wu wei zi extracts and the active chemical constituents, including schisandrin A, schisandrin B and schisandrol B are potent PXR agonists in reporter gene assays. Its hepato-protective effects are clinically documented and used for treatment of many ailments such as infections, cough and thirst. The therapeutically active hepatoprotective and immuno-modulating constituents are the lignans, schisandrin, deoxyschisandrin, gomisins and pregomisin. In addition to PXR activation, these constituents efficaciously induce the PXR target genes CYP3A4 and CYP2C9 in primary cultures of human hepatocytes and promote *in vivo* drug metabolism (Mu et al, 2006). It has also been shown that Wu wei zi increases the metabolism of co-administered drug warfarin in rat. Although the herb has hepato-protective property, it may cause drug-herbal interactions due to induction of detoxification system.

***Glycyrrhiza uralensis* (Gan cao)**

Gan cao (*Glycyrrhiza uralensis*) is another traditional Chinese medicine that has anti-inflammatory and hepato-protective effects. It activates PXR (Mu et al, 2006) but it remains to be determined if it would induce PXR target genes. Like Wu wei zi, Gan cao also promotes *in vivo* drug metabolism and increases metabolism of warfarin in rats. The activation of PXR by this herb may also provide beneficial effects because of hepato-protective action. One study has shown that PXR activation promotes bilirubin detoxification in mice (Synold et al, 2001). These studies highlight the dual nature of PXR activation:

i) the promotion of drug metabolism, leading to potential drug interactions and therapeutic failure, and ii) activation of detoxifying systems to protect our bodies from toxic insults.

***Taxus brevifolia* (Pacific yew)**

Paclitaxel (Taxol), a member of the taxane family of anti-microtubule agents, is isolated from the bark of the Pacific yew (*Taxus brevifolia*) and widely used in the treatment of several types of cancer such as ovarian, breast and lung carcinomas. Paclitaxel is metabolically inactivated by the hepatic cytochrome P450 enzymes CYP3A4 and CYP2C8. Both these enzymes hydroxylate paclitaxel thereby abolishing the anti-mitotic properties of the drug. In addition, paclitaxel is excreted from the intestine via P-glycoprotein efflux pump protein encoded by the gene MDR1 (Synold et al., 2001). Gene-targeting studies have demonstrated that P-glycoprotein is responsible for excretion of 85% of the orally administered paclitaxel. Earlier reports have shown that paclitaxel is an effective inducer of CYP3A expression in primary cultures of rat and human hepatocytes (Kostrubsky et al, 1997, 1998). Furthermore, investigations employing cell-based reporter assays have shown that paclitaxel strongly activates human PXR (Synold et al, 2001; Nallani et al, 2004). Mammalian two-hybrid assays revealed that paclitaxel-bound PXR recruits nuclear receptor co-activators and displaces corepressors. The Northern blot analysis in this study showed that paclitaxel induces the expression of CYP2C8, CYP3A4 and MDR1 in hepatocytes (Synold et al, 2001). These results were confirmed *in vivo* by employing PXR-null mice (Nallani et al, 2003). In view of these findings, the herbal drug paclitaxel could be a suspect in drug-herbal interactions.

***Coffea arabica* (Cafestol)**

Coffee bean (*Coffea arabica*) is a herbal remedy widely used as CNS stimulant and also has diuretic effect. It contains several active chemical constituents such as caffeine, cafestol, kahweol, etc. Cafestol, a diterpene, is the most potent cholesterol-elevating compound in coffee beans and may also act as an anti-carcinogen. Cafestol is abundantly present in unfiltered coffee brews as compared to espresso coffee. It is already known that PXR can also

inhibit CYP7A1 expression (Staudinger et al, 2001), and is activated by a variety of xenobiotics, and thus protects the liver from toxic compounds (Goodwin et al, 2003). It is also known that certain bile acids can inhibit CYP7A1 expression independently of small heterodimer partner (SHP) via PXR (Kerr et al, 2002; Wang et al, 2002; Saradhi et al, 2006). In this background, cafestol has been shown to regulate metabolic and detoxification genes in mice. It activates mouse and human PXR as well as FXR in the reporter-based transactivation assay. Cafestol enhances interaction of PXR with nuclear receptor coactivator SRC-1 and induces CYP3A4 promoter activity via PXR but to a lesser extent than its known ligand, rifampicin (Ricketts et al, 2007). Cafestol also induces the activity of several GST enzymes (Lam et al, 1982, 1987) and, therefore, is a potential suspect for drug-herbal interactions.

β- Carotene

β- Carotene belongs to the group of carotenoids and exhibits pro-vitamin A activity. Its major sources are green, yellow, orange and red vegetables. Tomatoes, spinach, carrots, apricot, grapefruit, cherry and papaya are rich in β-carotene. β-Carotene is endogenously present as several isomers: all trans–β, β´-carotene, the major β-carotene isomer, followed by 15-cis, 13 cis- and 9-cis –isomers (Stahl et al, 1992). Earlier studies adopting reporter cell assay have revealed that b-carotene is an activator of the human PXR even at physiological concentrations found in human plasma and organs. β-Carotene brings about PXR-mediated induction of drug metabolizing enzymes CYP3A as well as drug transporters MDR1 and MRP2 (Rühl et al, 2004). Induction of CYP3A genes and drug efflux proteins can increase the drug clearance and reduce the therapeutic efficacy of coadministered pharmaceutical drugs, ultimately causing drug-herbal interactions (Pal and Mitra, 2006).

Other herbal medicines

Various other herbal medicines have also been identified as activators of human PXR, as assessed by cell-based reporter assays (Chang, 2009). These include (**1**) aqueous extracts of various herbs in traditional Chinese medicines, such as *Rhei rhizoma* (da huang), *Radix angelicae Sinensis* (dang gui), and *R. astragali* (huang qi) (Mu et al, 2006); (**2**) Tanzanian plants, such as *Jatropha*

multifda, *Agauria salicifolia*, *Elaedendron buchananii*, *Turraea holstii*, *Clausena anisata*, *Sclerocarya birrea Sond*, *Cyphostemma hildebrandtii*, and *Sterculia africana* (van den Bout-van den Beukel et al, 2008); (**3**) Recent study showed that piperine, a major component extracted from the widely-used daily spice black pepper, could induce PXR-mediated expression of cytochrome P450 3A4 (CYP3A4) and multidrug resistance protein 1 (MDR1) in human hepatocytes, intestine cells, and a mouse model (Wang et al, 2013).

PXR-mediated drug-herbal interactions

Herbal ingredients are readily used by millions of people without prescription under the belief that anything natural is safe. Like allopathic (prescription) drugs, herbal medicines also have different pharmacokinetic and pharmacodynamic properties which ultimately lead to produce therapeutic responses, but sometimes cause adverse actions and/or drug-herbal interactions. The concurrent use of herbal medicines and conventional (prescription) drugs by patients suffering from different diseases has progressively increased. Co-administration of herbal medicines with conventional drugs increases the risk of undesirable interactions between the two. Recently, St. John's wort (*Hypericum perforatum*), an herbal drug traditionally used as a natural treatment for depression, represented a highlighted case that warranted its safety evaluation (Brazier and Levine, 2003). Many of the compounds present in herbal medicines can potentially interact with the co-administered conventional drugs, causing either serious side effects or decreased pharmacological effect of the conventional drugs of narrow therapeutic index. Although the consumption of herbal health supplements along with prescription drugs is increasing globally, adequate information is not available on the mechanisms and consequences of drug-herbal interactions.

Drug-herbal interactions can occur at the pharmaceutical, pharmacodynamic or pharmacokinetic (PK) levels (Beijnen and Schellens, 2004) but most of the interactions occur at PK level (Brazier and Levine, 2003) that involves changes in absorption, distribution, metabolism and excretion of the conventional drug, which in turn determine the bioavailability of the drug.

Binding of a herbal constituent as ligand to any of PXR activates or inhibits their transcriptional activity which would increase or decrease the metabolism or transport of the co-administered conventional drug(s) and lead to decreased therapeutic efficacy or increased toxicity of the drugs.

Molecular basis of drug-herbal interactions

The chemical constituents of herbs have the potential to interact with various classes of drugs. These interactions could be directly or indirectly mediated by induction or inhibition of enzymes involved in drug metabolism and drug efflux proteins (Maglich et al, 2002; Pal and Mitra, 2006). The primary mechanisms behind drug-herbal interactions involve either induction or inhibition of intestinal drug efflux pumps (efflux proteins such as P-glycoprotein and MRPs) and intestinal and hepatic metabolism by CYPs (Evans, 2000; Ioannides, 2002). PXR activation by various compounds modulates intestinal efflux proteins and intestinal and hepatic CYPs (especially CYP3A4) which results in altered drug concentrations in plasma, thereby, causing drug-drug interactions (Lehmann et al, 1998; Evans, 2000). Therefore, herbs which have the potential to modulate efflux proteins and CYP3A4 may cause drug-herbal interactions and alter bioavailability of therapeutic drugs (Fugh-Berman, 2000; Fugh-Berman and Ernst, 2001; Izzo and Ernst, 2001; Meijermanet al, 2006). Any inhibitory effect of herbs on efflux proteins and CYP3A4 may result in elevated level of plasma and tissue concentration of co-administered prescription drug that would lead to toxicity. On the other hand, the inductive effect may cause decrease in drug concentration that would lead to decrease in therapeutic efficacy and failure of treatment (Staudinger et al, 2001). Indirectly, PXR accounts for the breakdown of about half of the clinically used drugs by the activation of the main transcriptional regulators of CYP3A4 and P-glycoprotein which are extensively distributed in the human tissues such as liver, intestine, colon, lung, etc. (Blumberg et al, 1998). The enzyme CYP3A4 is involved in the metabolism of 50-60% of clinical drugs as well as compounds in herbal medicines. In addition to this, 25-30% of these compounds are metabolized by the CYP2B isoenzymes. The combined metabolic effects of CYP3A and CYP2B, upon their induction by xenobiotic

substrates such as compounds in herbal drugs, constitute a molecular basis for many drug-herbal interactions (Pichard et al, 1996). For example, if one drug activates PXR, it can encourage the elimination of other co-administered drugs that are also metabolized and eliminated by PXR-target gene products, thereby reducing the therapeutic efficacy of many drugs in combination therapy.

Pre-clinical modeling and prediction of PXR Activity

There are various methods, *in vivo* and *in vitro* to assess the pharmacological properties (therapeutic activity and safety) for screening of drugs. Due to lengthy experimental duration, high cost and intensive labor, only small number of drugs may be tested *in vivo* using animal models. In addition to this *in vivo* model offers limited predictive values because of species variations and other factors. Alternative methods such as *ex vivo* cell-based assays have gained more attention because of less time consumption, low cost, reproducibility of results and better adaptability for high throughput screening strategies (Naylor, 1999). In this context, a cell based screening approach for evaluating the therapeutic value and safety assessment of clinical or herbal drugs, xenobiotics and endobiotics are capable of being addressed at two main levels by engaging (i) PXR protein based or (ii) PXR-promoter based transcription assays (**Figure 1**).

Level 1. Drugs, xenobiotics and endobiotics that bind and activate PXR protein may be assayed to identify and eliminate the possibility of drug-drug, herbal-herbal, drug-herbal interactions during treatment regime.

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.

* * *

MATERIALS & METHODS

MATERIALS

All the necessary materials including chemical, reagents, bacterial and mammalian cell culture media, supplements and antibiotics, transfection reagents, plasticwares, herbal drugs, ligands, activators, kinase inhibitors, antibodies, enzymes, commercial kits, etc. are tabulated with details in **Table II**. Plasmids (recombinant constructs) are described in **Table III**. Primers used in this study are tabulated in **Table IV**.

Table II: List of all biochemical, reagents and supplies.

Bacterial and mammalian cell culture media and supplements		
Product name	**Company**	**Catalog. No.**
Agar	Himedia, INDIA	RM301
Ampicillin	Himedia, INDIA	RM645
Cell freezing medium	Sigma, St. Louis, MO, USA	C6164
Charcoal Stripped FBS	PAN biotech, GmbH, Germany	P30-2301
DMEM (high glucose)	Sigma, St. Louis, MO, USA	D7777
DMSO	Sigma, St. Louis, MO, USA	D2650
Escort III	Sigma, St. Louis, MO, USA	L3037
Escort IV	Sigma, St. Louis, MO, USA	L3287
FBS	PAN biotech, GmbH, Germany	3302
G418 disulfate salt	Sigma, St. Louis, MO, USA	A1720
HEPES	Sigma, St. Louis, MO, USA	H4034
Kanamycin	Himedia, INDIA	RM210
Luria-Bertani	Himedia, INDIA	M1245
Minimum Essential Medium	Sigma, St. Louis, MO, USA	56416C
PBS	Sigma, St. Louis, MO, USA	D-5652
PSA	Himedia, India	A002A
Serum free medium	Sigma, St. Louis, MO, USA	14610C
Sodium bicarbonate	Sigma, St. Louis, MO, USA	S5761
Sodium Pyruvate	Sigma, St. Louis, MO, USA	S8636
Trypsin-EDTA	Sigma, St. Louis, MO, USA	T3924
Plastic-wares		
0.5ml microcentrifuge tubes	Tarson, Kolkata, INDIA	500000
1.5ml microcentrifuge tubes	Tarson, Kolkata, INDIA	500010
0.5ml PCR tubes	Axygen, USA	AXY-PCR-05-L-C
1.5ml microcentrifuge tubes	Axygen, USA	MCT-150-R
2ml microcentrifuge tubes	Tarson, Kolkata, INDIA	500020
15ml falcons	Tarson, Kolkata, INDIA	546020
50ml falcons	Tarson, Kolkata, INDIA	546040
Cell-scrappers	Corning, NY, USA (Sigma)	CLS3020
Corning® cell culture flasks surface area 25 cm2	Corning, NY, USA (Sigma)	CLS430372
Corning® cell culture flasks surface area 75 cm2	Corning, NY, USA (Sigma)	CLS430725
Corning® cell culture flasks surface area 175 cm2	Corning, NY, USA (Sigma)	CLS431080

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® Costar® cell culture plates 48 well	Corning, NY, USA (Sigma)	CLS3548
Corning® Costar® cell culture plates 96 well	Corning, NY, USA (Sigma)	CLS3595
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
MicroAmp® Optical 96-Well Reaction Plate with Barcode	Applied Biosystems, International Inc., CA, USA	4306737
MicroAmp® Optical Adhesive Film	Applied Biosystems, International Inc., CA, USA	4311971
Micro tips (0.2-10 µl)	Tarson, Kolkata, INDIA	521000
Micro tips (2-200 µl)	Tarson, Kolkata, INDIA	521010
Micro tips (200-1000 µl)	Tarson, Kolkata, INDIA	521020
Micro tips (0.2-10 µl)	Axygen, USA	T-300
Micro tips (2-200 µl)	Axygen, USA	TE-204-Y-L
Micro tips (200-1000 µl)	Axygen, USA	TE-1004-B-L
PCR 0.5 ml tubes	Axygen, USA	PCR-05-C
PCR 0.2 ml tubes	Axygen, USA	PCR-02-C
PCR 0.2 ml tubes	Tarson, Kolkata, INDIA	B79001
Petridishes 35 mm	Tarson, Kolkata, INDIA	460035
Petridishes 100 mm	Tarson, Kolkata, INDIA	460095
Herbal drugs, ligands, activators, inhibitors		
Drug/ligand/Inhibitor	**Source**	**Catalog No.**
1,9-dideoxy forskolin	Sigma, St. Louis, MO, USA	D3658
5-Pregnen-3β-ol-20-one-16α-carbonitrile (PCN)	Sigma, St. Louis, MO, USA	P0543
Acacetin	Sigma, St. Louis, MO, USA	00017
AICAR	Sigma, St. Louis, MO, USA	A9978
Anacardic acid	Sigma, St. Louis, MO, USA	A7236
Anethole (trans)	Sigma, St. Louis, MO, USA	117870
Butein	Sigma, St. Louis, MO, USA	B178
C646	Sigma, St. Louis, MO, USA	SML0002
Camptothecin	Sigma, St. Louis, MO, USA	C9911
Capsaicin	Sigma, St. Louis, MO, USA	M2028
Catechins hydrate	Sigma, St. Louis, MO, USA	C1788
CITCO	Sigma, St. Louis, MO, USA	C6240
Colchicine	Sigma, St. Louis, MO, USA	C9754
Coumestrol	Sigma, St. Louis, MO, USA	27885
Curcumin	Sigma, St. Louis, MO, USA	C1386
Digitonin	Sigma, St. Louis, MO, USA	D141
Dorsomorphin	Sigma, St. Louis, MO, USA	P5499
Etoposide	Sigma, St. Louis, MO, USA	E1383
Eugenol	Sigma, St. Louis, MO, USA	E51791
Fisetin	Sigma, St. Louis, MO, USA	F4043
Forskolin	Sigma, St. Louis, MO, USA	F6886
FR180204	Sigma, St. Louis, MO, USA	SML0320

Genistein	Sigma, St. Louis, MO, USA	G6649
Glucagon	Sigma, St. Louis, MO, USA	G2044
Guggulsterone	Tocris Bioscience, Bristol, BS11 0QL, United Kingdom	2013
H89 dihydrochloride	Sigma, St. Louis, MO, USA	B1427
Hypericin	Tocris Bioscience, Bristol, BS11 0QL, United Kingdom	1520
Isoliquiritigenin	Sigma, St. Louis, MO, USA	I3766
Kaempferol	Sigma, St. Louis, MO, USA	K0133
LY294002	Sigma, St. Louis, MO, USA	L9908
MDL-12330A	Sigma, St. Louis, MO, USA	M182
Nicotinamide	Sigma, St. Louis, MO, USA	N3376
Piceatannol	Sigma, St. Louis, MO, USA	P0453
Quercetin hydrate	Sigma, St. Louis, MO, USA	337951
Resveratrol (trans)	Tocris Bioscience, Bristol, BS11 0QL, United Kingdom	1418
Rifampicin	Sigma, St. Louis, MO, USA	R8883
Silibinin	Sigma, St. Louis, MO, USA	S0417
Silymarin	Sigma, St. Louis, MO, USA	S0292
Sirtinol	Sigma, St. Louis, MO, USA	S7942
Staurosporine	Sigma, St. Louis, MO, USA	S5921
STO-609	Sigma, St. Louis, MO, USA	S1318
DL-Sulforaphane	Sigma, St. Louis, MO, USA	S4441
Taxol	Tocris Bioscience, Bristol, BS11 0QL, United Kingdom	1097
Verapamil	Sigma, St. Louis, MO, USA	V4629
Vinblastine sulfate	Tocris Bioscience, Bristol, BS11 0QL, United Kingdom	1256
Vincristine sulfate	Tocris Bioscience, Bristol, BS11 0QL, United Kingdom	1257
Enzymes		
Product name	**Company**	**Catalog. No.**
BamHI	NEB, England	R0136S
dNTP set	Fermentas Interanational Inc., Canada	R0181
EcoRI	NEB, England	R0101S
Kpn1	NEB, England	R0142S
Lysozyme	Sigma, St. Louis, MO, USA	L-6876
NotI	Fermentas Interanational Inc., Canada	ER0591
Pfu Polymerase	Fermentas Interanational Inc., Canada	EP0571
Ribonuclease A	Sigma, St. Louis, MO, USA	R6513
RNasin ribonuclease inhibitor	Promega, Madison, WI, USA	N21111
SalI	Fermentas Interanational Inc., Canada	ER0641
SYBR Green	Applied Biosystems, CA, USA	4367659
T4 DNA Ligase	Fermentas Interanational Inc., Canada	EL0015
T4 PNK	Fermentas Interanational Inc., Canada	EK0031
Taq DNA Polymerase	NEB, England	M0273L
XhoI	Fermentas Interanational Inc., Canada	ER0691
Antibodies (primary and secondary)		
Anti-mouse IgG HRP	Sigma, St. Louis, MO, USA	A4416
Anti-rabbit IgG HRP	Sigma, St. Louis, MO, USA	A0545
Anti-rabbit IgG-cy3	Sigma, St. Louis, MO, USA	C2306
Anti- human PXR rabbit polyclonal antibody	Generated in our laboratory (Saradhi et al, 2005a)	-

Anti-human β-actin antibody	Generated in our laboratory	-
Protein and DNA Standard Size Markers		
Prestained Protein Marker	Fermentas Interanational Inc., Canada	SM0671
Unstained Protein Marker	Sigma, St. Louis, MO, USA	SDS7
1Kb DNA Size Standard	Fermentas Interanational Inc., Canada	SM0311
100bp DNA Size Standard	Fermentas Interanational Inc., Canada	SM0241
Commercial Kits		
Annexin V FITC Apoptosis Detection kit	Calbiochem, USA	PF032
GenElute™ gel extraction kit	Sigma, St. Louis, MO, USA	NA1111
GenElute™ mammalian genomic DNA miniprep kit	Sigma, St. Louis, MO, USA	G1N10
Lipofectamine 2000	Invitrogen Life Tech., Carlsbad CA	11668019
Luciferase Assay System	Promega, Madison, WI, USA	E1501
Plasmid DNA mini prep kit	MDI Ambala, INDIA	MIPK50
RevertAid H Minus First Strand cDNA Synthesis Kit	Fermentas Life Sciences, USA	K1632
CB-X protein assay kit	G-Biosciences, St. Louis, MO, USA	786-12X
General Laboratory Chemicals		
3-Amino Phthalhydrazide (Luminol)	Biochemika Fluka	73660
Acetic Acid	Merck, INDIA	60006325001730
Acetone	Rankem, INDIA	A0110
Acrylamide	Sigma, St. Louis, MO, USA	A3553
Agar	Himedia, INDIA	RM 301
Agarose	Sigma, St. Louis, MO, USA	A9539
Ammonium persulphate	Sigma, St. Louis, MO, USA	A3678
β-Mercaptoethanol	Sigma, St. Louis, MO, USA	M7522
Boric Acid	Sigma, St. Louis, MO, USA	B6768
Briliant Blue G 250	Qualigens Fine Chemicals, INDIA	10401
Bromophenol Blue	Himedia, INDIA	RM117
BSA	Himedia, INDIA	RM105
Calcium Chloride	Sigma Aldrich, St. Louis, MO, USA	22231-3
Chloroform GR	Merck, INDIA	S13SF53306
Coomasie Briliant Blue R-250	Himedia, INDIA	RM344
Dextrose	Himedia, INDIA	RM077
Diethyl pyrocarbonate	Sigma, St. Louis, MO, USA	D5758
Di-Sodium Hydrogen Phosphate	Himedia, INDIA	RM1416
DTT	Sigma, St. Louis, MO, USA	D9163
EDTA disodium salt	Sigma, St. Louis, MO, USA	E5513
Equilibrated Phenol	Sigma, St. Louis, MO, USA	P4557
Ethanol	Merck, Germany	1009830511
Ethidium bromide	Himedia, INDIA	RM813
Formaldehyde	Ranbaxy, INDIA	F0070
Formamide	Qualigens Fine Chemicals, INDIA	24015
Freund's adjuvant complete	Sigma, St. Louis, MO, USA	F-5881
Freund's adjuvant incomplete	Sigma, St. Louis, MO, USA	F5506
Glycerol	Qualigens Fine Chemicals, INDIA	15455
Glycine	Sigma, St. Louis, MO, USA	G8898

Glycogen	Fermentas Interanational Inc., Canada	R0561
Guanidine hydrochloride	Sigma, St. Louis, MO, USA	G3272
Hoechst 33258	Sigma, St. Louis, MO, USA	86140-5
Hydrochloric Acid	Rankem, INDIA	H0070
Hydrogen Peroxide	Rankem, INDIA	H0120
IPTG	Sigma, St. Louis, MO, USA	I6758
Isopropanol	Rankem, INDIA	P0790
Lauryl Sulfate (SDS)	Sigma, St. Louis, MO, USA	L3771
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
Nickel NTA resins	G-Biosciences, St. Louis, MO, USA	786-281
N-Lauroyl Sarcosine sodium salt	Sigma, St. Louis, MO, USA	L9150
NP-40	Himedia, INDIA	RM 2352
ONPG	Sigma, St. Louis, MO, USA	N1127
Orthophosphoric acid	Qualigens Fine Chemicals, INDIA	29905
p-Coumaric acid	Sigma, St. Louis, MO, USA	C9008
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
Propidium Iodide	Sigma, St. Louis, MO, USA	81845
Protease Inhibitor Cocktail	Sigma, St. Louis, MO, USA	P8340
Saponin	Fluka BioChemika, Germany	47036
Sarcosine	Sigma, St. Louis, MO, USA	S7672
Skim milk powder	Titan Biotech Ltd., INDIA	651
Sodium Acetate	Sigma, St. Louis, MO, USA	S-2889
Sodium azide	Sigma, St. Louis, MO, USA	S2002
Sodium bicarbonate	Sigma, St. Louis, MO, USA	S5761
Sodium Chloride	Sigma, St. Louis, MO, USA	S5886
Sodium deoxycholate	Sigma, St. Louis, MO, USA	D6750
Sodium hydroxide	Rankem, INDIA	S0270
Sucrose	Sigma, St. Louis, MO, USA	S1888
TEMED	Sigma, St. Louis, MO, USA	T9281
Thiazolyl Blue Tetrazolium Bromide (MTT)	Sigma, St. Louis, MO, USA	M5655
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
Trypan blue	Sigma, St. Louis, MO, USA	T6146
Tween-20	Sigma, St. Louis, MO, USA	P5927
Urea	Sigma, St. Louis, MO, USA	U5378
Miscellaneous Materials		
Product name	**Company**	**Catalog No.**
Developer	Kodak, INDIA	4908216
Fixer	Kodak, INDIA	4908232
Membrane disc filter (0.22µ and 0.45µ)	MDI Ambala, INDIA	CN
Nylone membrane	MDI Ambala, INDIA	SNNPZ
PVDF Membrane	MDI Ambala, INDIA	SVF
Salmon sperm DNA (SS DNA)	Agilent Tech., USA	201190

Whatman Filter Paper 3MM	Whatman, USA	
Whatman Filter Paper No.1	Whatman, USA	100125
X-Ray Film	Kodak, INDIA	4910022
Parafilm	Fisher Scientific, USA	13-374-16

Table III: List of plasmids used in the present study.

Plasmids (Recombinant chimera)			
Plasmid	**Plasmid Details**	**Source**	**Reference**
pSG5-hPXR	Human PXR-1 gene sequences cloned into pSG5 mammalian expression vector	S. A. Kliewer, University of Texas Southwestern Medical Center, Dallas, USA	-
XREM-Luc (XREM-CYP-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	-
p-7975/7013-Tk (MDR-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	Geick et al, 2001
pVP16, pVP16-hPXR, pM (Gal4-DBD), GAL4-DBD-PBP, GAL4-DBD-SRC1, pFR-Luc	Mammalian two-hybrid expression vector for protein-protein interaction study. GAL4-responsive luciferase reporter gene vector used in mammalian two-hybrid assay	Jeff Staudinger, Department of Pharmacology and Toxicology, University of Kansas, USA	Ding and Staudinger, 2005a
pSV-βGal	β-galactosidase gene cloned in pSV vector	Jeff Staudinger, Department of Pharmacology and Toxicology, University of Kansas, USA	Ding and Staudinger, 2005a
pcDNA3.1	Mammalian expression vector containing the neomycin resistance gene	Invitrogen, Life Technologies, USA	-
pGL3-Basic Vector	Promoter-less basic luciferase reporter vector	Promega, Madison, WI, USA	-
p-1096/+43 Luc, p-594/+43 Luc, p-497/+43 Luc, p-397/+43 Luc, p-315/+43 Luc, p-197/+43 Luc, p-197/-83 Luc	Human PXR proximal promoter-reporter constructs (proximal PXR promoter fragments cloned in pGL3-Basic vector)	Generated in our laboratory	Saradhi, 2008

Table IV: List of primers used in the present study.

Primers			
Primer used for semi-quantitative RT-PCR and real time qPCR			
S.No.	**Primer name**	**Direction**	**Sequences (5′-3′)**
1.	PXR (human)	Forward	AGGATGGCAGTGTCTGGAAC
		Reverse	AGGGAGATCTGGTCCTCGAT
2.	CYP3A4 (human)	Forward	AGATCAATGGGATGTTCATTCC
		Reverse	CTTCTTGCTGAATCTTTCAGG
3.	MDR1 (human)	Forward	TGATGCTGCTCAAGTTAAAGG
		Reverse	CTTCAGTAGCGATCTTCCCA
4.	GAPDH (human)	Forward	GGCCTCCAAGGAGTAA
		Reverse	AGGGGAGATTCAGTGTGGTG
Primer used for characterization of PXR-promoter stable cell lines			
S.No.	**Primer name**	**Direction**	**Sequences (5′-3′)**
1.	-1096 F (human)	Forward	CGGGATCCAGGAAATCCCAGCCTCAAGTC
2.	-497 F (human)	Forward	CGGGATCCACGCGTTCAAAGTGGTGGGGTCAC
3.	+43 R (human)	Reverse	GAATTCAAGCTTTCCTCTTCCCGTCCTAGTCA
4.	Luc	Reverse	CAGCGGATAGAATGGCGC
5.	GAPDH (human)	Forward	CGAGATCCCTCCAAAATCAAG
		Reverse	GTCTTCTGGGTGGCAGTGAT

METHODS

Bacterial strains and growth conditions

E. coli strains DH10β and BL21 (DE3) were used for the amplification of plasmid DNA and overexpression of recombinant proteins respectively. *E. coli* strains harboring the desired plasmids or overexpressing the recombinant protein were grown in Luria-Bertani (LB) media (supplemented with 50 µg/ml kanamycin or 100 µg/ml ampicillin wherever needed) either at 30^{o}C or 37^{o}C as per the requirement.

Preparation of competent cells and bacterial transformation

The competent *E. coli* cells *(DH10β and BL21)* were prepared essentially by $CaCl_2$ method, as mentioned in Sambrook et al, (1989) and described briefly below. The *E. coli* cells were streaked on LB agar plate to obtain single colonies.

Five ml of LB medium was inoculated with a single colony and grown overnight at 37ºC with vigorous shaking at 250 rpm. 1 ml of this overnight culture (1% inoculum) was inoculated into 100 ml of LB medium and grown at 37ºC with shaking, until the OD_{600} is 0.3-0.4. The culture was transferred to a 50 ml Oakridge tube and centrifuged at 5,000 rpm for 5 min at 4ºC. The supernatant was discarded. The pellet was gently resuspended in 50 ml of freshly prepared ice-cold $CaCl_2$ solution (100 mM) and incubated on ice water for 30 min. The cells were pelleted by centrifugation at 5,000 rpm for 10 min at 4ºC, and were resuspended in 1/10 the original volume of ice cold 100 mM $CaCl_2$ and kept on ice for 2-3 h. Finally ice-cold glycerol was added drop wise with intermittent gentle mixing to the final concentration of 15% and stored in 100 µl aliquots at -80ºC for further use.

For bacterial transformation 100 µl of competent cells (*E. coli* cells) were used for each transformation. At first, the frozen cells were thawed on ice and 10 to 100 ng of DNA was added and incubated on ice for 45 min. The cells were subjected to heat shock at 42ºC for 90 s and further chilled for 5 min on ice. Then 1 ml of LB was added to it and incubated at 37ºC with gentle shaking for 1 hour. Subsequently, 100 µl of cells were plated on LB agar plates containing appropriate antibiotics and grown for 12 to 14 h at 37ºC to obtain the transformed cells. As a control, *E. coli* cells without addition of plasmid vector were treated under similar conditions and plated onto LB agar plates.

Mini-scale plasmid DNA preparation by alkaline lysis method

Plasmid DNA in small scale (10-20 µg) was prepared by alkaline lysis method as described previously (Sambrook et al 1989), with minor modifications. *E. coli* cells inoculated in 3-5 ml of LB medium containing appropriate antibiotics were grown overnight (12-16 h) with vigorous shaking at 37ºC. Cells were harvested by centrifugation at 12,000 rpm for 1 min and cell pellet was resuspended in 100 µl of resuspension solution [50 mM glucose, 25 mM Tris-Cl (pH 8.0), 10 mM EDTA (pH 8.0) and 20 µg/ml RNAase A] by thorough vortexing. Then 200 µl of lysis solution (0.2 N NaOH and 1% SDS) was added, mixed gently by inversion (6-8 times) and incubated at room temperature for less than 5 min. To this 150 µl of neutralization solution (3 M

potassium acetate and 2 M glacial acetic acid) was added, mixed by gentle inversion and cell debris was cleared by centrifugation at 12,000 rpm for 15 min. The clear supernatant was extracted with equal volume of phenol: chloroform (1:1) mixture by centrifugation at 12,000 rpm for 5 min. The upper aqueous phase was separated and re-extracted with chloroform by centrifugation at 12,000 rpm for 5 min. Then the upper aqueous phase was carefully separated and mixed with 2 volumes of absolute alcohol and incubated on ice for 15 min. The precipitated DNA was collected by centrifugation at 12,000 rpm for 10 min and washed twice with 500 µl of 70% ethanol. Finally, the plasmid DNA was pelleted by centrifugation, air dried and resuspended in water or TE buffer [10 mM Tris-Cl (pH 8.0), 1 mM EDTA].

Isolation of genomic DNA

Genomic DNA was isolated from HepG2, Hepx-1096/+43 and Hepx-497/+43 cell lines by using GenElute™ mammalian genomic DNA miniprep kit according to the manufacturer's protocol. In brief, cells were grown in 100 mm culture plates. When reached to 80-90% confluency cells were trypsinized and pelleted by centrifuging for 5 min at 1000 rpm. The pellet was resuspended thoroughly in 200 µl of resuspension solution and 20 µl of RNase A solution was added to remove RNA contamination and incubated for 2 min at RT. Then, 20 µl of proteinase K solution (20 µg/µl) was added to the resuspended cells and incubated for 2 min at RT. To this 200 µl of lysis solution C was added, vortexed thoroughly and incubated at 70°C for 10 min. Then to this lysate, 200 µl of absolute ethanol was added and mixed thoroughly by vortexing 5-10 s. In the meantime, GenElute miniprep binding column in a collection tube was assembled and 500 µl of column preparation solution was added to the binding column and centrifuged at 12,000 rpm for 1 min. The flow-through was discarded and ethanol-lysate mixture was loaded onto binding column and centrifuged at 10,000 rpm for 1 min. The binding column was transferred into a fresh collection tube and washed with 500 µl of wash solution by centrifugation at 10,000 rpm for 1 min. Again, the binding column was transferred into a fresh collection tube and washed with 500 µl of wash solution by centrifugation at 13,000 rpm for 5 min. The flow-

through was discarded and again re-centrifuged at 13,000 rpm for 5 min to ensure the removal of wash solution completely. The binding column was transferred to a fresh collection tube and genomic DNA was eluted with the addition of 200 µl of elution solution to the binding column and centrifugation at 10,000 rpm for 1 min. The elute containing pure genomic DNA was stored at -20°C in aliquots till further use.

Quantification of plasmid and genomic DNA or RNA

The quantity of isolated plasmid and genomic DNA or RNA was determined by measuring the OD at 260 nm and 280nm in spectrophotometer. The reading at 260nm allows calculation of the concentration and purity of nucleic acid. An OD of 1.0 corresponds to approximately 50 µg/ml for double stranded DNA, 40 µg/ml for single stranded DNA/RNA and ~20 µg/ml for single stranded oligonucleotides. The ratio between the reading at 260nm and 280nm (OD_{260}/OD_{280}) provides an estimate of the purity of the nucleic acids. Pure preparation of DNA has an OD_{260}/OD_{280} value of 1.8. If there is contamination with protein or phenol, the OD_{260}/OD_{280} will be significantly less than 1.8. On the other hand, high quality of RNA has an OD_{260}/OD_{280} value of 2.0. The concentration of DNA or RNA was calculated with the following equation:

DNA concentration (ng/µl) = OD at 260 nm x 50 x dilution factor

Agarose gel electrophoresis

Agarose gel electrophoresis was performed as described by Sambrook et al (1989). For plasmid DNA samples, 1% agarose was melted in 1X TAE buffer by heating, cooled to 37°C and ethidium bromide (0.5 µg/ml) was added before casting the gel on the gel tray. One-sixth volume of DNA gel loading buffer was mixed with samples and loaded onto the wells. The electrophoresis was performed at 5V/cm in TAE buffer (40 mM Tris-Acetate, 1.0 mM EDTA, pH 8.0) and the plasmid DNAs were visualized, on an UV transilluminator.

Maintenance of mammalian cell-cultures

American Type Culture Collection (ATCC) cell lines COS-1 (kidney cell line from African green monkey), HepG2 (human hepatocellular carcinoma cell

line), were obtained from National Centre for Cell Science repository (Pune, India). Human intestinal colon adenocarcinoma cell line LS180 directly purchased from ATCC. HepXR cell line (HepG2 cells stably transfected with human PXR) were generated in our laboratory and described earlier (Saradhi et at, 2005b). These cell lines are immortalized and also known as transformed cells i.e. cells whose growth properties have been altered. For the convenience, the term cell(s) were used at many places instead of cell line(s). COS-1 and HepG2 cells were grown in DMEM supplemented with 10% FBS, 100 µg/ml penicillin and 100 µg/ml streptomycin (complete medium), according to ATCC recommendation while LS180 cells were grown in MEM with 10% FBS supplemented with 1X sodium pyruvate plus antibiotics. HepXREM (HepG2 cells stably transfected with human PXR and XREM-CYP-Luc) cell line, PXR-promoter stable cell lines; Hepx-1096/+43 and Hepx-497/+43 (HepG2 cells stably transfected with human PXR proximal promoter-reporter constructs, p-1096/+43 Luc and p-497/+43 Luc respectively) were generated and characterized in the present study. HepXREM, Hepx-1096/+43, Hepx-497/+43 and HepXR cell lines were maintained in the same DMEM complete medium containing 400µg/ml of G418 (selective medium). The cultures were maintained in a humidified incubator maintained at 5% CO_2 and 95% air atmosphere at 37°C. The cells were routinely maintained in monolayer culture.

For regular subculturing, cells were detached from culture plates by trypsinization with trypsin–EDTA [0.5 g/l porcine trypsin and 0.2 g/l EDTA.4Na, in Hank's balanced salt solution with phenol red]. During cell trypsinization, medium was discarded and cells were washed with sterile PBS. Then 1 ml of trypsin-EDTA solution was added to the 100 mm plate for a few minutes and removed. The cells were allowed to stand for a few minutes in CO_2 incubator and then observed under phase contrast microscope to check if the cells have rounded up and detached. Once trypsinized, complete medium was added to re-suspend and recover the cells by gentle pipetting and further sub-cultured in fresh plates.

For cryopreservation, trypsinized scraped cells were collected in a centrifuge tube and pelleted by centrifugation at 1,000 x g for 5 minutes. The supernatant was discarded and the cell pellet was gently suspended in

complete medium. Depending on the cell line, 5% or 10% DMSO was added to medium containing the cells. Then the cell suspension was transferred to 1 ml cryovials and incubated at -80ºC for overnight. The cooling rate of 1ºC per minute was achieved by using Tarson cryo-cooler with 100% isopropyl alcohol. After 24 h the cryovials were transferred to the liquid nitrogen container (-196ºC) for long term storage.

Liposome-mediated transient transfections

Transient DNA transfections in COS-1 and HepG2 cells were performed using Escort III & IV reagent respectively at ~60-70% confluency according to the instructions provided by the manufacturer. Sometimes if needed, transfection was also done using Lipofectamine 2000 reagent which gives better transfection efficiency. The day before transfection, exponentially growing cells were seeded into 24 well, 12 well plate and 35 mm tissue culture dishes as per the experimental requirement in complete medium. The following day, cells were incubated in DMEM only (without serum and antibiotics) (250 µl/well for 24-well plate, 500 µl/well for 12-well plate and 1.0 ml for 35mm plate) for 30 min prior to transfection. For transfection, appropriate amount of DNA (250 ng/well of 24-well plate, 250-500 ng/well of 12-well plate and 0.5-1 µg for 35 mm plate) was added to 25 µl, 50 µl and 100 µl of DMEM only respectively in a microfuge tube and incubated at RT for 5 min. Simultaneously, transfection reagent (4 µl transfection reagent for 35 mm, 2.0 µl/well for 12-well and 1.0 µl/well for 24-well plate) was added to 100 µl, 50 µl and 25 µl of DMEM only respectively in another microfuge tube and incubated at RT for 5 min. The contents of two tubes were mixed properly by gentle pipetting and further incubated at room temperature for 30-45 min. Following the incubation, the DNA-lipid complex was added to the culture plate and incubated at 37ºC in CO_2 incubator for 12-15 h (Escort III/IV) or 7-8 h (Lipofectamine 2000). After transfection period the medium was replenished with complete DMEM medium and cells were allowed to express the proteins of transfected plasmids for 24 h. If required transfected cells were also treated with herbal drugs and incubated further for 24-48 h in CO_2 incubator,

depending on experimental requirements. These transiently transfected cells were further processed according to experimental plans.

Preparation of luciferase mammalian cell culture lysis reagent

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

Luciferase reporter gene assay

Firefly luciferase assay is widely used as a reporter for studying gene regulation and function, and for pharmaceutical screening. The firefly luciferase is 62 kDa monomeric protein and its enzymatic activity provides a sensitive, rapid means to assay transcriptional activity of regulated activation sequences of DNA when fused to the protein coding sequence of the luciferase gene. The luciferase enzyme catalyzes ATP-dependent D-luciferin oxidation by oxygen into oxyluciferin with emission of light.

HepG2 cells were seeded into culture plates at 70-80% confluency and follow up transfection according to the manufacturer's protocol. Following transfection period (12 h), cells were supplemented with complete DMEM and subsequently, the cells were treated with different drugs and further incubated for 24 h. To determine the reporter gene activities, cells were harvested and luciferase assays were performed according to the kit protocol (Promega, Madison, WI, USA). In brief, at the end of treatment period, the culture medium was discarded and cells were washed twice with 1 ml of PBS by gentle swirling to remove any residual growth medium. Then cells were lysed by adding 100 µl or 200 µl of reporter lysis buffer (RLB) to each well of 24-well or 12-well culture plates respectively and allowed to remain in contact with cells for 10 min. Then cell lysate was prepared by manually scraping the cells with a rubber police man. A homogenous cell lysate was obtained by vortexing the sample for 10-15 s. To clear the cell extract, lysate was centrifuged at 12,000 rpm for 1 min. The clear supernatant was separated and used for the reporter gene assay. If required the samples were stored at -80°C. Prior to determining

the luciferase reporter gene activity, whole cell lysate and luciferase assay reagent were equilibrated to the room temperature and luminometer was programmed to perform a 3 s pre-measurement delay followed by a 15 s measurement period for each reporter assay. Then, 25 µl of luciferase assay reagent (luciferin) was mixed with 5.0 µl of cell lysate and luminescence was measured in the TD-20/20 DLReady™ luminometer (Turner Designs).

For determining the β-galactosidase activity, 50 µl of cell lysate was mixed with 50 µl of β-galactosidase assay 2X buffer (200 mM sodium phosphate buffer pH 7.4, 2 mM MgCl2, 100 mM β-mercaptoethanol and 1.33 mg/ml o-Nitophenyl-beta-galactopyronoside). Samples were incubated at 37°C until a faint yellow color was developed (2-3 h) and the reaction was stopped by adding 150 µl of 1 M sodium carbonate. The contents were mixed by pipetting and transferred to a flat bottom 96-well plate. Absorbance of the samples was recorded at 415 nm using a microplate reader (Bio-Rad, CA, USA).

Optimization of promoter-reporter assay for HepXREM and PXR-promoter reporter (Hepx-1096/+43 and Hepx-497/+43) stable cell line in 48 well culture plates

To standardize an efficient and reproducible protocol for promoter-reporter assay, cells were propagated in 48-well culture plate with complete DMEM containing 5% FBS and antibiotics then allowed to proliferate up to ~ 60% confluency. Then cells were treated with different drugs for 24 h. All the drugs used were prepared in drug solvent DMSO:ethanol (in 1:1 ratio). After 24 h of incubation period, luciferase reporter activity was determined. Briefly, to determine the luciferase activity, medium from each well was removed and washed twice with PBS. Then 50 µl of RLB (reporter lysis buffer) was added in each well and incubated the plate on ice for 15-20 min followed by repetitive pipeting to ensure complete lysis. 5.0 µl of cell lysate was taken in 1.5 ml microcentrifuge tube and then mixed with 25 µl luciferase assay substrate, luciferin. The luciferase activity was measured by placing the reaction in a pre-programmed TD-20/20 DLReady™ luminometer (Turner Designs, USA) and

the activities expressed as 'relative luciferase activity' (Ausubel et al, 2002 and Naylor, 1999).

Isolation of total RNA from cultured mammalian cells

Isolation of total RNA from cultured cells was performed with TRI REAGENT™ (a mixture of guanidine thiocynate and phenol in a monophase solution), a single-step method reported by Chomczynski and Sacchi et al, (1987) for total RNA isolation. In short, after washing twice with PBS, cultured mammalian cells (propagated in 100 mm culture plate at 70-80% confluency) were lysed directly using 1 ml of TRI reagent per 100 mm culture dish. After homogenization and lysis, cells were allowed to stand for 5 min at RT. Then, 200 µl of chloroform was added to homogenized samples and vortexed vigorously for 15 s and allowed to stand for 10 min at RT. After that 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 200 µl of isopropanol to the aqueous phase, mixed and allowed to stand for 10 min at RT followed by microfugation at 12,000 rpm for 10 min at 4°C. Supernatant was decanted and the RNA pellet was washed with 1 ml of 75% ethanol. The samples were vortexed and microfuged at 10,000 rpm for 5 min at 4°C. After discarding the supernatant, RNA pellet was air dried. Then the RNA pellet was dissolved in appropriate volume of DEPC (diethylpyrocarbonate) treated water with mixing at 55-60°C for 5 to 10 min. The RNA samples were stored at -80°C until used for RT-PCR.

Preparation of lysate from the cultured mammalian cells

The cells were washed twice with PBS to remove culture media and then mechanically detached from the surface using a cell scraper. The cells suspended in PBS were collected in a microcentrifuge tube and centrifuged at 5,000 rpm for 2 min to pellet the cells. The cell pellet was lysed in lysis buffer (20 mM Tris pH 7.6, 0.5 mM DTT, 1 mM EDTA, 1 mM PMSF, 0.1% NP-40 and protease inhibitor cocktail) and incubated on ice for 30 minutes with intermittent tapping. After half hour of incubation, 5 M NaCl was added

dropwise to the final concentration of 400 mM with further incubation on ice for 30 min. The whole cell lysate was collected as supernatant following centrifugation at 12,000 rpm for 15 min at 4°C and aliquots were rapidly frozen at -80°C. Protein concentration of cell lysate was estimated by CB-X™ Protein assay kit (G-Biosciences) according to the manufacturer's protocol and subjected to western blot analysis.

Cytoplasmic and nuclear fractionation

For cytoplasmic and nuclear extract preparation, cells were resuspended in lysis buffer (20 mM Tris pH 7.6, 0.5 mM DTT, 1 mM EDTA, 1 mM PMSF, 0.1% NP-40 and protease inhibitor cocktail) and incubated for 30 min on ice with intermittent tapping. Cell lysates were pelleted by centrifugation at 12,000 rpm for 5 min at 4ºC and the supernatant (cytoplasmic fraction) was retained. Pellet were resuspended once in the same lysis buffer containing 0.4 M NaCl and incubated for an additional 30 min. After incubation for 30 min, nuclear fraction was collected by centrifugation at 18,000 rpm for 15 min at 4ºC and aliquots were rapidly frozen at -80 ºC. Protein in the samples was estimated by CB-X™ Protein assay kit (G-Biosciences) as per manufacturer's instructions and subjected to western blot analysis.

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

Polyacrylamide gel electrophoresis under denaturating condition (in the presence of 0.1% SDS) was performed according to Laemmli's method (Laemmli, 1970). The proteins were stacked at pH 6.8 in a stacking gel containing 4% acrylamide, 0.106% N, N'-methylene bisacrylamide, 0.125 M Tris-Cl (pH 6.8), 0.01% TEMED and 0.1% ammonium persulfate. The separating gel consisted of 8 or 10% acrylamide, 0.33% N, N'-methylene bisacrylamide, 0.375 M Tris-Cl (pH 8.8), 0.01% TEMED and 0.1% ammonium persulfate. The protein samples were electrophoresed in a running buffer composed of 0.025 M Tris-base, 0.192 M glycine (pH 8.3) and 0.1% SDS. Protein samples for electrophoresis were prepared in SDS-PAGE sample buffer containing 0.0625 M Tris-HCl (pH 6.8), 2% SDS, 10% glycerol and 5%

β-mercaptoethanol (Laemmli, 1970) and immersed in a boiling water bath for 5 min. After a brief spin, the samples were loaded directly on the gel. Standard molecular weight markers were electrophoresed alongside with the sample proteins to determine their molecular size.

Western blot analysis

For western blotting, equal amount of proteins were finally dissolved in SDS-PAGE sample buffer, denatured by heating at 95°C for 5 min and resolved on a 10% SDS-PAGE. Proteins were electro-blotted onto the polyvinyldifluoridine (PVDF) membrane using semi-dry transfer system (Amersham Biosciences, USA) or wet-transfer system (Life tech., invitrogen, USA). After protein transfer, the blot was blocked with 5% non-fat dry milk in TBST for 2 h at room temperature and then incubated overnight with PXR antiserum at dilution of 1:2,500 at 4°C. The membrane was then washed three times with TBST (TBS with 0.1% Tween-20) and incubated for 1 h with 1:10,000 dilution of horseradish peroxidase-conjugated anti-rabbit antibody. The bound antibody complexes were detected using the Enhanced Chemi-Luminescence (ECL) method.

Development of a stably transfected cell line constitutively expressing wild type human PXR and XREM-CYP-Luc reporter (HepXREM)

To obtain a stable cell line expressing functional wild type human PXR and the XREM-Luc promoter-reporter construct, HepG2 cells, a human liver cell line, were seeded in 35 mm culture dish in DMEM containing 10% FBS and antibiotics. At ~60% confluence, the cell medium was changed to DMEM only and were transfected with a mixture containing the XREM-CYP-Luc reporter construct, pSG5-hPXR expression plasmid and pCDNA.3-neo in a ratio of 10:2:1 using Lipofectamine 2000 reagent. After the cells had been exposed to the precipitated DNA for 8 h, the medium was removed and replaced with fresh DMEM containing 10% FBS and antibiotics. The cells were allowed to double once under non-selective conditions. After 24 h incubation, this medium was then replaced by DMEM containing 400 µg/ml G418

(selective medium). The medium was renewed every 2-3 days for about two weeks until small colonies were visible. After 2-3 weeks of selection period, individual colonies were isolated and further propagated under selective conditions. Positive clones that expressed both hPXR and XREM-Luc were identified by their ability to respond to human PXR-specific agonist, rifampicin in luciferase assay. The PXR stable cell line was further characterized by western blotting, RT-PCR, luciferase assay and immunocytochemistry and designated as HepXREM.

Development of stably transfected cell lines with PXR proximal promoter-reporter constructs p-1096/+43 Luc and p-497/+43 Luc (Hepx-1096/+43 and Hepx-497/+43)

For preparation of PXR-promoter integrated stable cell lines, PXR proximal promoter-reporter constructs, p-1096/+43 Luc and p-497/+43 Luc were selected on the basis of functional analysis of different deletion constructs of PXR promoter characterized in our laboratory (Saradhi, 2008). PXR proximal promoter-reporter constructs, p-1096/+43 Luc and p-497/+43 Luc were linearized with restriction enzyme SalI and BamHI respectively. DNA transfection was performed with Lipofectamine as per manufacturer's protocol. HepG2 cells ($2.4x10^5$) were seeded in 35 mm culture plate and co-transfected with PXR proximal promoter-reporter construct and a vector that contains neomycin resistance gene (pcDNA3.1) in 10:1 molar ratio. Cells were allowed to double (up to 80% confluent) in complete medium. Later, cells were supplemented with complete medium containing 400 µg/ml of G418 (selective medium). The medium was replaced every third day. After two weeks of selection period, proliferating individual colonies were isolated and further propagated in separate culture dishes under selective conditions. Individual clones were screened and compared for the stable integration and response reflected by luciferase activity of PXR promoter. To determine the PXR promoter activities, cells were harvested and luciferase assay was performed according to the established protocol and values for luciferase activity were recorded in luminometer (Turner Designs TD-20/20, Promega, Madison, WI, USA). The activity was expressed as relative luciferase activity (RLA). On the

basis of RLA value, the clones of stably integrated hepatic cell lines of PXR promoter-reporter constructs were selected and named 'Hepx-1096/+43' and 'Hepx-497/+43'.

Immunocytochemistry and fluorescence microscopy

For indirect immunodetection, HepG2, HepXR and HepXREM cells were seeded over sterile glass cover slips in 35mm culture plates at ~60% confluency and incubated in CO_2 incubator. After 24 h of incubation, the cells were washed thrice with 10 mM phosphate buffered saline (PBS) and then fixed with chilled methanol (-20°C) containing 5% (v/v) acetic acid for 20 min on ice. Following fixation, cells were incubated further for 5 min in ice-cold PBS containing 0.5% Triton X-100. The cells were then blocked in 3% bovine serum albumin (BSA) in PBS for 1 h. For immunodetection, the cells were incubated overnight with primary PXR polyclonal antiserum at a dilution of 1:500 at 4°C. After three washes with PBS, the cells were incubated with Cy3-conjugated sheep anti-rabbit IgG (1:300 dilution) prepared in PBS with 3% BSA. Hoechst-33342 was included with the secondary antibody preparation for staining and visualization of the nucleus. The cells were rinsed three times in PBS and the cover slips were mounted on a glass slide with 20% glycerol in PBS and visualized under the fluorescence microscope. The edges of the cover slips were sealed with transparent nail polish.

The fluorescent cells were viewed and imaged through a Nikon upright fluorescence microscope (model 80i) equipped with water immersion objectives and connected to cooled CCD digital camera (model Evolution VF, Media cybernatics, USA). Cell images were captured and analysed with Image ProPlus version 5.0 software (Media cybernatics, USA). Cell images were recorded with a 40X objective. The images were processed using standard image processing techniques. For long-term storage these slides were stored at 4°C.

Cell viability analysis for herbal drugs by MTT assay

To evaluate the cytotoxic effect of different herbal drugs on HepG2 cells, MTT assay was performed. MTT assay is colorimetric assay that indicates the formation of purple formazan which is directly proportional to the number of

metabolically active cells. This assay is based on the detection of NAD signals proportional to colorimetric metabolic activity produced by reduction of Yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) to purple formazan in the mitochondria of living cells. The purple formazan crystals are solubilized with acidified isopropanol and the intensity is measured colorimetrically at 570 nm by a spectrophotometer. This reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversion can be directly related to the number of viable (living) cells. When the amount of purple formazan produced by cells treated with an agent is compared with the amount of formazan produced by untreated control cells, the effectiveness of the agent in causing death of cells can be deduced.

HepG2 cells were seeded in 96-well culture plate with complete culture medium containing 5% FBS and antibiotics and allowed to proliferate up to ~60% confluency. Then cells were treated with different drugs at the final concentration of 10 μM for 24 h. After 24 h of incubation period, medium was removed and 100 μl DMEM only was added in each well. Then MTT (1:10 dilution of the 5mg/ml stock in PBS) was added in each wells and further incubated at 37°C for 3 h. When the purple precipitate is clearly visible under microscope then medium was removed and 100 μl isopropanol was added in each well and further incubated at 37°C for 1 h. At the end of incubation period plate cover was removed and absorbance was measured in each well at 570 nm with reference wave length at 650 nm. Data are interpreted as absorbance values that are lower than the control cells indicate a reduction in the rate of cell proliferation. Conversely, a higher absorbance rate indicates an increase in cell proliferation.

Cell morphology analysis by phase contrast microscopy

To study the effect of herbal drugs on cell morphology, HepG2 cells were seeded into 35 mm culture plates approximately at 60% confluency. Next day the cells were treated with different herbal drugs (10 μM concentration) and control (DMSO:ethanol, 1:1 ratio) and allowed to incubate in CO_2 incubator for 24 h. After 24 h period, the cells were analyzed and imaged under Apotome

fluorescence microscope (Carl Zeiss) equipped with appropriate filters sets and Axiovision 3.1 software (Carl Zeiss). Cell images were recorded with a 40X objective using phase contrast filter. The images were processed using standard image processing techniques.

Cell counting by trypan blue

Cell counting was done to seed accurate number of cells as per experimental requirement. Equal volumes of 0.4% trypan blue stain and a well mixed cell suspension were mixed and allowed to stand for 5 min at room temperature. Trypan blue/cell mix (approximately 10 µl) 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 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 per ml 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 cycle phase analysis by FACS

The cell cycle was analyzed by flow cytometry in HepG2 and HepXR (stably integrated PXR in HepG2) cell lines. For cell cycle analysis, $1x10^6$ cells/well were seeded in 6-well culture plate, followed by treatment with different herbal drugs at 10 µM concentration and then harvested 24 h and 48 h post-treatment. Briefly, cells were trypsinized and washed twice with ice-cold PBS, fixed in 70% ethanol in ice-cold PBS for 2 h at 4°C. Then cells were washed with PBS and incubated with 0.1 mg/ml RNase A at 37°C for 1 h. After

incubation period, cells were stained with 50 µg/ml of propidium iodide for 15-20 min in dark at 4°C and then measured by flow cytometry by using FACS Calibur (Becton–Dickinson) and Cell Quest software for data acquisition and analysis. A minimum of 20,000 events were recorded for each sample.

Cell apoptosis assay by FACS

Cell apoptosis studies were performed in HepG2 and HepXR cell lines using Annexin V FITC Apoptosis Detection kit according to the manufacturer's protocol with minor standardizations. For apoptosis assay, $1x10^6$ cells/well were seeded in 6-well culture plate, followed by treatment with different herbal drugs for 24 h. Cells were also treated with 10 µM camptothecin and 1.0 µM staurosporine for 24 h as a positive control for apoptosis. Briefly, cells were trypsinized and added 10 µl media binding buffer, 0.5 µl of annexin V-FITC (200 µg/ml) for 15 min at RT in the dark. After incubation period, centrifuged at 1000 x g for 5 min at RT and media was removed. Cells were gently resuspended in 0.5 ml cold binding buffer, incubated with 2.0 µl of propidium iodide (30 µg/ml) for 10-15 min in dark at 4°C and then measured by flow cytometry by using FACS Calibur (Becton–Dickinson) and Cell Quest software for data acquisition and analysis. A minimum of 20,000 events were recorded for each sample.

Genomic DNA PCR

Genomic DNA of HepG2, Hepx-1096/+43 and Hepx-497/+43 was used for amplification to characterize stable PXR-promoter reporter cell line. Amplification was achieved by PCR using 50 ng of genomic DNA (template) in Taq buffer containing 250 µM dNTP mix, 10 pmol of each forward and reverse primers and 2.5U of Taq polymerase. After initial denaturation at 94°C for 5 min, amplification was conducted for 30 cycles at 94°C for 30 s, primer annealing at desired temperature for 45 seconds (65°C for -1096 F and +43 R, -497 F and +43 R primer set; 60°C for -1096 F and Luc, -497 F and Luc primer set; 60°C for GAPDH), and extension at 72°C for required time (60 s for GAPDH; 1:15 s for -1096 F and +43 R; 40 s for -497 F and +43 R; 1:20 s for -1096 F and Luc; 45 s for -497 F and Luc primer set). Final extension was

carried out for 7 min at 72ºC. The PCR products were resolved on 1.5% agarose gel in TAE buffer (pH 8.0) and visualized by ethidium bromide staining. In parallel, amplification of GAPDH (housekeeping gene) was taken as an internal control. The set of primers to characterize PXR-promoter reporter stable cell lines that generated an amplified fragment size of 1139 bp in -1096 F and +43 R, 550 bp in -497 F and +43 R PXR-promoter specific primers, 1158 bp in -1096 F and Luc primer set; 569 bp in -497 F and Luc primer set and 323 bp for human GAPDH have been tabulated in **Table IV**. The gel pictures were photographed using AlphaEaseFC software (AlphaImager HP, version 5.0.1, AlphaInnotech Corporation, San Leandro, CA) having motorized zoom lens.

Reverse transcriptase-PCR

Total RNA from cultured cells was isolated using TRI-reagent according to the manufacturer's instructions briefly described. Genomic DNA contamination from RNA sample was removed by treatment with DNase I. The concentration of total RNA was determined by reading the O.D. at 260 nm. Two microgram of total RNA was used as template for reverse transcription by using the RevertAid first strand cDNA synthesis kit (Fermentas). Oligonucleotide primers (ranging from 21 to 24 mers) were designed for PCR (amplification) from areas conserved in the published sequences of the human PXR, CYP3A4, MDR1 and GAPDH cDNA sequences. Amplification was achieved by PCR using 2.0 µl of cDNA (template) in Taq buffer containing 250 µM dNTP mix, 10 pmol of each forward and reverse primers and 2.5U of Taq polymerase. After initial denaturation at 94ºC for 5 min, amplification was conducted for 30 cycles at 94ºC for 30 s, primer annealing at desired temperature for 45 seconds (55ºC for PXR and GAPDH; 50ºC for CYP3A4 and MDR1), and extension at 72ºC for 20 s. Final extension was carried out for 5 min at 72ºC. The PCR products were resolved on 1.5% agarose gel in 1X TAE buffer (pH 8.0) and visualized by ethidium bromide staining. In parallel, amplification of GAPDH (housekeeping gene) was taken as an internal control. The set of primers in cDNA amplification that generated an amplified fragment size of 171 bp for human PXR, 121 bp for CYP3A4, 238 bp for MDR1 and 121 bp for

human GAPDH have been tabulated in **Table IV**. The gel pictures were photographed using AlphaEaseFC software (AlphaImager HP, version 5.0.1, AlphaInnotech Corporation, San Leandro, CA) having motorized zoom lens.

Quantitative Real Time PCR

HepG2 cells were treated with different drugs for 24 h followed by isolation of total cellular RNA using TRI reagent as per manufacturer's protocol. After removal of potentially contaminating genomic DNA by DNAse digestion, 1 µg of total RNA was reverse transcribed using First stand cDNA synthesis kit following the manufacturer's recommended protocol. Relative quantification of PXR, CYP3A4 and MDR1 mRNA expression was performed using primer sets tabulated in **Table IV**. The expression of these genes was measured by SYBR Green PCR Master Mix using 7500 Real-Time PCR System (Applied biosystem, Foster City, CA, USA). Data were analyzed in Applied Biosystems SDS v2.0 software using auto threshold and auto baseline settings. All samples were quantitated by the comparative cycle threshold C(t) method for relative quantitation of gene expression, normalized to GAPDH.

Mammalian two-hybrid assay

HepG2 cells were seeded in 12-well plates and transiently co-transfected by Escort III as shown in the scheme with the plasmids pVP16 vector, pM (Gal4-DBD) vector, VP16-PXR (encoding VP16 transactivation domain fusion protein with PXR) together with Gal4-DBD-SRC1 or Gal4-DBD-PBP (encoding Gal4 DNA binding domain fusion protein with SRC1/PBP) along with promoter-reporter plasmid pFR-Luc (in 1:1:4 ratio), containing the luciferase reporter gene with upstream five tandem repeats of the 17-bp GAL4-binding element, as a read out for interaction. Promoter reporter plasmid pFR-Luc was used at 400 ng/well and rests of the plasmids were used at 100 ng/well. Following the transfection period, cells were treated either with vehicle (DMSO:ethanol) or 10 µM of rifampicin, acacetin, resveratrol, piceatannol, kaempferol, sulforaphane and coumestrol. Stimulation in luciferase activity indicated the interaction between the two proteins. Luciferase activities were assayed as described above.

Molecular Modeling and Docking Studies

The structures of all drugs were obtained in 3D format from the PubMed database (http://www.ncbi.nlm.nih.gov). These SDS format drug structures were minimized and convert into MOL2 format using Marvin Sketch version 5.2.3. The hPXR-LBD protein crystal structure was obtained from the RCSB Protein Data Bank (http://www.rcsb.org) in PDB format (Protein Data Bank; 1ILH). All water molecules and ligands from the protein structures were removed using the molecular graphics system Molsoft ICM-Browser (http://www.molsoft.com/icm_browser). The active site of LBD was defined using residues known to line the site. These residues consist of 17 hydrophobic residues (Leu-206, Leu-209, Val-211, Leu-240, Met-243, Phe-251, Phe-281, Phe-288, Trp-299, Leu-308, Met-323, Leu-324, Leu-411, Ile-414, Phe-420, Met-425, and Phe-429), five polar residues (Ser-208, Ser-247, Cys-284, Gln-285, and Tyr-306), and four charged residues (Glu-321, His-327, His-407, and Arg-410) (Liu et al, 2011). The PDBQT files for docking were generated in AutoDockTools (ADT) version 1.5.4 (http://mgltools.scripps.edu), which was used to add polar hydrogens and Compute Gasteiger charges to the protein structures. The search space with XYZ dimensions 24 Å × 24 Å × 24 Å was centered at coordinates 8.934 (x), 30.182 (y), 25.546 (z). Docking of drugs to hPXR-LBD was carried out with the program AutoDock Vina version 1.1.1 (Trott and Olson, 2010) with the number of GA runs=10; population size=150; maximum number of evals=2500000 and maximum number of generations=27000. The docking results were analyzed and the figures created in Molsoft ICM-browser.

Statistical analysis

Most of the experiments were done at least 3 times in duplicates and values represent the means ± SD of three separate experiments. Statistical analysis was done by unpaired student t-test and analysis of variance (ANOVA) and asterisks (*, **, ***/ #, ##, ###) 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

Modulation of PXR-mediated transactivation function by prospective anti-cancer herbal drugs

INTRODUCTION

Nuclear Receptor PXR is a highly promiscuous ligand-activated transcription factor that acts as xenobiotic sensor and regulates the drug/xenobiotic clearance in liver and intestine through activation of genes involved in drug and xenobiotic metabolism. It is activated by structurally diverse groups of xenobiotic, endobiotic compounds, numerous clinical drugs, phytochemicals, and dietary constituents (Willson and Kliewer, 2002; Wang et al, 2013). Upon ligand binding, PXR forms heterodimer with Retinoid X receptor (RXR) and binds to the promoters of PXR regulated genes to control their expression (Chen, 2008). Ligand binding to PXR leads to the recruitment of nuclear receptor coactivators to induce the expression of the target genes, in turn which encode proteins involved in xenobiotic detoxification and endobiotic metabolism, such as drug-metabolizing enzymes and transporters (Ihunnah et al, 2011; Wang et al, 2013). Abnormal activation of PXR by xenobiotics may lead to unwanted adverse drug-drug or food-drug interactions, a plausible liability concern in drug development and clinical therapy (Chang and Waxman, 2006; Chang, 2009). Though the effect of clinical drugs on PXR activity has been greatly investigated, significantly less is known about the effect of dietary food supplements and herbal drug constituents on PXR activity and its regulated genes.

Cytochrome P450 3A4 (CYP3A4) and multidrug resistance protein1 (MDR1, also known as P-glycoprotein or P-gp), both expressed in the liver and intestine, are the major PXR regulated genes. CYP3A4, a monooxygenase, mainly functions in catalyzing the first step of detoxification of xenobiotics by hydroxylation reaction (Poulton et al, 2013, Wang et al, 2013). MDR1, a member of the ATP-binding cassette transporter family, acts as an efflux pump to limit the absorption of xenobiotics and contributes to the extrusion of many drugs in the intestine (Crowe and Tan, 2012). Since CYP3A4 and MDR1 together contribute to the metabolism and transportation of more than 50% of clinically used drugs and a great number of xenobiotics (Guengerich, 1999; Veith et al, 2009), induction or inhibition of CYP3A4 and MDR1 may cause drug-drug and dietary-drug interactions.

Today, cell-based assays are used in more than half of all high-throughput drug screenings for target validation and ADMET (absorption, distribution, metabolism, elimination and toxicity) in the early stage of drug discovery. Stable transfection and integration of gene(s) of interest in hepatic cell line(s) will be used to generate stably integrated promoter-reporter hepatic cell line that will serve as valuable tools for *ex vivo* screening and evaluation of various herbal drugs or xenobiotics for PXR activity and CYP3A4 and MDR1 modulators.

In the present study, we have developed and characterized a stable promoter-reporter cell line (HepXREM) that expresses human PXR and a commonly used PXR promoter-reporter i.e. XREM-Luciferase. This cell lines offered high-throughput *in vivo* analysis of PXR influencing factors. Among other applications, this cell line can be used to evaluate uncharacterized ligands, extracellular stimuli, and upstream events in the PXR signalling pathway. Further, to find PXR activators/antagonists we screened various anticancer herbal drugs by using HepXREM stable cell line. Interestingly, we have seen that acacetin, resveratrol, piceatannol, kaempferol, guggulsterone, forskolin, genistein, butein and isoliquiritigenin strongly transactivate PXR in HepXREM cell line. Quercetin, vincristine, vinblastine and hypericin activated PXR moderately. These drugs also modulated MDR1 promoter activity. Acacetin and resveratrol also induced the endogenous mRNA expression of CYP3A4 and MDR1. Acacetin, resveratrol, piceatannol and kaempferol increase the nuclear receptor coactivator SRC1 and PBP interaction with PXR in cells and consequently inducing the gene transcription. Molecular docking studies also supported that these drugs could bind directly to PXR-LBD. Additionally, anethol, etoposide, and eugenol inhibited the PXR transcriptional activity and emerged as novel PXR antagonists. Furthermore, we have shown that SIRT1 inhibitors nicotinamide and sirtinol inhibited basal and induced PXR transcriptional activity. In conclusion, the present study provides screening system and method that facilitate the identification of compounds with potential to activate or inhibit PXR transcriptional functions. The study also reports some potentially novel activators and antagonists of PXR.

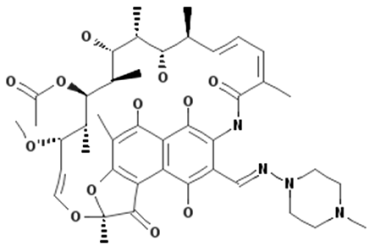
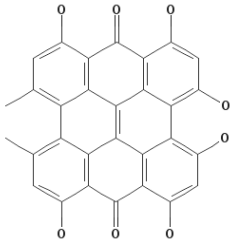
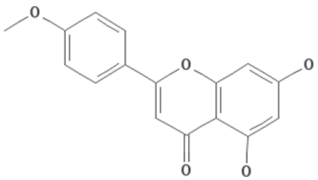
RESULTS

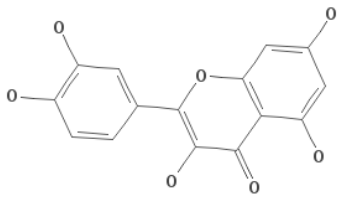
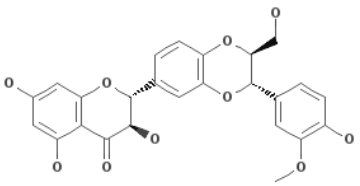
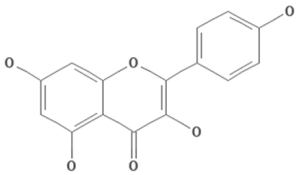
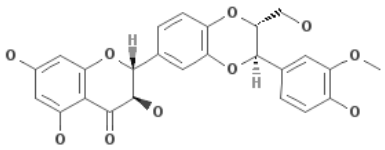
Herbal drugs are readily used by millions of people without prescription on belief that anything natural is safe. Like allopathic (prescription) drugs, herbal medicines also have different pharmacokinetic and pharmacodynamic properties that ultimately lead to different therapeutic responses, and also have adverse or beneficial actions due to drug-herbal interactions. The concurrent use of herbal medicines and conventional (prescription) drugs by patients suffering from different diseases has progressively increased. Co-administration of herbal medicines with conventional drugs increases the risk of undesirable interactions between the two. Interactions between drugs can affect the pharmacokinetics of concomitantly administered chemotherapeutic agents. An important mechanism that underlies these interactions is the induction of drug metabolizing enzymes and efflux transporters (CYP3A4 and MDR1) through the activation of PXR by herbal drugs (Harmsen et al, 2007). For example, if one drug activates PXR, it can be predicted that administration of this drug will promote the elimination of other co-administered drugs that are also metabolized and eliminated by PXR-target gene products, thereby reducing the efficacy of multi-drug therapies in patients on combination therapy. In this context, studies on PXR interactions with herbal drugs in changing physiological environment both under normal and pathogenic conditions may give important clues in evaluating the herbal drugs. In this study various anticancer herbal drugs (**Table V**) were selected on the basis of their therapeutic category. These selected herbal drugs were screened on the basis of their effect on PXR transcriptional activity.

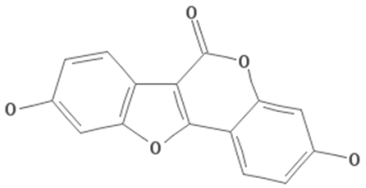
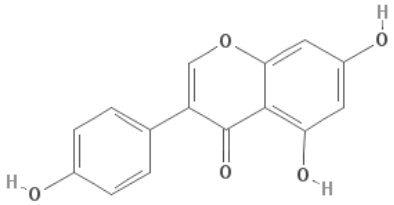
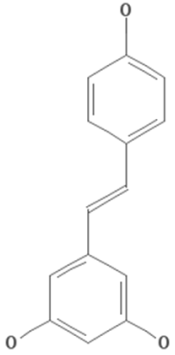
Generation and characterization of a liver cell line with stable integration of human PXR and CYP3A4 promoter-reporter useful for screening of herbal drugs

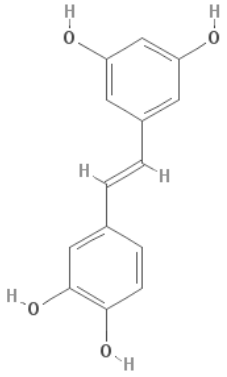
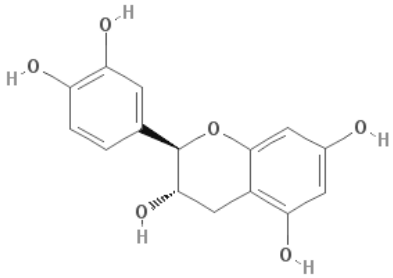
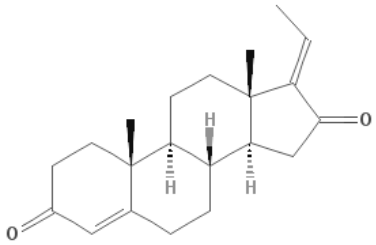
PXR is activated by diverse group of steroids, dietary compounds, drugs and xenobiotics that in turn are substrates for the PXR induced metabolizing enzymes, cytochrome P450 (CYP). Screening of ligands that can specifically activate or inhibit the PXR activity will indicate the limitations or benefits of prospective drug molecules. Preparation of cell lines that expresses stable level

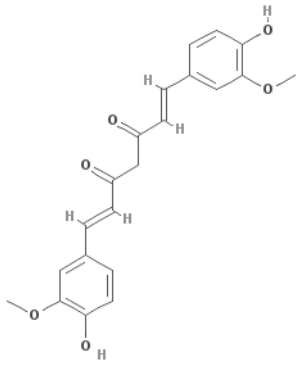
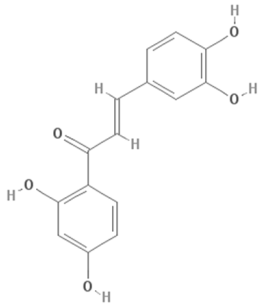
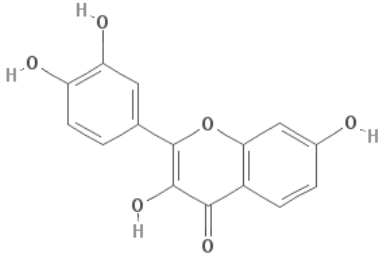
Table V: List of herbal drugs used in the present study. The drugs are described with their source, category, structure, mode of action and therapeutic use.

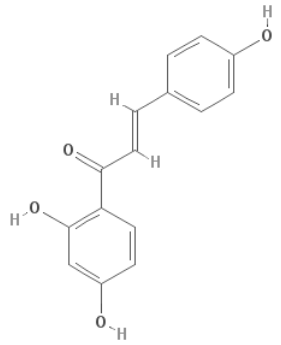
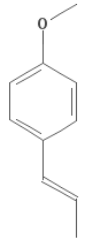
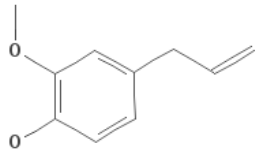
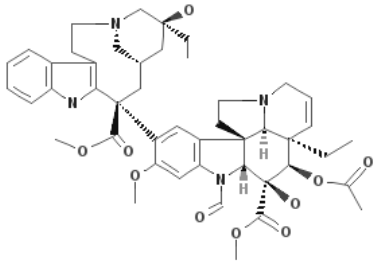
S. No.	Drugs	Biological source	Category	Structure	Therapeutic/ Potential use	Mode of action	Reference
1.	Rifampicin	*Streptomyces mediterranei*	Semisynthetic antibotic		Anti-tubercular	Prototypical ligand of human PXR. Inhibit DNA dependent RNA synthesis.	Bertilsson et al, 1998
2.	Hypericin	*Hypericum perforatum* (St. John's wart)	Bioflavonoid		Anti-depressant	Hyperforin, not hypericin contribute to CYP3A4 induction. Induction of CYP3A4 mRNA by hypericin was reported previously but it was observed at high concentration.	Gutmann et al, 2006 Godtel-Armbrust-Godtel et al, 2007
3.	Acacetin	*Robinia pseudoacacia* (black locust)	Flavonoid		Anti-cancer effect	Inhibits cell growth and cell cycle progression, and induces apoptosis in human prostate cancer cells. Inhibits VEGF expression, tumor angiogenesis and growth through AKT/HIF-1α pathway.	Singh et al, 2005 Liu et al, 2011 Watanabe et al, 2012

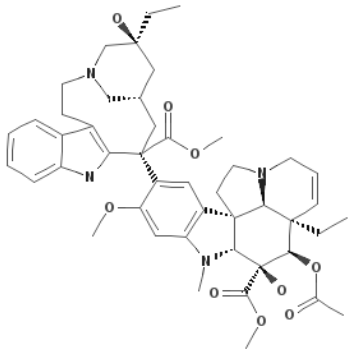
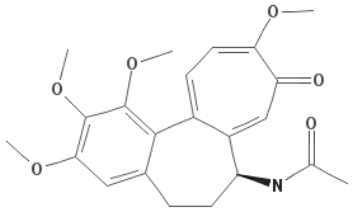
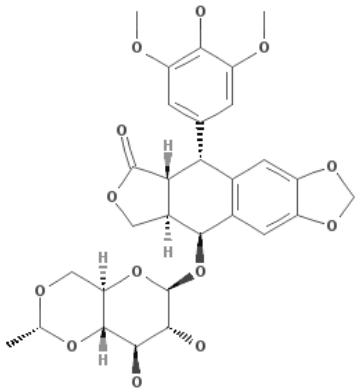
4.	Quercetin	*Ginkgo biloba*	Flavonoid		Antioxidant antihistamine and an anti-inflammator. Protect against heart disease and cancer	Cell-based reporter assays in HepG2 revealed that quercetin activates PXR, CAR, and AhR. Induced the expression of UGT1A1 and CYP1A2 in HepG2 cells but not in primary human hepatocytes.	Li et al, 2009 Wang et al, 2013
5.	Silymarin	Milk thistle (*Silybum marianum*)	Flavonolignan (flavonoid)		Hepatoprotective, Anti-inflammatory, Anticarcinogenic	Induce CYP3A4 mRNA in human hepatocytes. Potentiates TRAIL-induced apoptosis in human colon adenocarcinoma and in derived TRAIL-resistant metastatic cells.	Raucy et al, 2003 Souza et al, 2012 Kauntz et al, 2012
6.	Kaempferol	*Ginkgo biloba*	Flavonoid		Antioxidant, antihistamine and an anti-inflammatory. Protects against heart disease and cancer	Kaempferol suppresses cell metastasis via inhibition of the ERK-p38-JNK and AP-1 signalling pathways.	Li et al, 2009 Chen et al, 2013
7.	Silibinin	*Sylibum marianum*	Flavonoid		Hepatoprotective, Anticancer	Silibinin inhibits Wnt/β-catenin signalling in human prostate and breast cancer cells. It also protects cells from UVB-induced apoptosis through the simultaneous inhibition of ATM-p53 pathway and autophagy.	Lu et al, 2012 Liu et al, 2013

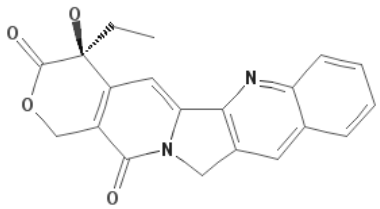
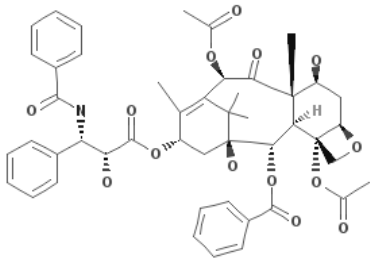
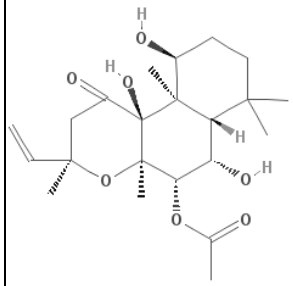
8.	Coumestrol		Isoflavonoid		Phytoestrogen Anticancer	Antagonizing the action of the human nuclear xenobiotic receptor pregnane X receptor (PXR). Induces senescence through protein kinase CKII inhibition-mediated reactive oxygen species production in human breast cancer and colon cancer cells.	Wang et al, 2008a Lee et al, 2013
9.	Genistein	*Genista tinctoria*	Isoflavonoid		Phytoestrogen Anticancer	Prevents the growth of ovarian cancer cells induced by 17ß-estradiol or bisphenol A via the inhibition of cell cycle progression. Induce G2 PHASE arrest in human and murine cell lines and inhibits protein-tyrosine kinase.	Sakamoto et al, 2010 Hwang et al, 2013
10.	Resveratrol	mainly in red grapes (key ingredient of red wine)	Stilbenoid, a type of natural polyphenolic compund		Anti-oxidant Anti-cancer Anti-inflammatory	Inhibition of PKC-mediated induction of COX-2. Up-regulation of the endogenous defense system via the Nrf2/Keap1 system. SIRT1 activator	Kluth et al, 2007 Hubbard et al, 2013

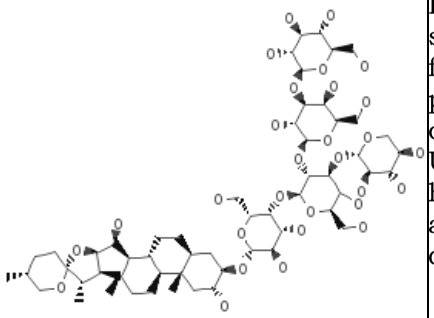
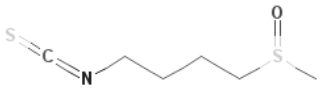
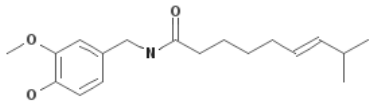
11.	Piceatannol	metabolite of resveratrol found in red wine.	Stilbenoid, a type of natural polyphenolic compound		Anti-oxidant Anti-cancer	piceatannol suppresses TNFα shedding, leading to inhibition of TNFα/NFκB pathway. Piceatannol treatment induced p38 MAPK phosphorylation but inactivation of Akt and ERK.	Wolter et al, 2002 Liu and Chang, 2012
12.	Catechins	Predominantly in green tea (*Camellia sinensis*)	Polyphenol (Flavonoid)		Antioxidant, Anticancer, Cardioprotective effect	Four polyphenol catechins in green tea include gallocatechin (GC), epigallocatechin (EGC), epicatechin (EC), and epigallocatechin gallate (EGCG).	Kluth et al, 2007
13.	Guggulsterone	Guggal (*Commiphora mukul*)	Polyphenol		Cholesterol lowering agent, Anti-inflammatory, Anti-rheumatic, Anti-tumor and Anti-angiogenesis	Anti-tumor and anti-angiogenesis activity by suppressing nuclear factor-κB and STAT3 activity. Inhibits cell proliferation and induce apoptosis through the activation of JNK, suppression of Akt, and downregulation of antiapoptotic protein expression.	Shishodia et al, 2007 Moon et al, 2011

14.	Curcumin	*Curcuma longa*	Natural polyphenol		Antioxidant, Anti-inflammatory, Antitumor, Hepatoprotective	Induce apoptosis in cancer cells. Interfere with the activity of the transcription factor NF-κB. Anti-inflammatory properties may be due to inhibition of eicosanoid biosynthesis.	Srivastava et al, 1995 Aggarwal and Shishodi, 2006 Strofer et al, 2011
15.	Butein	*Toxicodendron vernicifluum*	Chalconoids, a natural phenol		Anticancer	Induces apoptosis and inhibits prostate tumor growth *in vitro* and *in vivo*. Induction of glutathione synthesis and heme oxygenase 1 is mediated through the ERK/Nrf2 pathway and protects against oxidative stress.	Yang et al, 2011 Khan et al, 2012
16.	Fisetin	Found in many plants. It serves as a colouring agent.	Flavonoid, group of polyphenols		Anti-aging, Anti-inflammatory, Anticancer	Activation of reactive oxygen species/AMP activated protein kinase signalling mediates fisetin-induced apoptosis in multiple myeloma U266 cells.	Jang et al, 2012

17.	Isoliquiritigenin	*Glycyrrhiza glabra*	Chalconoid, a type of natural phenols		Anticancer	Prevents 6-hydroxydopamine-induced apoptosis in dopaminergic neurons.	Hwang and Chun, 2012
18.	Anethole	Main constituent of fennel, anise and camphor	Phenylpropene, a type of aromatic compound		Anti-inflammatory, Anticancer	Anethole is a potent anti-metastatic drug that functions through inhibiting MMP-2/9 and AKT/mitogen-activated protein kinase (MAPK)/NF-κB signal transducers.	Choo et al, 2011
19.	Eugenol	Mainly in clove	Phenylpropene, a type of aromatic compound		Analgesic, Anti-inflammatory, Anticarcinogenic	Eugenol exerts its anticancer activity via apoptosis induction.	Hussain et al, 2011
20.	Vincristine	*Catharanthus roseus* also known as *Vinca rosea*	Alkaloid		Anti-cancer	Mitotic inhibitor (Disruption of the microtubules arrests mitosis in metaphase) Activate PXR-mediated Pgp induction.	Harmsen et al, 2010

21.	Vinblastine	*Catharanthus roseus* also known as *Vinca rosea*	Alkaloid		Anti-cancer	Mitotic inhibitor (Disruption of the microtubules arrests mitosis in metaphase) Activate PXR-mediated Pgp induction.	Harmsen et al, 2010
22.	Colchicine	*Colchicum autumnale*	Alkaloid		Therapeutic use for treatment of gout and rheumatism. Antitumor agent.	Ability to disrupt microtubule networks.	Dvorak et al, 2007
23.	Etoposide	Semisynthetic derivative of podophyllotoxin, a substance extracted from *Podophyllum peltatum*	Alkaloid		Anticancer	Type II topoisomerases inhibitor.	Wu et al, 2011a

24.	Camptothecin	*Camptotheca acuminate*	Alkaloid		Anticancer	Selectively inhibits the nuclear enzyme DNA topoisomerases type I. Attenuates cytochrome P450 3A4 induction by blocking the activation of human pregnane X receptor.	Chen and Liu, 1994 Chen et al, 2010
25.	Taxol (paclitaxael)	Pacific yew tree (*Taxus brevifolia*)	Diterpenoid		Anti-cancer	It stabilizes microtubules in their polymerized form leading to cell death. Activates PXR-mediated Pgp induction.	Brito et al, 2008 Harmsen et al, 2010
26.	Forskolin	*Coleus forskohlii*	Diterpenoid		Fat burning property, used to treat various diseases such as hypothyroidism, heart disease, and respiratory disorders.	Activates adenyl cyclase, thereby increasing intracellular concentration of cAMP and thus activating the protein kinase A (PKA) signal transduction pathway.	Sidhu and Omiecinski, 1996 Ding and Staudinger, 2005a Pavlikova et al, 2010

27.	Digitonin	*Digitalis Purpurea*	Glycoside		Digitonin is a Detergent solution useful for permeabilizing cells. Useful in hyperglycaemia and dyslipidemia.	Digitonin enhanced glucose tolerance and had beneficial effects on serum lipids by improve antioxidant activity.	Ebaid et al, 2006
28.	Sulforaphane	Cruciferous vegetables (abundantly in broccoli)	Phytochemical, classified as isothiocyanate		Anticarcinogenic agents	SFN can induce apoptosis and cell cycle arrest in human cancer cells. Anti-cancer effects with the induction of phase II xenobiotic metabolism enzymes via activation of the Keap1/Nrf2 antioxidant response pathway. Sulforaphane induced cell cycle arrest in the G2/M phase via the blockade of cyclin B1/CDC2 in human ovarian cancer cells.	Zhou et al , 2007 Chang et al, 2013
29.	Capsaicin	Active component of chili peppers, which belongs to the genus Capsicum	Capsaicinoid		Anticancer	Capsaicin in combination with cisplatin induced higher apoptotic cell death in gastric cancer.	Huh et al, 2011

of PXR can be used for reporter assay without transfecting the exogenous PXR. Furthermore, the results obtained from transient transfection are not always consistent due to variability in expression levels or over expression. So, a preferred choice is to have cell-based screening system that can provide more reproducible assays. This led us to develop a human cell line derived from HepG2 cells stably co-transfected with expression plasmid of human PXR and its most commonly used regulatory gene promoter, XREM-Luc. For convenience reason we term this cell line HepXREM. This cell lines will offer high-throughput *in vivo* analysis of drug molecules and other influencing factors. Among other applications, this cell line can be used to evaluate uncharacterized ligands, extra-cellular stimuli, and upstream events in the PXR signalling pathway.

To obtain a stable cell line expressing functional wild type human PXR and the XREM-Luc promoter-reporter construct, HepG2 cells, a human liver cell line, was seeded in 35-mm culture dishes in DMEM containing 10% FBS and antibiotics. At ~60% confluence, the cell medium was changed to DMEM only and were transfected with a mixture containing the (XREM-Luc) reporter construct, pSG5-hPXR expression plasmid and pCDNA.3-neo in a ratio of 10:2:1 using Lipofectamine 2000 reagent. After the cells had been exposed to the precipitated DNA for 18 h, the DMEM was removed and replaced with fresh DMEM containing 10% FBS and antibiotics. After a further 24 h, this medium was then replaced by DMEM containing 400 mg/ml G418. The medium was renewed every 2-3 days for about two weeks until small colonies were visible. Positive clones that expressed both hPXR and XREM-Luc were identified by their ability to respond to human PXR-specific ligand, rifampicin.

The stable integration and expression of PXR was verified by western blot analysis. Cytoplasmic and nuclear cell lysates from stable HepXREM cells, HepXR cells and HepG2 cells were subjected to western blot using the PXR polyclonal antiserum developed in our laboratory (Saradhi et al, 2005b). A major protein band having an expected molecular mass of PXR (50 kDa) was detected with other less prominent bands (possible isoforms of PXR) in nuclear extract having different expression levels. HepXR showed the highest expression of PXR followed by HepXREM. A faint band of PXR was detected in

normal HepG2 confirming its low endogenous expression level. Furthermore, the cytoplasmic fractions of these cell lines did not shown similar levels of PXR suggesting that PXR is a predominant nuclear protein (**Figure 10A**). The stable HepXREM cell line is further characterized by PXR mRNA expression levels using semi-quantitative RT-PCR analysis. The band of PXR mRNA in HepXREM cell line was more intense than in HepG2 cells and less intense as in HepXR cell line (**Figure 10B**). To address the question whether HepXREM cell lines is transcriptionally responsive, a dose dependent curve was generated by analyzing the level firefly luciferase activity with increasing concentration of ligand, rifampicin. Rifampicin efficiently activated PXR with maximal luciferase activity induced at 10 µM. Approximately, 3-fold activation was observed in presence of 10 µM rifampicin confirming that this cell line is transcriptionally responsive and active (**Figure 10C**). The agonist activities of PXR-specific inducer measured using our stable model was consistent with those measured in transient transfectants in presence of rifampicin. In transient transfection, ~6-fold activation was observed in presence of 10 µM rifampicin as comparison to control (**Figure 10D**). Further, a normal expression and nuclear localization of PXR was further confirmed by indirect immunofluorescence staining with anti-PXR antibody (**Figure 10E**). These data confirms that we have successfully generated stable reporter cell line HepXREM. The significance of this cell line lies in its ability to be used in a standard high-throughput system that permits the ability to simultaneously screen numerous agents for induction of PXR. Pre-screening compounds with this cell line may permit identification of potent inducers of human CYP450s that can be essentially 'screened out' in process of drug development.

Modulatory effect of different herbal drugs on PXR transcriptional activity

Stably integrated hepatic cell line (HepXREM) that expresses human PXR and most commonly used PXR activated promoter-reporter; XREM-Luc is a valuable tool to obtain more reproducible results during drug screening. In the present study, PXR response to herbal drugs has been evaluated by screening

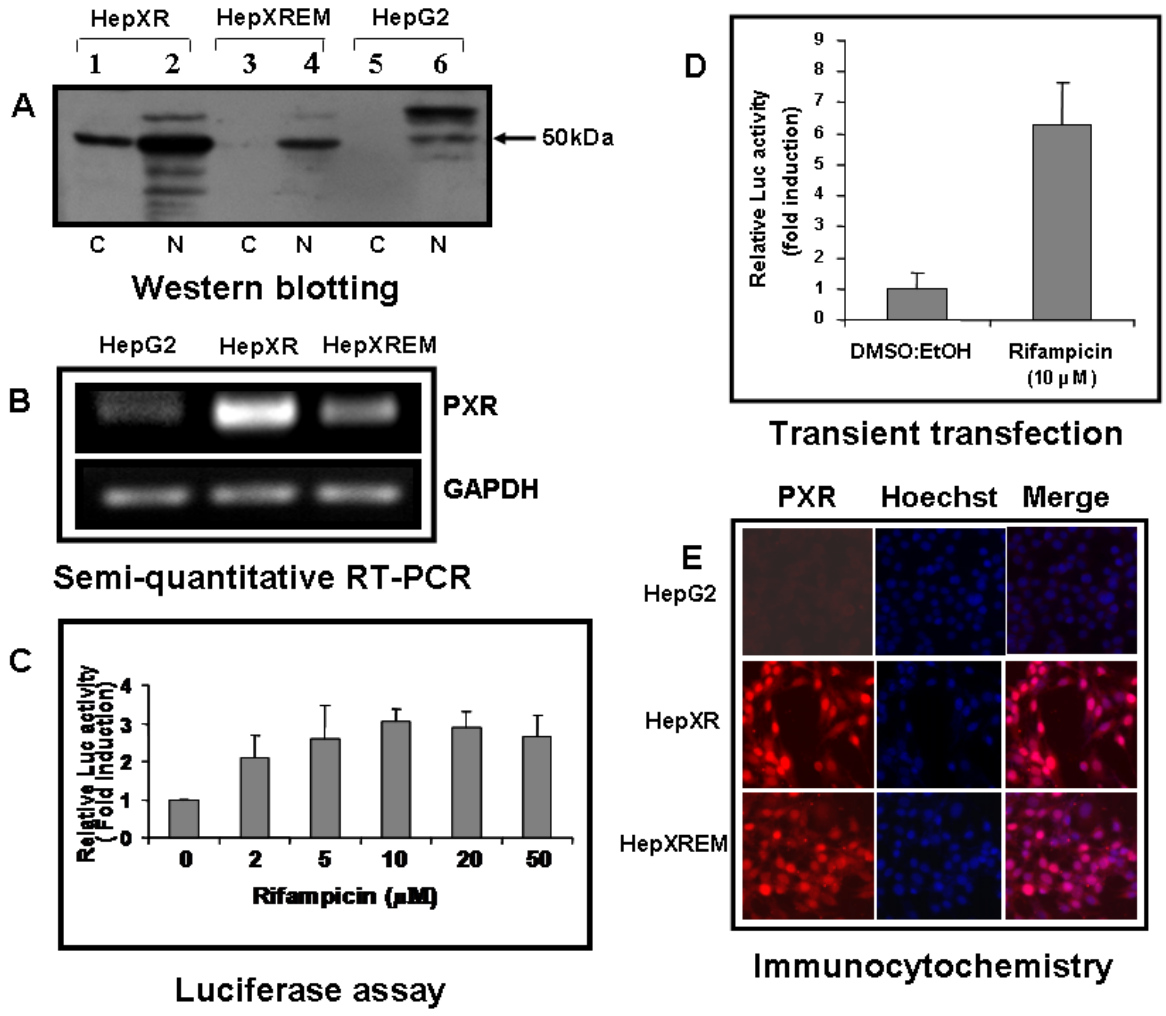


Figure 10: Characterization of HepXREM stable cell line. *Western blot analysis for detection of PXR in stable HepXREM cell line **(A)**. Cytoplasmic and nuclear extracts from HepXR, HepXREM and HepG2 cells were prepared and electrophoresed through 10% SDS-PAGE, blotted on to PVDF membrane and probed with anti-PXR antibodies at 1:1000 dilutions. A major immuno-reactive band of 50 kDa relative molecular weight was detected with other less prominent bands. Lane 1 Cytoplasmic extract of HePXR, Lane 2 Nuclear extract of HePXR, Lane 3 Cytoplasmic extract of HepXREM, Lane 4 Nuclear extract of HepXREM, Lane 5 Cytoplasmic extract of HepG2, Lane 6 Nuclear extract of HepG2. Semi-quantitative RT-PCR analysis to assess the expression level of PXR transcript in stable HepXREM cell line **(B)**. Total RNA was isolated from HepG2, HepXR and HepXREM cell line. Total RNA obtained from these cell lines were reverse transcribed using RevertAid first strand cDNA synthesis kit (Fermentas). Amplification of PXR and GAPDH gene from cDNA was performed as per standard protocol using Taq DNA polymerase. PCR products (an amplified fragment of 171 bp for PXR and 121 bp for GAPDH) was separated on 1.5% agarose gel in TAE buffer [pH 8.0] and visualized by ethidium bromide staining. In parallel, GAPDH (housekeeping gene) cDNA was amplified as control. Rifampicin mediated transcriptional response of PXR in stable HepXREM cell line **(C)**. HepXREM cells were incubated for 24 h in the presence or absence of different concentration of rifampicin ranging from 0 to 50 μM and luciferase*

assay was done. At 10 µM concentration maximum PXR activation was observed in comparison to DMSO-treated control cells. Relative fold activity was calculated in comparison to DMSO induced luciferase activity. The values represent the means ± SD from three separate experiments. Transient transfection assay to analyze the effect of rifampicin (10 µM) on PXR transcriptional activity ***(D)****. HepG2 cells were seeded and then co-transfected with pSG5-PXR expression plasmid and XREM-Luc promoter-reporter construct in a ratio of 1:8. After 10-12 h of incubation complete DMEM was added and further incubated for 24 h with or without 10 µM rifampicin. After 24 h of expression period luciferase activity was taken. Relative fold activity was calculated in comparison to DMSO:ethanol induced luciferase activity. The values represent the means ± SD from three separate experiments. Indirect immunofluorescence staining of PXR in HepXREM cell line* ***(E)****. HepG2, HepXR and HepXREM cells were seeded on sterile cover slip, after 24 h the cells were fixed, processed for immunodetection with rabbit anti-PXR antibodies (1:500 dilutions). Then cells were washed with PBS and probed with cy3-conjugated anti-rabbit secondary antibody and imaged under fluorescent microscope. The left panel shows the distribution patterns of receptor in immunodetected cells. The middle panel shows the Hoechst staining for visualizing the nuclei and the right panel shows the merge images of the two fluorescences.*

various active herbal ingredients in HepXREM cell line. In addition to this application, the cell line can be used to evaluate uncharacterized ligands, extra-cellular stimuli, and upstream events in the PXR signalling pathways.

With the help of following observations, numbers of herbal drug bioactive ingredients (**Table V**) were screened by using stably integrated liver cell line, HepXREM by luciferase assay for the search of PXR agonist(s) or antagonist(s). The cells were propagated in 48-well culture plate with complete DMEM containing 5% FBS and antibiotics and allowed to proliferate up to ~60% confluency. Then cells were treated with different drugs for 24 h. All the drugs used were prepared in drug solvent DMSO:ethanol (in 1:1 ratio) and were studied for their effect on PXR transcriptional activity at the final concentration of 0.1, 1.0 and 10 µM. After 24 h of incubation period, luciferase reporter activity was determined by using known standard procedures (Ausubel et al, 2002).

Briefly, to determine the luciferase activity, cells were lysed and used for luciferase assay using the substrate, luciferin (Promega, Madison, WI, USA) (Ausubel et al, 2002). The luciferase activity was measured in a pre-programmed TD-20/20 DLReadyTM luminometer (Turner Designs, USA) and

the activities expressed as 'relative luciferase activity'. Luciferase reporter activities of the drug-treated cells were compared with their corresponding cell line treated with drug solvent alone (DMSO:ethanol in 1:1 ratio) and referred as control. Since the cell line has a significant basal luciferase reporter activity the solvent treated cells were taken as 100% for comparison purposes. The promoter-reporter responses derived from the HepXREM cell line following treatment with different drugs are shown in **figure 11** and **Table VI**. Our results indicated that acacetin, resveratrol, piceatannol, guggulsterone, forskolin, kaempferol, genistein, butein and isoliuiritigenin increased PXR transcriptional activity at higher level and comparable with rifampicin; the well-known agonist of PXR. Coumestrol, quercetin, hypericin, vincristine, vinblastine and colchicine also increased PXR transcriptional activity at moderate levels. Anethol, capsaicin, catechin, curcumin, eugenol, fisetin, silibinin, silymarin and taxol did not have any significant effect on PXR transcriptional activity. Camptothecin, digitonin, etoposide and sulforapane apparently showed suppression of PXR transcriptional activity. Whether this suppression is due to general cell toxicity, cell cycle arrest or antagonistic property needs to be ascertained by further studies. 10 μM of acacetin, resveratrol, piceatannol, guggulsterone, forskolin, kaempferol, genistein, butein and isoliuiritigenin increased PXR transcriptional activity nearly by 305%, 252%, 213%, 169%, 208%, 224%, 354%, 163%, and 164% respectively. Rifampicin increased the PXR transcriptional activity by 228% at the same concentration. Hypericin and vinblastine transactivated PXR at low concentrations i.e. 1.0 μM and 0.1 μM respectively. Taken together, all these results concluded that acacetin, resveratrol, piceatannol, guggulsterone, forskolin, kaempferol, genistein, butein and isoliuiritigenin are potent PXR activators while camptothecin, digitonin, etoposide and sulforapane are antagonists of PXR.

Acacetin, resveratrol and piceatannol trigger PXR transcriptional activity in dose dependent manner

Acacetin, resveratrol, picetannol, forskolin, kaempferol, genistein, butein

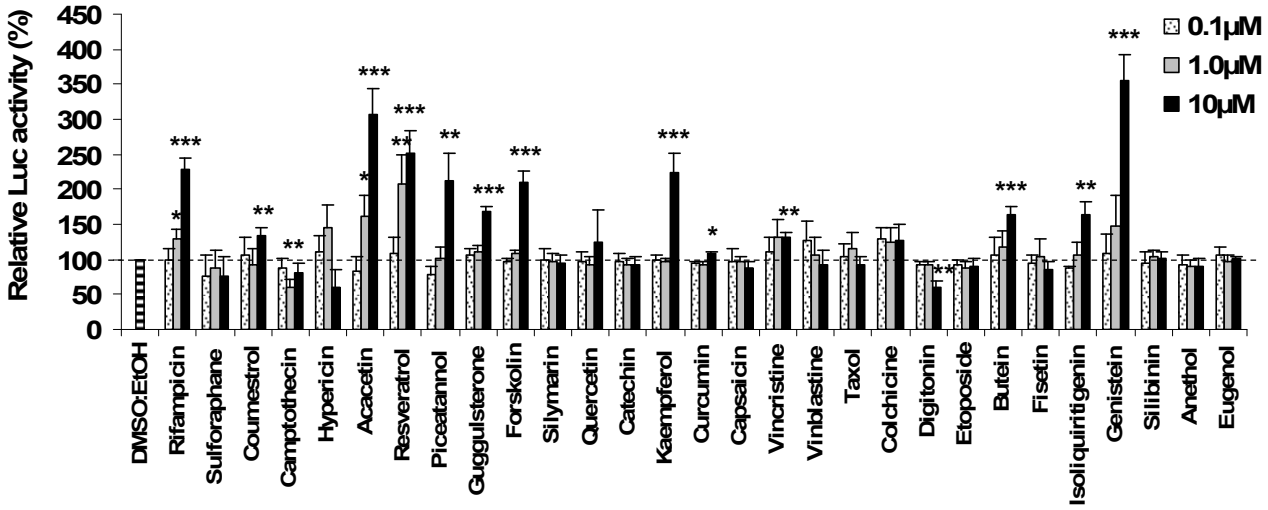


Figure 11: Effect of herbal drugs on PXR transcriptional activity in HepXREM cell line by luciferase-reporter gene assay. *HepXREM cells were seeded in 48-well culture plate with complete DMEM containing 5% FBS and antibiotics and allowed to proliferate up to ~60% confluency. Than the cells were treated with indicated herbal compounds at different concentration (0.1 µM, 1.0 µM and 10 µM,). After 24 h of incubation period, cell lysate were prepared and luciferase activity was determined. Luciferase values are expressed as percentage of the activity of control sample (DMSO:ethanol). Data represent the mean ± S.D. 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). Here, rifampicin was used as a standard PXR ligand for comparison purpose with other herbal ingredients as it is a well-established PXR activator.*

Table VI: Classification of anti-cancer herbal drugs based on their effects on PXR transcriptional activity in HepXREM cell line.

Serial No.	Classification of drug response	Herbal Drugs
1.	High activation (High activators)	Rifampicin, Acacetin, Resveratrol, Piceatannol, Genistein, Forskolin, Kaempferol, Guggulsterone, Butein and Isoliquiritigenin
2.	Moderate activation (Moderate activators)	Coumestrol, Quercetin, Hypericin, Vincristine, Vinblastine and Colchicine
3.	No effect	Anethol, Capsaicin, Catechin, Curcumin, Eugenol, Fisetin, Silibinin, Silymarin and Taxol
4.	Repression (Repressors)	Camptothecin, Digitonin, Etoposide and Sulforapane

isoliquirtigenin, coumestrol, curcumin and vincristine increased the PXR transcriptional activity in HepXREM cells. Further to see the effect of higher dose of drugs on PXR transcriptional activity, we assessed the dose-dependent effect of selected herbal drugs on PXR transcriptional activity. To achieve this, HepXREM cells were propagated in 48 well culture plate with complete culture medium and allowed to proliferate up to ~60% confluency. Then cells were treated with different concentrations of acacetin, resveratrol and picetannol ranging from 0.1-100 µM and incubated for 24 h. After 24 h of incubation period, PXR transcriptional activities of different samples were determined by luciferase assay. **Figure 12** shows the effect of acacetin, resveratrol and picetannol on PXR transcriptional activity in dose-dependent manner. Data obtained from this dose-dependent activation study showed the maximum PXR transcriptional activity by acacetin, resveratrol and picetannol at 10 µM concentration (**Figure 12**). PXR transcriptional activity decreased at higher concentrations (more than 10 µM).

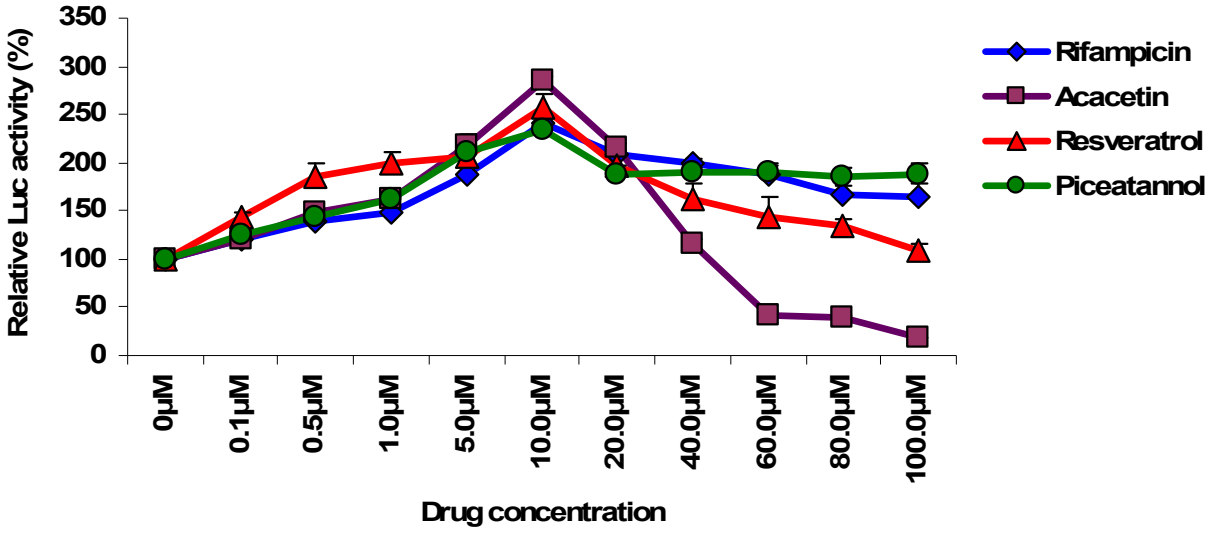


Figure 12: Dose dependent effect of acacetin, resveratrol and piceatannol on HepXREM cell line. *HepXREM cells were seeded in 48-well culture plate and incubated for 24 h in the presence or absence of different concentration of herbal drug ranging from 0 to 100 µM. After 24 h of incubation period luciferase assay was done as described under 'optimization protocol in 48-well culture plate'. A dose dependent activation was observed with highest activity at 10 µM concentration in comparison to control (DMSO:ethanol). Relative Luc activity was calculated in comparison to DMSO:ethanol induced luciferase activity. Data represent the mean ± S.D. of three independent experiments.*

Effect of herbal drugs on cell viability (cell proliferation)

To evaluate the cytotoxic effect of different herbal drugs on HepG2 cells, MTT assay was performed as describe in 'Materials and Methods'. HepG2 cells were grown in 96-well culture plate and treated with different drugs at the final concentration of 10 μM for 24 h. Staurosporine (0.1 μM) was used as a control for cell apoptosis. After 24 h of incubation period, medium was removed and 100 μl DMEM only was added in each well. Then MTT (1:10 dilution of the 5mg/ml stock in PBS) was added in each wells and further incubated at 37°C for 3 h. When the purple precipitate is clearly visible under microscope then medium was removed and 100 μl isopropanol was added in each well and further incubated at 37°C for 1 h. At the end of incubation period plate cover was removed and absorbance was measured in each well at 570 nm with reference wave length at 650 nm. Data are interpreted as absorbance values that are lower than the control cells indicate a reduction in the rate of cell proliferation. Conversely, a higher absorbance rate indicates an increase in cell proliferation (**Figure 13**). **Figure 13** shows the effect of herbal drugs on cell viability. Only camptothecin, hypericin, vincristine, vinblastine, taxol, colchicine, digitonin and etoposide inhibited the cell proliferation by causing apoptosis at 10 μM concentration as compared to control cells while other drugs did not show any cell toxicity. Staurosporine (0.1 μM), a positive control for apoptosis, inhibited the cell proliferation nearly 72% (**Figure 13**). Camptothecin, hypericin, vincristine, vinblastine, taxol, colchicine, digitonin and etoposide significantly inhibited cell proliferation nearly 60%, 28%, 37%, 39%, 24%, 54%, 60% and 49% respectively. Fisetin also inhibited the cell proliferation approximately 11% but non-significantly. Taken together, the inhibition of PXR transcriptional activity in HepXREM cells at 10 μM by camptothecin, hypericin, vinblastine, taxol, digitonin and etoposide may be contributed by inhibiting the cell proliferation causing apoptosis. However inhibition of PXR transcriptional activity by sulforaphane is not caused by cell toxicity since sulforaphane did not affect the cell proliferation (**Figure 11** and **Figure 13**).

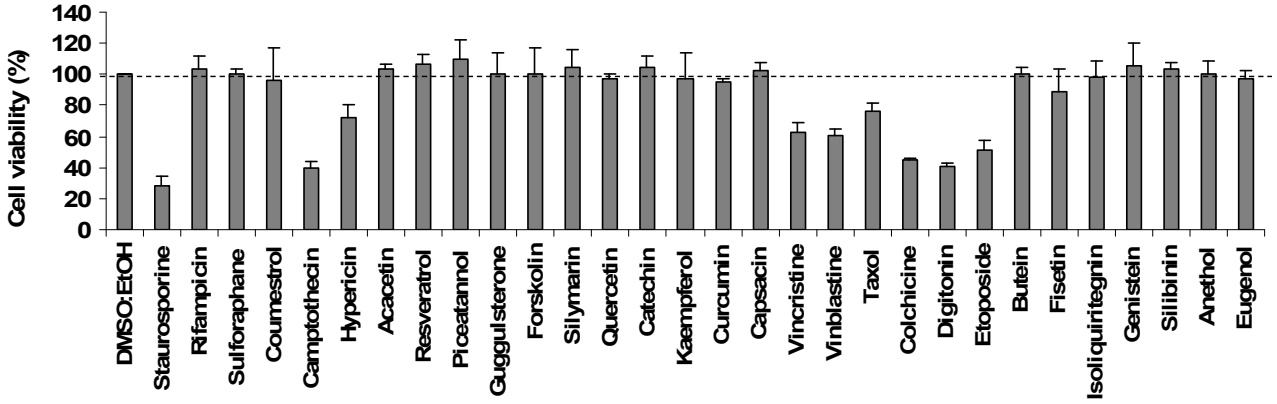


Figure 13: Effect of herbal drugs on cell viability. HepG2 c*ells were propagated in 96 well culture plate and allowed to proliferate up to ~60% confluency. Then cells were treated with different herbal drugs at the concentrations of 10 μM for 24 h. Cell viability was determined using the MTT assay as described in 'Materials and Methods'. Here, staurosporine at 0.1 μM concentration was used as a control which induces cell apoptosis. Data are expressed as the mean ± S.D. of three independent experiments.*

Analysis of drug effect on cells morphology by phase contrast microscopy

HepG2 cells (hepatoma), an anchorage-dependent cell line, are epithelial in morphology and frequently used in in vitro models for human biotransformation studies. To evaluate this parameter HepG2 cells were propagated in 35 mm culture plate with complete DMEM and allowed to proliferate up to ~60% confluency. Then cells were treated with different drugs for 24 h. All the drugs used were studied for their effect on the HepG2 cell morphology at the optimized concentration of 10 μM. After 24 h of incubation period, cultured cells were viewed under phase contrast microscope. Morphology of the drug-treated cells were compared with their corresponding cell line treated with drug solvent alone (DMSO:ethanol in 1:1 ratio) (referred as control). **Figure 14** shows representative cell images following herbal treatments at 10 μM for 24 h. When compared to control (DMSO:ethanol treatment) a few herbal drugs produced dramatic morphologic changes while others did not produce any such change in cellular morphology of HepG2 cells (**Figure 14**). The addition of taxol at 10 μM concentration for 24 h caused alteration in cell shape with fragmented nucleus. All the cells in culture were

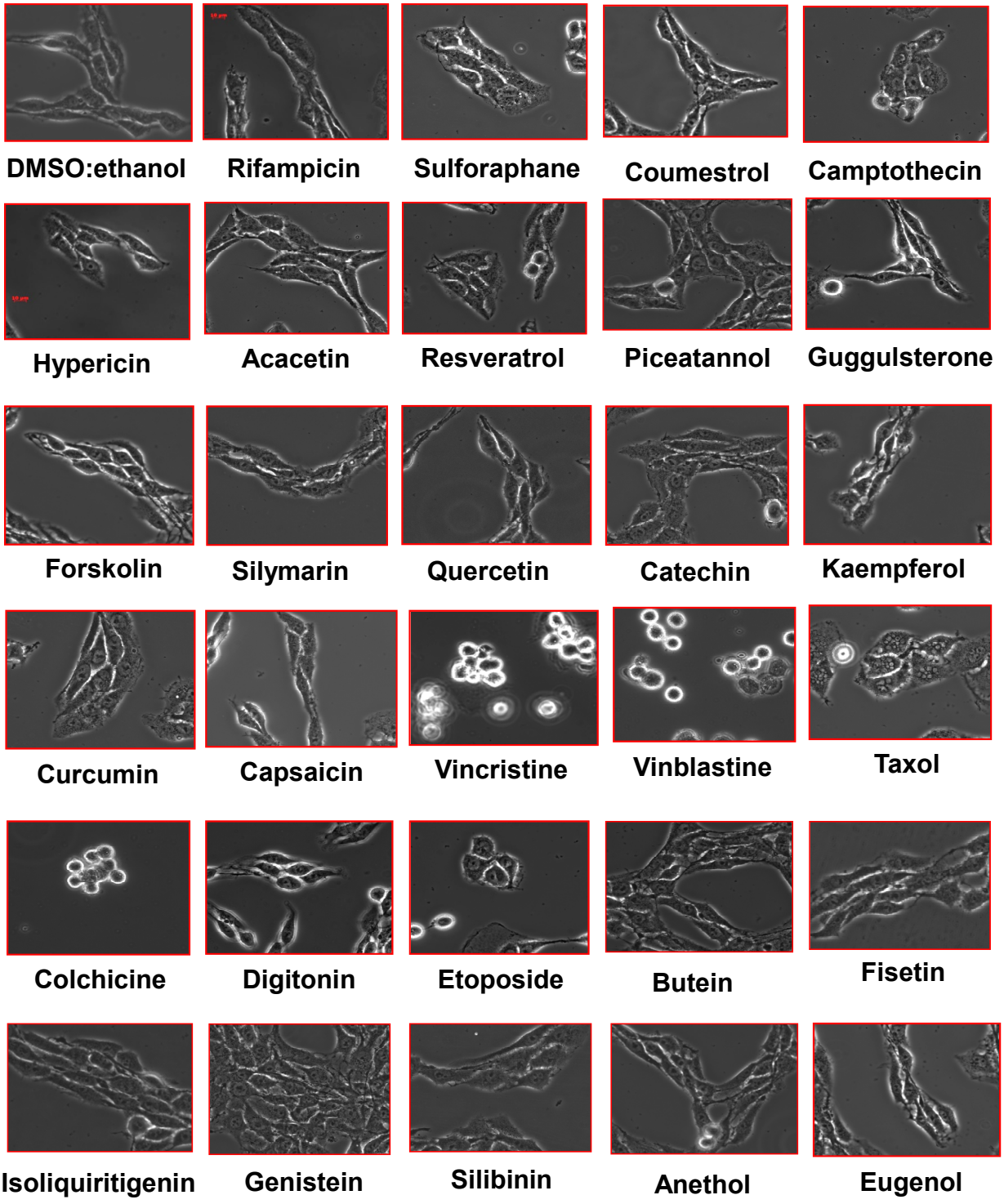


Figure 14: Effect of different herbal drugs on the morphology of human liver cells, HepG2. *HepG2 cells were seeded onto coverslips in 35 mm culture plates and propagated up to ~60% confluency and then treated with different herbal drugs including prescription drug rifampicin, a prototypical ligand of PXR at 10 µM for 24 h. After 24 h of drug treatment, morphological evaluation of cells was done under phase-contrast microscopy.*

rounded and arrested in mitosis by vincristine, vinblastine or colchicine. Camptothecin, etoposide and digitonin also altered the cell morphology. Rest of the drugs used in this study did not provoke any changes in HepG2 cell morphology at 10 µM concentration following 24 h incubation (**Figure 14**).

Activation of PXR by herbal drugs also modulates MDR1 promoter

Since PXR is a key regulator of the genes involved in xenobiotic detoxification and elimination of drugs, xenobiotics including CYP3A4, CYP2B10, CYP2C, drug transporter genes; MDR1, MRP2 and MRP3 etc. We have screened the effect of herbal drugs on CYP3A4 promoter by PXR activation or repression. Further to see if the activation of PXR by these drugs is not promoter dependent we used another PXR regulated gene promoter, MDR1 for our study. To see the effect of herbal drugs on PXR transcriptional activity on MDR1promoter, HepG2 cells were transiently co-expressed with pSG5-PXR expression plasmid and MDR1-Tk-Luc promoter-reporter construct. The cells were treated with vehicle or 10 µM of the herbal drugs and further incubated for 24 h. After 24 h of expression period luciferase activity was taken.

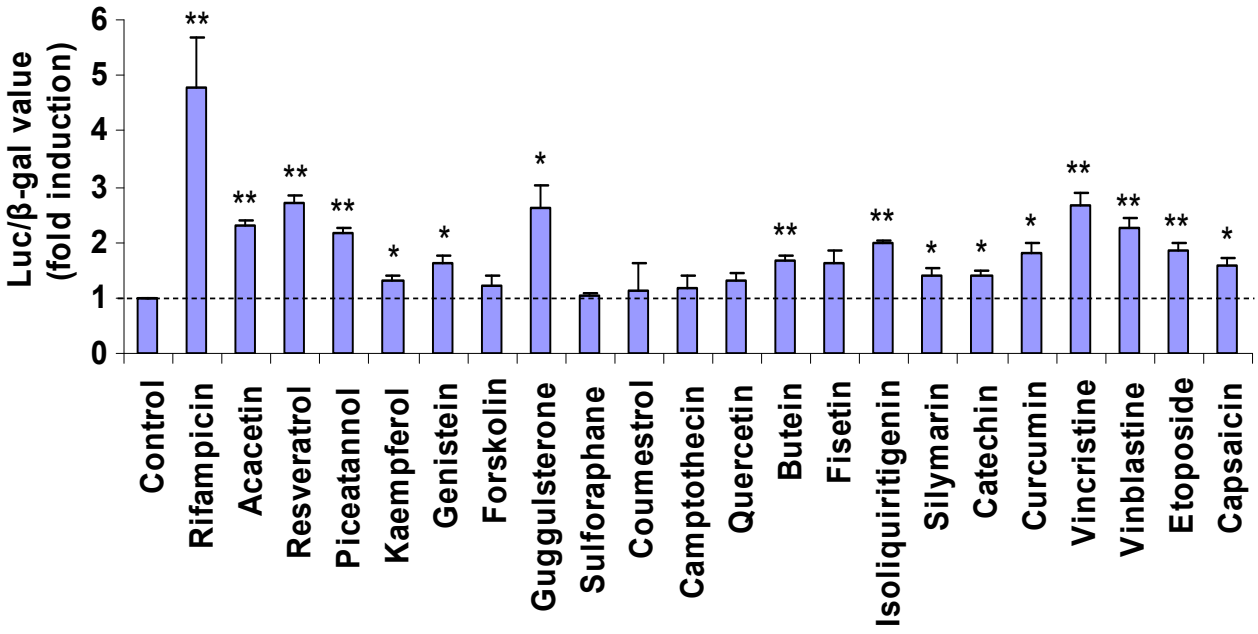


Figure 15: Activation of PXR by herbal drugs also modulates MDR1promoter in luciferase assay. *HepG2 cells were seeded in 24-well plate and then co-transfected with pSG5-PXR expression plasmid and MDR1-Tk-Luc promoter-reporter construct (p-7975/7013/Tk) in a ratio of 1:8. After 10-12 h of incubation period, complete DMEM was added and the cells were treated with*

vehicle or 10 μM of indicated drugs and further incubated for 24 h. After 24 h of expression period luciferase activity was taken. Relative fold activity was calculated in comparison to DMSO:ethanol induced luciferase activity. Data are expressed as the mean ± S.D of three independent experiments. Asterisks (and **) signify luciferase values that differed significantly from the scores of corresponding controls (P<0.05 and P<0.01 respectively in Student's t-test).*

Relative fold activity was calculated in comparison to DMSO:ethanol induced luciferase activity. **Figure 15** showed the effect of herbal drugs on PXR transcriptional activity on MDR1 promoter. Acacetin, resveratrol, piceatannol, guggulsterone, genistein, butein, isoliquiritigenin, vincristine, vinblastine, curcumin and etoposide increased the PXR transcriptional activity strongly. Rifampicin produced highest PXR activation among all the drugs. Kaempferol, catechin, capsaicin and silymarin transactivated PXR significantly. However, forskolin, coumestrol, quercetin, and fisetin did not affect PXR transcriptional activity. Our results indicated that the activation of PXR by these drugs was not only restricted to CYP3A4 promoter but also these drugs modulated the MDR1 promoter by PXR activation but differently.

Acacetin and resveratrol induce CYP3A4 and MDR1 mRNA expression in HepG2 cells

Acacetin and resveratrol along with other drugs increased the PXR transcriptional activity on CY3A4 and MDR1 promoters in luciferase assay. We further checked the effect of acacetin and resveratrol on the endogenous expression levels of CYP3A4 and MDR1 gene in HepG2 cells. To execute the effect of acacetin and resveratrol on CYP3A4 and MDR1 gene expression, HepG2 cells were seeded into 60 mm plates and next day cells were treated with vehicle control or 10 μM rifampicin, acacetin and resveratrol and allowed to incubate for 24 h. After 24 h, total RNA was isolated as described into 'material and methods' and quantitative real time PCR was performed using human CYP3A4 and MDR1 primers with syber green. **Figure 16** showed the effect of acacetin and resveratrol on the endogenous expression levels of CYP3A4 and MDR1 gene. Our results indicated that acacetin and resveratrol induced the CYP3A4 expression 2.2 folds and 2.5 folds respectively. However rifampicin induced CYP3A4 nearly 3.7 folds (**Figure 16A**). Further, acacetin

and resveratrol induced the MDR1 expression nearly 1.5 folds and 2.3 folds respectively. Rifampicin induced MDR1 expression nearly 3.5 folds (**Figure 16B**).

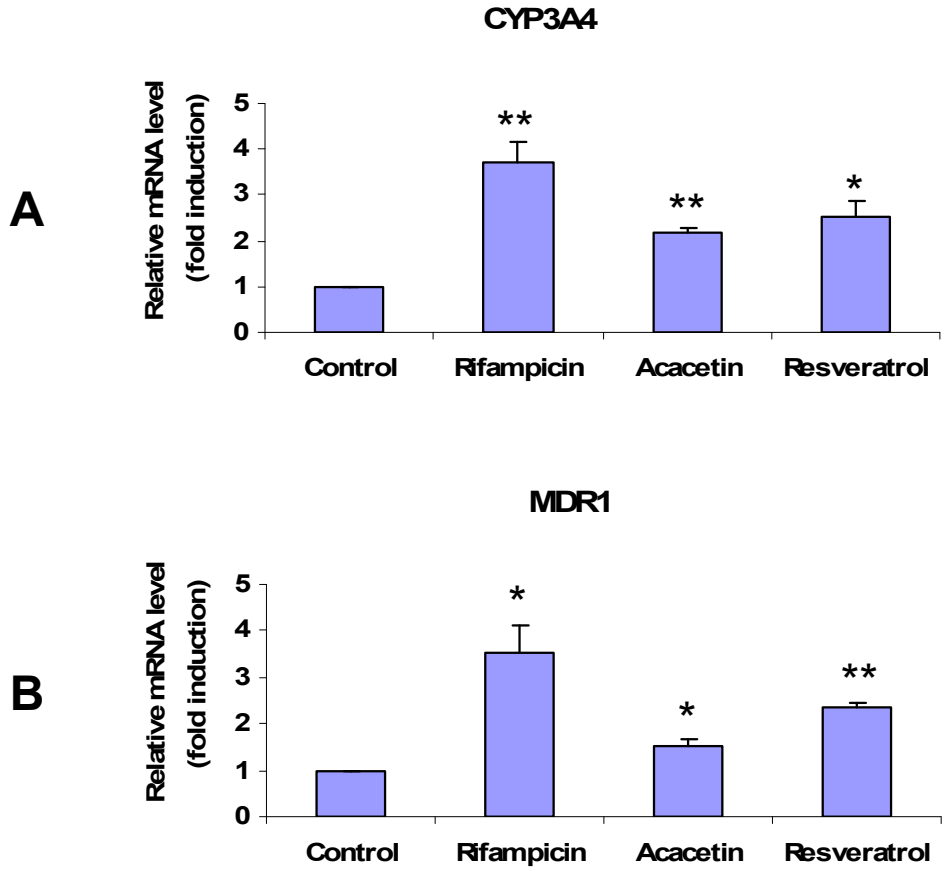


Figure 16: Acacetin and resveratrol induced CYP3A4 and MDR1 mRNA expression in HepG2 cells. ***A)*** *Human CYP3A4 and* ***B)*** *MDR1 mRNA expression were analyzed by quantitative real-time PCR in HepG2 cells after the treatment of vehicle control or 10 μM rifampicin, acacetin and resveratrol for 24 h. Data were normalized with GAPDH. Results were presented as fold increase over the vehicle control. Data represent the mean ± S.D of three independent experiments. Asterisks (* and **) signify values that differed significantly from the scores of corresponding controls (P<0.05 and P<0.01 respectively in Student's t-test).*

Anethol, etoposide, eugenol and camptothecin inhibit rifampicin-mediated PXR transcriptional activity and act as PXR antagonists

PXR antagonists would be useful to study the molecular basis of receptor function. In addition, clinically they may prevent drug-drug interactions and adjust the efficacy of therapeutics that serves as PXR agonists. To date, only five PXR antagonists have been reported that include

ecteinascidin-743 (ET-743) (Synold et al, 2001), ketoconazole (Huang et al, 2007), sulforaphane (Zhou et al, 2007), coumestrol (Wang et al, 2008a), and recently camptothecin (Chen et al, 2010). Therefore, to check if the drugs that have given low or no activity or suppressed PXR activity have PXR antagonistic property, we have done PXR transcriptional assay treated with drugs in combination with rifampicin. In this context, HepXREM cells were grown in 48-well culture plates. On reaching ~60% confluency, cells were either vehicle treated (control) or treated with rifampicin 10 μM alone or indicated herbal drugs alone at 1.0 μM concentration (**Figure 17**) or rifampicin in combination with indicated drugs and further incubated in a CO2 incubator. After 24 h of incubation period cell were lysed and luciferase activities were determined as described under 'optimized protocol in 48-well culture plate'. **Figure 17** showed that 1.0 μM of anethol, etoposide and camptothecin suppressed the PXR transcriptional activity significantly even when treated alone. Interestingly, 1.0 μM of anethol, etoposide, eugenol and camptothecin inhibit the rifampicin-induced PXR transcriptional activity significantly (**Figure 17**).

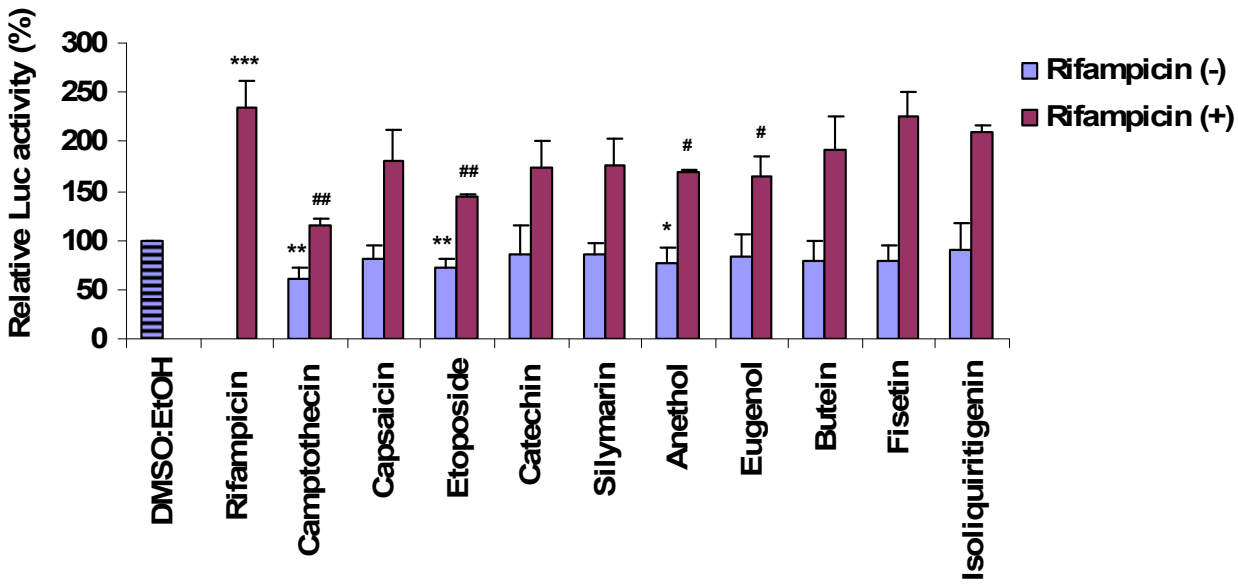


Figure 17: Inhibition of rifampicin-mediated PXR transcriptional activity by herbal drugs in HepXREM cell line. *HepXREM cells were seeded in 48-well culture plates. On reaching ~60% confluency, cells were either vehicle treated (control) or treated with rifampicin 10 μM alone or indicated herbal drugs alone (1.0 μM) or rifampicin in combination with indicated drugs and further incubated in a CO_2 incubator. After 24 h of incubation period cells were lysed*

and luciferase activities were determined as described under 'optimized protocol in 48-well culture plate'. Luciferase values are expressed as relative Luc activity of control cells. Data represent the mean ± S.D. of three independent experiments. Asterisks (, ** and ***) signify luciferase values that differed significantly from the score of corresponding control (P<0.05, P<0.01 and P<0.001 respectively in Student's t-test). While the symbol (# and ##) signify luciferase values that differed significantly from the score of rifampicin alone (P<0.05 and P<0.01 respectively).*

However, 1.0 μM of capsaicin, catechin, silymarin, butein and isoliquiritigenin also inhibit the rifampicin-induced PXR transcriptional activity but non-significantly (**Figure 17**). These results indicated that anethol, etoposide and eugenol are novel PXR antagonists. Additionally, camptothecin also worked as PXR antagonists in our experiments however it was recently reported as PXR antagonist when we were in progress with our research work (Chen et al, 2010).

Molecular docking of herbal drugs on PXR

The large and promiscuous ligand binding pocket of PXR accepts molecules of widely varying sizes and is likely capable of binding small molecules in multiple orientations. To determine whether these herbal drugs activate PXR by directly binding to it, we tested acacetin, resveratrol, piceatannol, kaempferol, genistein, forskolin, guggulsterone, curcumin, coumestrol, butein, fisetin, isoliquiritigenin, etoposide, anethole, nicotinamide and sirtinol molecular docking study in human PXR-LBD using AutoDock Tool. AutoDock Tool predicted the mode of herbal drugs binding to human PXR-LBD. Herbal drugs were positioned in the binding pocket (**Figure 18**). The top pose was selected based on the AutoDock score. Binding energy of different drugs and amino acid residues involved in the binding of individual drugs to hPXR LBD were tabulated in **Table VII**. The binding energies of different drugs are comparable with the binding energy of rifampicin, a well-known PXR agonist (**Table VII**). These results indicated that these herbal drugs could directly interact with PXR to promote coactivator recruitment, thus activating PXR as agonists.

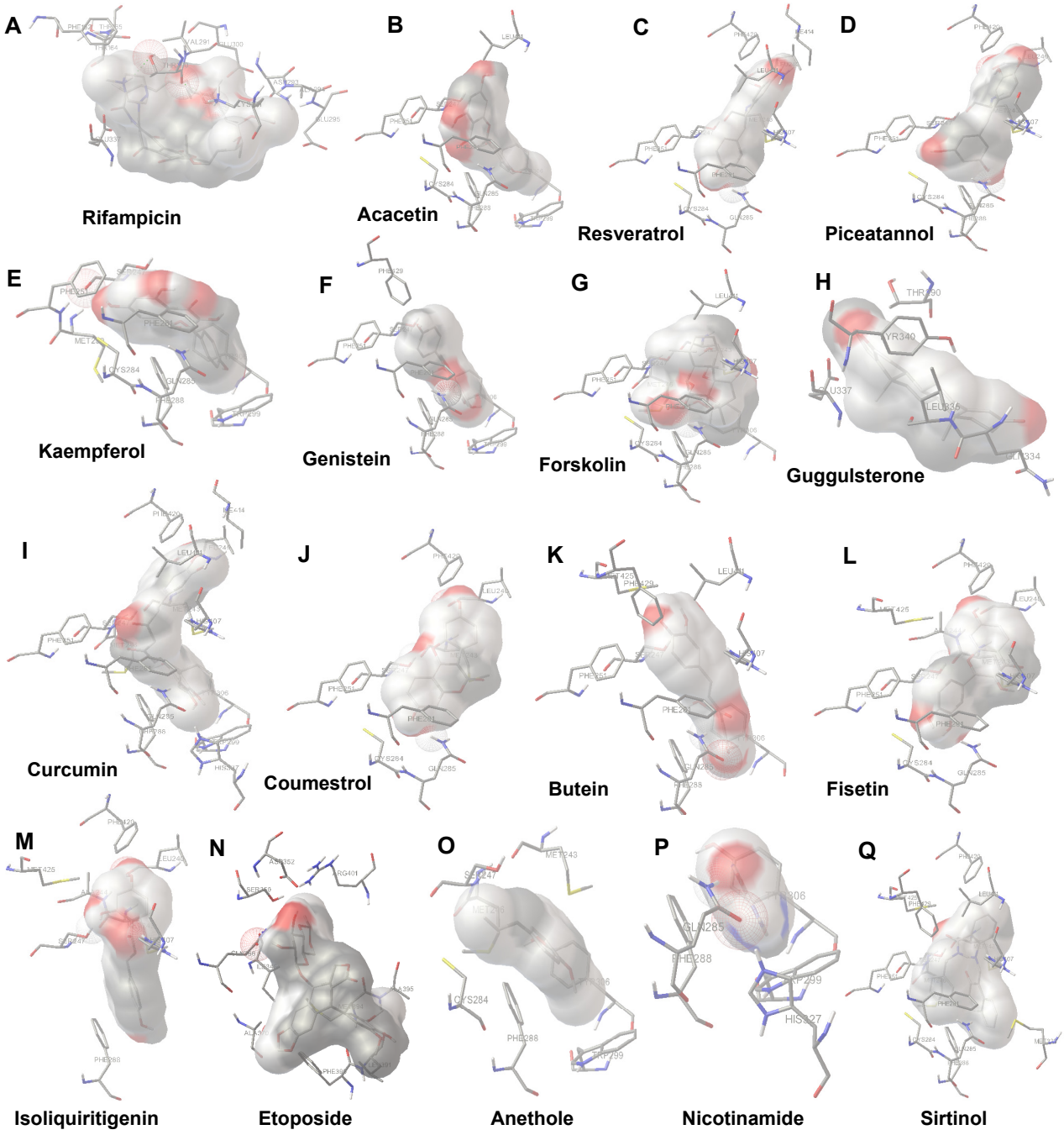


Figure 18: Molecular docking of different herbal drugs to the ligand-binding domain of hPXR. *Rifampicin (A), acacetin (B), resveratrol (C), piceatannol (D), kaempferol (E), genistein (F), forskolin (G) , Guggulsterone (H), Curcumin (I), Coumestrol (J), Butein (K), Fisetin (L), Isoliquiritigenin (M), Etoposide (N), Anethole (O), Nicotinamide (P) and Sirtinol (Q) were docked to the ligand-binding domain of hPXR. Shown are the key amino acids involved in the interactions and hydrogen bonding between drugs and the ligand-binding domain of hPXR.*

Table VII: Binding energy of different drugs and amino acid residues involved in the binding of individual drugs to hPXR LBD.

S. No.	Drugs	Binding energy	Amino-acid in close contact
1.	Rifampicin	-5.13	PHE152, THR164, THR165, Val211, Leu239, Leu240, Met243, Ala244, Met246, Met250, Phe251, Phe281, Cys284, Phe288, THR290*, VAL291, Trp299, ASN293, ALA294, GLU295, GLU300, Tyr306, Met323, Leu324, LYS331*, GLU337, Leu411, Ile414, Phe420, Met425
2.	Acacetin	-6.57	SER247, PHE251, PHE281, CYS284, GLN285, PHE288, TRP299, TYP306, LEU411
3.	Resveratrol	-5.76	LEU240, MET243, SER247, PHE251, PHE281, CYS284, GLN285*, HIS407, LEU411, ILE414, PHE420
4.	Piceatannol	-5.61	LEU240*, MET243, SER247, PHE251, CYS284, GLN285*, PHE288, HIS407, PHE420
5.	Kaempferol	-6.53	SER247*, MET250, PHE251, PHE281, CYS284, GLN285, PHE288, TRP299, TYP306
6.	Genistein	-6.5	SER247, PHE251, PHE281, GLN285*, PHE288, TRP299, TYP306, PHE429
7.	Forskolin	-6.71	MET243, MET246, SER247, PHE251, PHE261, CYS284, GLN285*, PHE288, TYP306, HIS407, LEU411
8.	Curcumin	-8.45	LEU240, MET243, MET246, SER247, PHE251, PHE281, GLN285, PHE288, TRP299, TYP306, HIS327, HIS407, LEU411, ILE414, PHE420
9.	Guggulsterone	-3.84	THR290, GLN334, LEU335, GLU337, TYR340
10.	Coumestrol	-6.6	LEU240, MET243, SER247*, PHE251, PHE281, CYS284, GLN285*, PHE420
11.	Butein	-6.32	SER247, PHE251, PHE281, GLN285*, PHE288, TYP306, HIS407, LEU411, MET425, PHE429
12.	Fisetin	-6.39	LEU240*, MET243, ALA244, SER247*, PHE251, PHE281, CYS284, GLN285, HIS407, PHE420, MET425
13.	Isoliquiritigenin	-6.15	LEU240*, ALA244, SER247, PHE288, HIS407*, PHE420, MET425
14.	Etoposide	-5.3	ILE346, SER350, ASP352, GLN366*, ALA370, PHE390, LEU391, MET394, ALA395, ARG401
15.	Anethole	-4.35	MET243, MET246, SER247,CYS284, PHE288, TRP299, TYP306
16.	Nicotinamide	-4.27	GLN285*, PHE288, TRP299, TYP306, HIS327
17.	Sirtinol	-10.37	MET243, MET246, SER247*, PHE251, PHE281, CYS284, GLN285, PHE288, MET323, HIS407, LEU411, PHE420, MET425, PHE429

* indicates *amino acid involved in H-bond formation with drugs.*

Acacetin, resveratrol, piceatannol and kaempferol increase PXR interaction with nuclear receptor coactivators SRC1 and PBP

Nuclear receptor coactivators are one of the major factors to determine the transcriptional activity of nuclear receptors. The common nuclear receptor coactivators includes SRC1, SRC2, SRC3, PBP (peroxisome proliferator-activated receptor (PPAR)-binding protein), CBP, etc. These have been shown to contribute to the ligand-induced activation of PXR along with other nuclear receptors (Ding et al, 2005). To find out the possible molecular mechanism how these drugs induce the CYP3A4 and MDR1 gene by PXR activation in cells, we tested whether treatment of these drugs could recruit nuclear receptor coactivators SRC1 and PBP to PXR. We performed mammalian two-

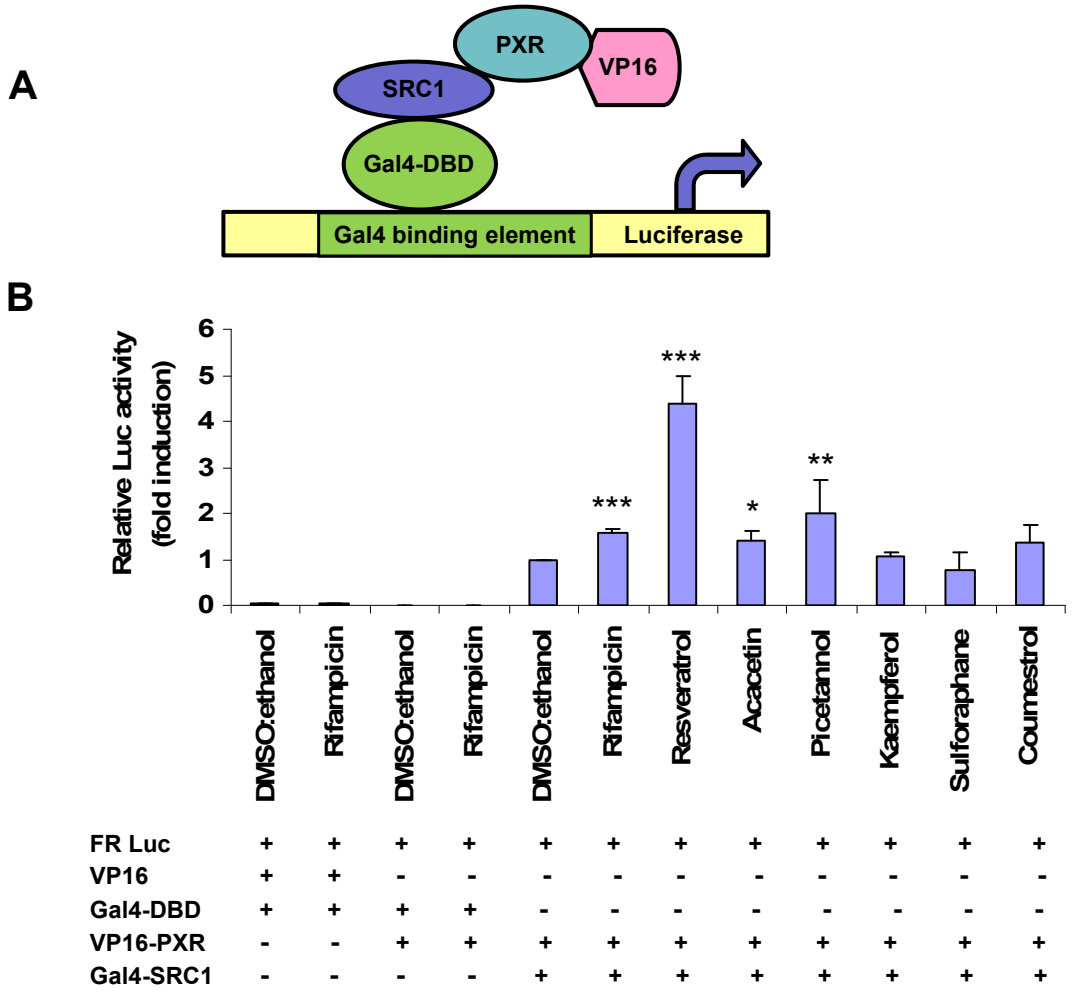


Figure 19: Effect of herbal drugs on PXR and steroid receptor activator-1 (SRC1) interaction. *HepG2 cells were seeded into 12-well culture plates. Next day, cells were cotransfected with the Gal4-responsive pFR-Luc reporter gene,*

Gal4 vector, Gal4-SRC1, VP16 vector, VP16-hPXR plasmids as per shown in scheme. Following the transfection period, the cells were treated with vehicle or 10 μM of the indicated drugs and allowed to incubate for 24 h. After 24 h luciferase assays were performed as described in 'Materials and Methods'. Data are expressed as the mean ± S.D. 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).*

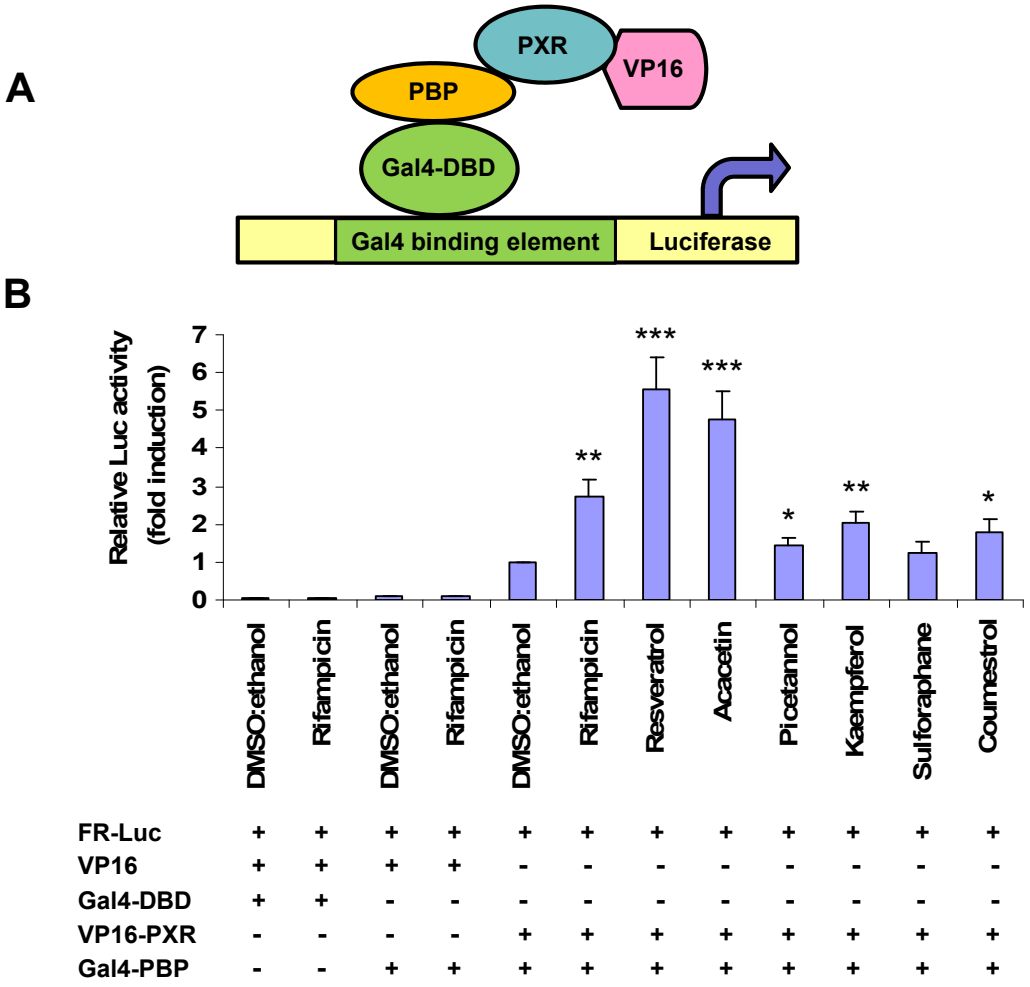


Figure 20: Effect of herbal drugs on PXR and PPAR-binding protein (PBP) interaction. *HepG2 cells were cotransfected with the Gal4-responsive pFR-Luc reporter gene, Gal4 vector, Gal4-PBP, VP16 vector, VP16-hPXR plasmids as per shown in scheme. Following the transfection period, the cells were treated with vehicle or 10 μM of the indicated drugs and allowed to incubate for 24 h. After 24 h luciferase assays were performed as described in 'Materials and Methods'. Data are expressed as the mean ± S.D. 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).*

hybrid analysis with PXR and coactivators SRC1 and PBP. HepG2 cells were cotransfected with the expression vectors encoding the receptor interacting domains from the coactivator protein SRC1 and PBP fused to GAL4, together with VP16-tagged human PXR and the GAL4-responsive luciferase reporter gene pFR-LUC as shown in the scheme (**Figure 19** and **Figure 20**). Acacetin, resveratrol, piceatannol, and kaempferol increased the interaction between PXR and all coactivator proteins SRC1 and PBP in HepG2 cells (**Figure 19** and **Figure 20**). Rifampicin the well-known human PXR ligand was used as positive control. These results imply that acacetin, resveratrol, piceatannol, and kaempferol recruits nuclear receptor coactivators to PXR in live cells and consequently they triggers the gene transcription of PXR regulated gene CYP3A4.

SIRT1 inhibitors nicotinamide and sirtinol inhibit both basal and induced PXR transcriptional activity

Here, in this part of the study, we have analyzed the effect of SIRT1 inhibitors, nicotinamide and sirtinol on drug-mediated transcriptional activity of PXR. SIRT1 inhibitors are included under the category of Class III HDAC inhibitor. Activity of Class III HDACs is not affected by class I, II and IV HDACs inhibitors (Xu et al, 2007). HDACs are implicated in diverse cellular processes including regulation of transcription (Minucci and Pelicci, 2006; Xu et al, 2007). In our study, resveratrol (known SIRT1 activator) and its metabolite piceatannol strongly transactivate the PXR transcriptional activity. In this context, we asked an obvious question whether SIRT1 have some role in PXR transactivation or not. If yes, then resveratrol and picetannol mediated activity may be suppressed by SIRT1 inhibitors nicotinamide and sirtinol. Therefore, we performed luciferase assay in HepXREM cell line with SIRT1 inhibitors/activators alone or in combination. HepXREM cells were propagated in 48-well culture plate and allowed to propagate to ~60 % confluency. Then cells were treated with 10 μM sulforaphane (**Figure 21A**), 10 μM camptothecin (**Figure 21B**) known PXR antagonists and 5.0 mM concentration of nicotinamide (**Figure 21C**), 50 μM sirtinol (**Figure 21D**) in absence or presence of 10 μM concentration of PXR activators, rifampicin, acacetin, resveratrol or

piceatannol and incubated for 24 h. After 24 h of incubation, PXR transcriptional activity was estimated by luciferase assay. Our results indicated that nicotinamide inhibited PXR transcriptional activity alone as well as it inhibited rifampicin, acacetin, resveratrol and piceatannol mediated PXR transcriptional activity (**Figure 21C**). Alternatively, sirtinol did not inhibit basal transcriptional activity of PXR but it inhibited the PXR activators mediated activity (**Figure 21D**). This study delineates that SIRT1 inhibitors, nicotinamide and sirtinol act as a novel antagonists of the nuclear xenobiotic receptor PXR (**Figure 21 C** and **D**) and also indicates the possibility of role of SIRT1 in PXR transactivation.

DISCUSSION

PXR is an important target in drug discovery and development studies. It plays an essential role in regulation of critical sets of genes encoding certain drug metabolizing enzymes and transporters involved in drug metabolism. Originally, the human PXR (hPXR) was shown to regulate the expression of the CYP3A gene (Bertilsson et al, 1998), encoding the major phase I enzyme in human drug metabolism (Thummel and Wilkinson, 1998). In addition, other CYP450 drug metabolizing enzymes, certain phase II conjugating enzymes and a number of transmembrane transporters are induced via PXR activation (Maglich et al, 2002). PXR is highly promiscuous in nature therefore binds to a broad spectrum of structurally distinct ligands including naturally occurring and synthetic compounds (Lehmann et al, 1998; Jones et al, 2000). Although various methods are available to assess the pharmacological properties for drug screening, but due to their high cost, lengthy experimental duration they offers limited use. Animal experiments are often not sufficiently meaningful or predictive for these PXR ligands due to their species-specific PXR binding and subsequent regulation of CYP3A subfamily members. Primary human hepatocyte cultures have been considered to be the 'standard model system' for in vitro induction assessment (Madan et al, 2003). There are some disadvantages associated with the use of primary cultures, including limited access to human liver specimens and considerable variability among donors and samples. Thus other in vitro systems are investigated for their usefulness.

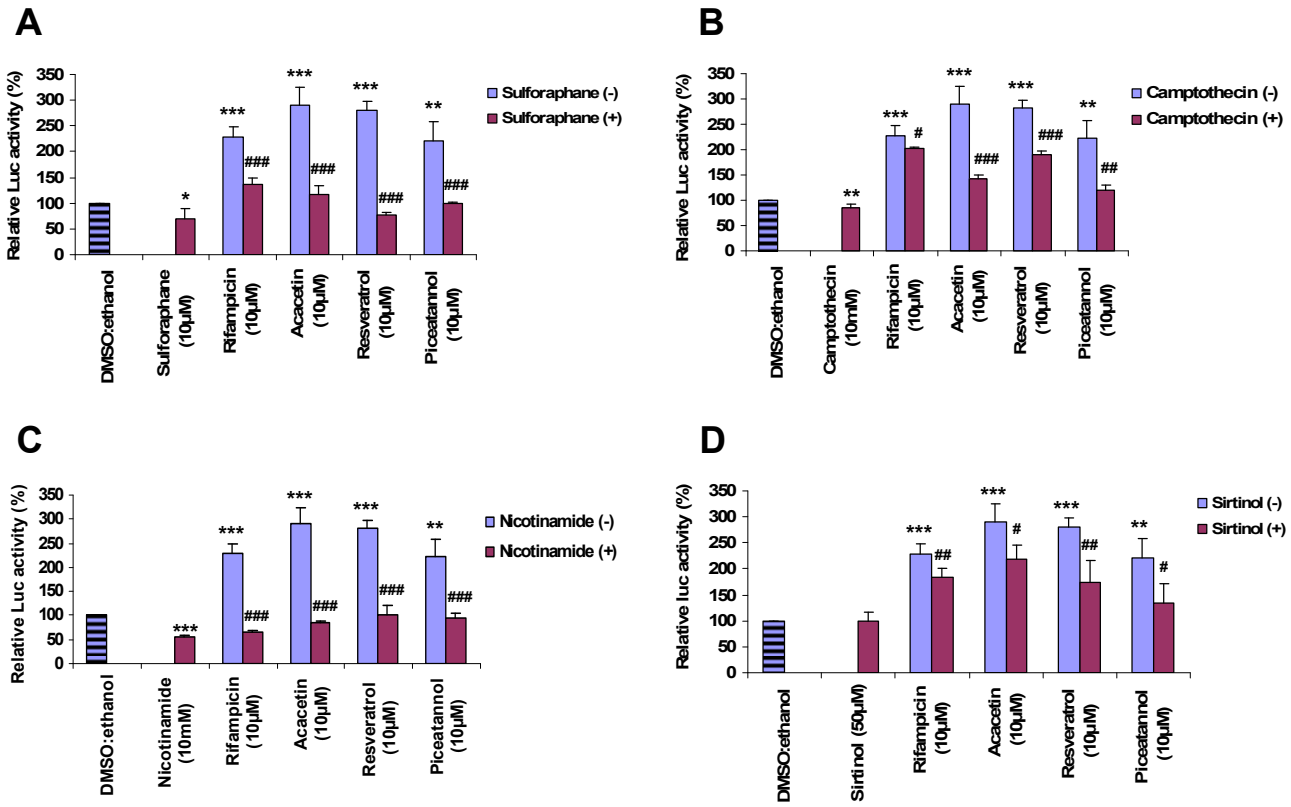


Figure 21: Effect of SIRT1 inhibitors on rifampicin, acacetin, resveratrol and piceatannol-mediated PXR transcriptional activity in HepXREM cell line. *Graph showing transcriptional activity of PXR in HepXREM cell line treated with indicated drugs. HepXREM cells were seeded in 48-well culture plates. On reaching ~60% confluency, they were either vehicle treated (control) or treated with different drugs (rifampicin, acacetin, resveratrol or piceatannol) at 10 μM without or with PXR antagonist, sulforaphane (10 μM) (**A**) or camptothecin (**B**), SIRT1 inhibitors, nicotinamide (5.0 mM) (**C**) and sirtinol (50 μM) (**D**) and further incubated in a CO_2 incubator. After 24 h of incubation period, cells were lysed and luciferase activities were determined as described under 'optimized protocol in 48-well culture plate'. Luciferase values are expressed as relative Luc activity of control cells. Data represent the mean ± S.D. 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). While the symbol (#, ## and ###) signify luciferase values that differed significantly from the score of rifampicin, acacetin and resveratrol alone (P<0.05, P<0.01 and P<0.001 respectively).*

Most common approach is by transient transfection of promoter-reporter gene construct into a suitable cell line. Transient transfection of a xenobiotic response enhancer module (XREM) driven reporter gene into human hepatoma cells was found to be a suitable tool for the detection of species differences in PXR-dependent induction of gene expression in the case of the drug development

candidate EMD 392949 (Mueller et al, 2010). Furthermore, the potential for PXR activation by environmental pollutants among the family of brominated flame retardants could be identified (Fery et al, 2009) by this system. However, there may be considerable biological and experimental variation within this system. Additionally transient transfections are costly and time-consuming. In this context, we needed alternative methods, which provide faster, easier and more reproducible results. For this purpose, we have generated stably integrated hepatic cell line that served as a useful tool for the screening of herbal drugs, xenobiotics and endobiotics.

In the present study, we generated stable cell line in HepG2 cells, stably integrated with human PXR and a commonly used CYP3A4 promoter-reporter i.e. XREM-Luc and named as HepXREM. The result obtained in HepXREM cell line is in agreement with previous findings, i.e., showing that 'prototypical' PXR activator rifampicin produces enhanced PXR-transcriptional activity by rifampicin. Further, in search for PXR activators/antagonists we screened various structurally diverse anticancer herbal drugs by using the stable cell line HepXREM. Interestingly, our results showed that acacetin, resveratrol, kaempferol, guggulsterone, forskolin strongly transactivate PXR in HepXREM cell line. Quercetin, vincristine, vinblastine and hypericin activated PXR moderately. Additionally, MTT assay and morphological assessment by phase contrast microscopy indicated that acacetin, resveratrol, kaempferol and guggulsterone do not have any cytotoxic and morphological effect on HepG2 cells. Furthermore, acacetin and resveratrol induced the endogenous mRNA expression of PXR regulated CYP3A4 and MDR1 genes. The results obtained in HepXREM cell line need further investigation to confirm whether the transcriptional activity is a result of direct interaction between PXR and drugs or it is mediated through some other signalling pathway(s). Further, to understand the mechanism of action of these selected drugs at molecular level, interaction studies of PXR with its coactivators/corepressors carried out. Acacetin, resveratrol, picetannol, kaempferol, etc. increased the PXR interaction with nuclear receptor coactivator, SRC1 and PBP. Moreover, molecular docking of herbal drugs with the ligand binding domain of PXR indicated the possibility of direct interactions of these herbal drugs with LBD of PXR.

Moreover, it will be interesting to examine if these drug molecules are having some role in chromatin remodeling at promoters of PXR regulated genes. In our study, resveratrol (known SIRT1 activator) and its metabolite piceatannol strongly transactivated the PXR transcriptional activity. In this context using SIRT1 inhibitors nicotinamide and sirtinol, we investigated whether SIRT1 have role in PXR transactivation or not? If yes, then resveratrol and picetannol mediated activity may be suppressed by SIRT1 inhibitors nicotinamide and sirtinol. Our results indicated that nicotinamide inhibits PXR transcriptional activity alone as well as it inhibits rifampicin, acacetin, resveratrol and piceatannol mediated PXR transcriptional activity. Alternatively, sirtinol could not alter basal transcriptional activity of PXR but inhibited the induced PXR transcriptional activity. These results imply that SIRT1 inhibitors, nicotinamide and sirtinol act as a novel antagonist of the PXR and also indicate the possibility of a role of SIRT1 in PXR mediated transactivation. It needs future investigation to confirm the role of SIRT1 in PXR transcription or PXR-regulated gene transcription by performing 'gain of function' or 'loss of function' experiments in HepXREM cell line. In conclusion, the analysis of PXR expression and inducibility of drug-metabolizing enzymes in HepXREM cell line exerts a pattern very similar to previous reports in tissues, primary cells and transient transfection assays. In particular, the major xenobiotic receptors and target genes among the CYPs are expressed making these cell lines a suitable tool for testing strategies. The use of stably integrated cell lines prepared in this study provides a tool for the screening and classification of test chemicals. The results with prototype inducer are in very good agreement with previous reports on non-transfected and transiently transfected cell models. These data demonstrate that the aforementioned approach provides a versatile and reliable tool for the evaluation of potency of test compounds with regard to the PXR signalling pathway. In conclusion, the present study provides screening system and method that facilitate the identification of compounds with potential to activate or inhibit PXR transcriptional functions. The study also reports some potentially novel activators and antagonists of PXR.

* * *

CHAPTER-II

Transcriptional regulation of PXR promoter by prospective anti-cancer herbal drugs

INTRODUCTION

The Pregnane & Xenobiotic Receptor (PXR), a member of the nuclear receptor super-family is a well-known xenobiotic sensing receptor. The highly promiscuous nature of PXR allows it to interact with a wide range of structurally diverse chemicals. Ligand-activated PXR up-regulates the expression of drug-metabolizing enzymes and drug transporters to protect the cell from chemical insults (Wilson and Kliewer, 2002; Zhou et al, 2009; Ihunnah et al, 2011; Gao and Xie, 2012). However, the physiological importance of PXR extends beyond xenobiotic protection. Subsequent studies have also shown the involvement of PXR in normal physiology and diseases such as hepatic steatosis, vitamin D homeostasis, bile acids homeostasis, steroid hormones homeostasis, inflammatory bowel diseases, cancer, etc. (Zhou et al, 2009; Ihunnah et al, 2011; Gao and Xie, 2012; Pondugula and Mani, 2013). PXR has emerged as a transcriptional activator of at least 40 genes, including sets of genes that are biologically important drug transporters and drug-metabolizing enzymes. Among the critical target genes it regulates phase I (CYP450), phase II (GST, SULT and UGT) and phase III (MDR, MRP and OATP) genes involved in metabolism and clearance of xenobiotics (Ihunnah et al, 2011; Gao and Xie, 2012). While PXR is known to transcriptionally activate many genes, its own transcriptional mechanisms remains inadequately explored. *In silico* analysis by Aouabdi et al have shown the presence of putative protein/DNA interaction sites within the 2 kilobases (kb) 5′ to the putative transcription start site of human PXR proximal promoter (Aouabdi et al, 2006). NR family members like GR, PPARα and HNF4α also has been shown to bind to these sites and may regulate PXR gene expression which is predominantly mediated by its proximal promoter (Pascussi et al, 2000; Aouabdi et al, 2006; Zhou et al, 2006; Gibson et al, 2006; Iwazaki et al, 2008). Further, to unravel the molecular mechanism of PXR gene regulation in details, its proximal promoter (upto -1096 upstream of transcription start site) was characterized in our laboratory (Saradhi, 2008). Our laboratory study has shown the mechanism of PXR regulation where PXR promoter acts as a transcriptional target for Sp1 protein family member(s) and heterogenous nuclear ribonucleoprotein K (hnRNP K) (Saradhi, 2008).

Herbal drugs and dietary active constituents are involved in the regulation of various transcription factors and cascade of signalling proteins in normal physiology and disease (Ding and Staudinger, 2005a; Dong et al, 2010; Gupta et al, 2011; Hsu et al, 2011; Whitlock and Baek, 2012). Several herbal drugs have been shown to regulate the transcriptional activity of nuclear receptors viz. ERα, PPARα, PPARγ, HNF4α, AR, GR, PR, PXR, CAR, LXR, FXR etc. (Brobst et al, 2004; Chang and Waxman, 2006; Chang, 2009; Harmsen et al, 2009, Li et al, 2012). However, there are only a few nuclear receptor (ERα, CAR, etc.) are exploited for their own transcriptional regulation (gene expression) by herbal drugs or dietary active constituents. Various herbal drugs have been shown to transactivate the master regulator PXR and subsequently up-regulating the drug metabolism enzymes of Phase I, II and III (Meijerman et al, 2006; Negi et al, 2008; Satsu et al, 2008; Chang 2009). However, a little is known about the transcriptional regulation of PXR gene by herbal drugs or other ligands. Since PXR is emerging as a multifunctional protein including its role in energy homeostasis, inflammatory bowel disease, cancer, etc. (Zhou et al, 2009; Ihunnah et al, 2011; Gao and Xie, 2012; Pondugula and Mani, 2013) it will be interesting to explore the novel insights underlying the transcriptional regulation of PXR by herbal drugs for better understanding the roles of PXR in the human normal physiology and pathophysiology. Simultaneously, it will also be exciting to find out PXR transcriptional modulators (activators/inhibitors) to control its gene expression in normal physiology and disease.

Therefore, to explore the transcriptional regulation of PXR gene by anti-cancer herbal drugs, we have generated two PXR-promoter cell lines; Hepx-1096/+43 and Hepx-497/+43 by stably integrating two selected regions of proximal PXR promoter-reporter constructs (characterized in our laboratory) in a liver cell line HepG2. Both the cell lines were characterized and used for the screening of PXR-promoter modulators using 29 anti-cancer herbal drugs. We observed that acacetin, resveratrol, piceatannol, genistein and kaempferol regulated PXR-promoter positively (up-regulation) while forskolin, sulforaphane, etoposide, hypericin, vinblastine regulated negatively (down-regulation). Additionally, forskolin and sulforaphane also inhibited the induced

PXR-promoter activity. Camptothecin, curcumin, taxol and colchicine showed inverse response to PXR-promoter in both the cell lines. In conclusion, our study provided the evidences for PXR-promoter regulation by herbal drugs and indicates the possibilities for involvement of different signalling pathways in PXR gene regulation utilized by herbal drugs.

RESULTS

For the systematic and specific study of the PXR gene regulation by herbal drugs, one need a cell based highly reproducible system which can be used for the initial screening of regulatory molecules, drugs, activators, inhibitors, etc. However, up to date, there is no stable cell line available for the study of PXR gene regulation. In this context, to full fill the requirements of our study for the PXR gene regulation and to find out PXR gene regulatory molecules (activators/inhibitors), we thought to generate a stable cell line of PXR-promoters which was initially characterized in our laboratory (Saradhi, 2008).

Preparation and characterization of cell lines with stable integration of proximal PXR promoter-reporter constructs

For preparation of PXR-promoter integrated stable cell lines, PXR proximal promoter-reporter constructs, p-1096/+43 Luc and p-497/+43 Luc were selected on the basis of functional analysis of different deletion constructs of PXR promoter (**Figure 22A** and **22B**) generated in our laboratory (Saradhi, 2008). HepG2 cells were transiently transfected with equimolar amounts of each construct and following 24 h incubation period, luciferase activities were determined (**Figure 22B**). When HepG2 cells were transfected with PXR promoter-reporter construct, p-1096/+43 Luc and compared to cells transfected with the empty luciferase vector, about 100% increase in the promoter activity was observed. A deletion of about 502 bases from the 5'-end (p-594/+43 Luc) showed a marginal increase in reporter activity. A further deletion of 97 bp (p-497/+43 Luc), however, showed a modest increase in reporter gene activity. Surprisingly, a further deletion of 100 bp (p-397/+43

Luc) resulted in a decrease in promoter activity suggesting, presence of putative activation element(s) between –497 and –397. Further progressive deletions resulted in first an increase in activity (in case of p-315/+43 Luc) followed by a decrease in promoter activity (p-197/+43 Luc). A surprising finding of this deletion analysis was the reporter gene activity of a construct encompassing bases from –197 to -83 that was devoid of TATA region. Taken together, these results suggest the presence of multiple putative regulatory elements on the proximal promoter especially in the sequence stretch between coordinates –497 and –397. Based on the results derived from luciferase assay of different PXR proximal promoter-reporter constructs, p-1096/+43 Luc and p-497/+43 Luc were selected for making stable cell line since p-1096/+43 Luc was full length and p-497/+43 Luc showed highest promoter activity.

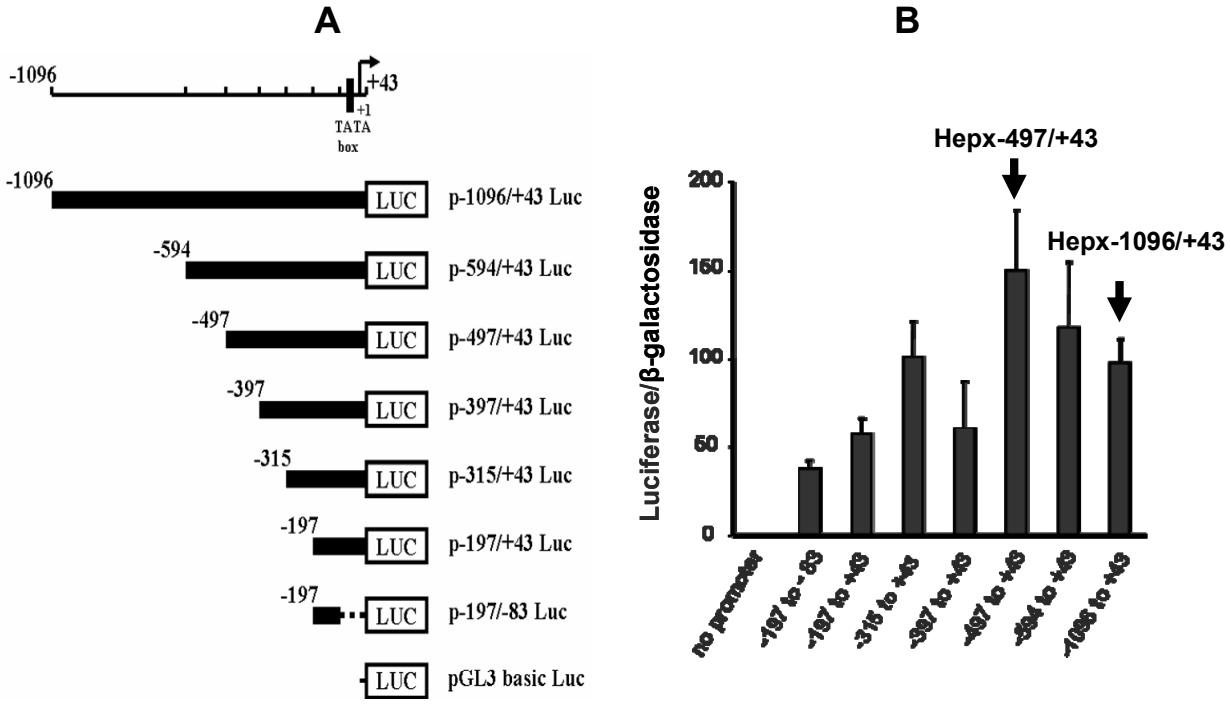


Figure 22: Deletion analysis of the proximal PXR promoter by luciferase reporter gene assays. *Schematic representation of the PXR proximal promoter and its various deletion constructs* **(A)**. *The relative positions of TATA box and transcription start site are indicated. Plot showing relative promoter activity of the various deletion constructs* ***(B)***. *The 'promoter-less basic luciferase reporter vector' and PXR-promoter-reporter constructs were transiently transfected along with ß-galactosidase as an internal control reporter gene into HepG2 cells. After 24 h of incubation period, cell lysate were prepared and luciferase and ß-galactosidase activities were determined. Luciferase values were normalized to*

ß-galactosidase values and shown as Luciferase/β-galactosidase (Luc/β-gal). Graph is plotted after subtracting the background Luc/β-gal values of 'promoter-less basic luciferase reporter vector' from Luc/β-gal value of PXR promoter-reporter constructs. Data represent the mean ± S.E. of three independent experiments. (Adapted and modified from Saradhi, 2008). The two constructs shown with arrow (Hepx-497/+43 and Hepx-1096/+43) were used for generation of stable cell lines used in the present study.

Generation of human liver cell lines with stably integrated PXR-promoter-reporter constructs

PXR proximal promoter-reporter constructs, p-1096/+43 Luc and p-497/+43 Luc were linearized with restriction enzyme SalI and BamHI respectively. DNA transfection was performed with Lipofectamine as per manufacture's protocol. HepG2 cells ($2.4x10^5$) were seeded in 35 mm culture plate and co-transfected with PXR proximal promoter-reporter construct and a vector that contains neomycin resistance gene (pcDNA3.1) in 10:1 molar ratio. Cells were allowed to double (up to 80% confluent) in complete medium. Later, cells were supplemented with complete medium containing 400 µg/ml of G418 (selective medium). The medium was replaced every third day. After two weeks of selection period, proliferating individual colonies were isolated and further propagated in separate culture dishes under selective conditions. Individual clones were screened and compared for the stable integration and response reflected by luciferase activity of PXR promoter. To determine the PXR promoter activities, cells were harvested and luciferase assay was performed as described in 'Materials and Methods'. The activity was expressed as relative luciferase activity (RLA). On the basis of RLA value, the clones of stably integrated hepatic cell lines of PXR promoter-reporter constructs, named 'Hepx-1096/+43' and 'Hepx-497/+43' were selected.

Selection of clones of human liver cell line stably integrated with PXR-promoter-reporter constructs

Clones with stably integrated PXR-promoter-reporter construct were selected with complete culture medium containing drug G418 (neomycin selective medium) as described in 'Materials and Methods'. In all, 22 clones of Hepx-1096/+43 and 29 clones of Hepx-497/+43 proliferating in selective

culture medium were collected, propagated and checked for presence of luciferase-reporter activity. For luciferase reporter assay of individual clones 3.0 x 10^4 cells/well in 48-well plate were seeded and cultured overnight in complete culture medium. Comparative results of different clones exhibiting modest to high PXR-promoter activity measured by luciferase-reporter are shown in **Table VIII**. Based on high luciferase reporter activities clone 11 and clone 9 of Hepx-1096/+43 and Hepx-497/+43 respectively were selected for further evaluation.

Table VIII: Luciferase reporter activities of different human liver cell clones having stably integrated PXR- promoter-reporter constructs.

S.No.	Hepx-1096/+43		Hepx-497/+43	
	Clone No.	Luc Activity	Clone No.	Luc Activity
1.	2	129.30	1	16.34
2.	7	41.38	3	44.38
3.	10	218.80	4	57.86
4.	**11**	**310.30**	6	95.02
5.	13	25.91	**9**	**1529.00**
6.	14	19.69	18	137.20
7.	16	22.23	--	--
8.	20	178.10	--	--

Characterization of Hepx-1096/+43 and Hepx-497/+43 (human liver cell lines stably integrated with PXR-promoter-reporter constructs)

To further characterize the selected clones (Clone no. 11 and 9) of Hepx-1096/+43 and Hepx-497/+43 stable cell lines, first, we checked the integration of PXR-promoter-reporter constructs (p-1096/+43 Luc or p-497/+43 Luc) in both the cell lines by PCR amplification of genomic DNA using specific primers of PXR-promoter. For this experiment, equal number of HepG2, Hepx-1096/+43 and Hepx-497/+43 cells were seeded in 60 mm culture plates in

complete DMEM medium for 24 h and following the incubation, genomic DNA was isolated as described in 'Materials and Methods'. PXR-promoter was amplified using specific primers of PXR-promoter. We observed amplified PCR products of expected sizes 550 bp and 1139 bp in HepG2, Hepx-497/+43 and Hepx-1096/+43 cell line respectively (**Figure 23A**) for human PXR-promoter. Faint band was observed in the lanes of HepG2 cells as compared to both the cell lines which showed very prominent bands of PXR-promoters (**Figure 23A**).

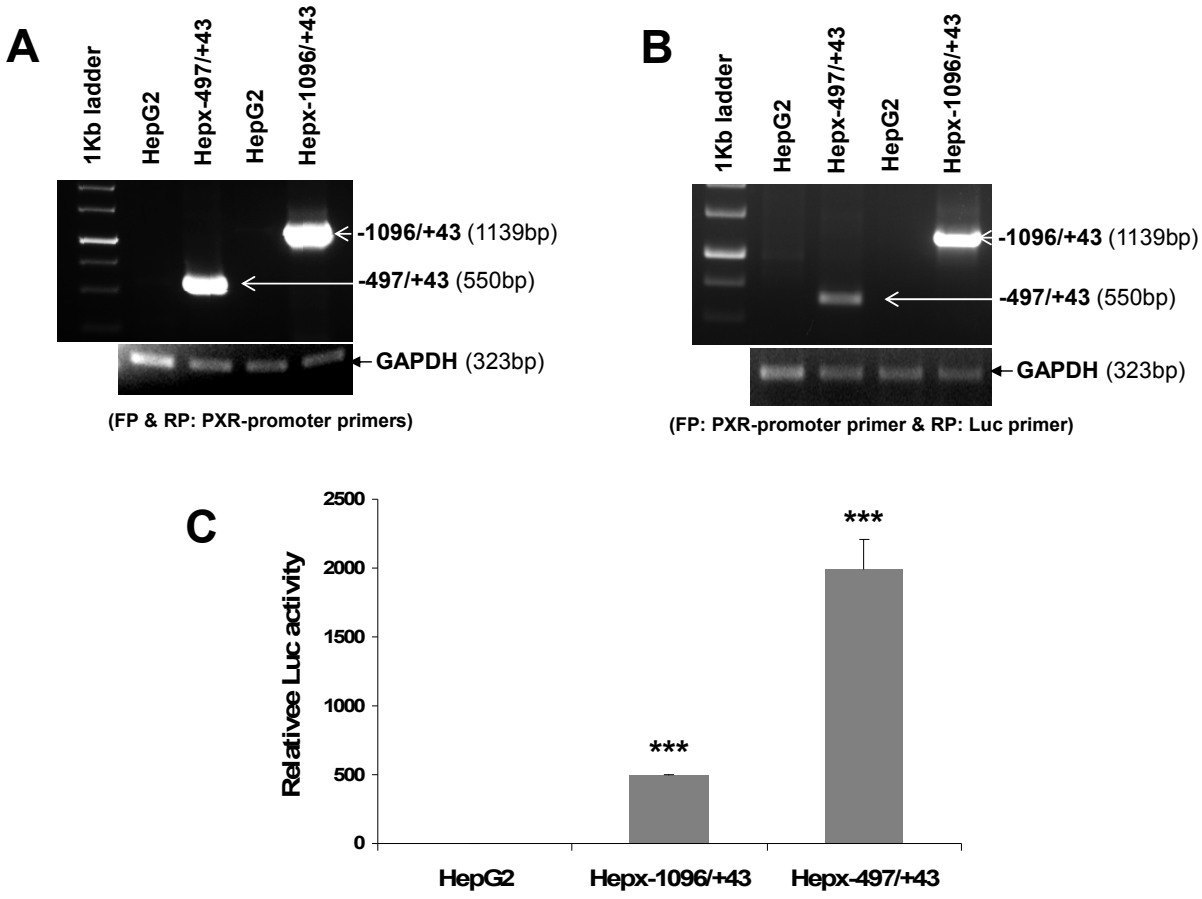


Figure 23: Characterization of Hepx-1096/+43 and Hepx-497/+43 stable cell lines. *(**A**) and (**B**) show PCR analysis of genomic DNA for PXR-promoter in HepG2, Hepx-1096/+43 and Hepx-497/+43 cell lines. GAPDH was used as an internal control. We observed amplified PCR products of expected sizes 550 bp and 1139 bp in Hepx-497/+43 and Hepx-1096/+43 cell line respectively (**A**) for human PXR-promoter. (**B**) indicates the band sizes of 569 bp and 1158 bp in Hepx-497/+43 and Hepx-1096/+43 cell line respectively. The differences between the band sizes of PXR-promoter in both the figures (**A** and **B**) are due to the primers. In (**B**), the reverse primers are overlapping the Luc gene. The band of 323 bp product size of human GAPDH gene was detected in all the cell lines. (**C**) demonstrates the transcriptional activity of PXR-promoter in Hepx-1096/+43 and*

Hepx-497/+43 cell lines. Equal number of HepG2, Hepx-1096/+43 and Hepx-497/+43 cells were seeded in 12-well culture plates and incubated for 24 h in CO_2 incubator. After 24 h of incubation, cells were harvested for luciferase assay. The relative values for reporter luciferase are plotted. The values represent the means ± SD of three independent experiments. Asterisks (***) signify luciferase values that differed significantly from the scores of corresponding control (p<0.001 in Student's t-test).

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For confirming our observations more stringently, we used another set of primers in which the reverse primer was of Luc gene and forward primer was the same (PXR-promoter). This set of primers will show band only when Luc gene is integrated with PXR-promoter. Therefore, there will be no band amplification in HepG2 samples. As expected, our results were in agreement to this logic. **Figure 23B** indicate the band sizes of 569 bp and 1158 bp in lanes of Hepx-497/+43 and Hepx-1096/+43 cell line respectively while there is no band in lanes of HepG2 cells. The differences between the band sizes of PXR-promoter in both the figures (**Figure 23A** and **23B**) are due to the different set of primers. Human GAPDH gene was used as internal control. The band of GAPDH was detected in all the cell lines with product size of 323 bp.

Further to determine the PXR promoter activities for functionality of PXR-promoter reporter in both the cell lines, equal number of HepG2, Hepx-1096/+43 and Hepx-497/+43 cells were seeded in 12-well culture plates and incubated for 24 h in CO_2 incubator. After 24 h of incubation, cells were harvested and luciferase assay was performed as described in 'Materials and Methods'. The relative values for reporter luciferase were plotted. Our results showed that the relative luc activities of Hepx-1096/+43 and Hepx-497/+43 cells were 500 and 2000 times more than the activity of HepG2 cells (**Figure 23C**). These results were extremely significant (p<0.001).

Modulation of PXR-promoter activity by prospective anticancer herbal drugs

Herbal drugs have been shown to activate nuclear receptor family members including PXR. However, it is not clear that herbal drugs only regulate drug metabolism enzymes and transporters by activating PXR or affect PXR gene expression. A little is known about PXR gene promoter regulation by

herbal drugs. In this context, to study the regulation of PXR-promoter by herbal drugs and to find out the modulators of PXR gene we utilized the PXR-promoter stable cell lines Hepx-1096/+43 and Hepx-497/+43 (stably integrated with PXR promoter-reporter constructs) in conjugation with luciferase assay. Initially, 28 herbal drugs namely acacetin, resveratrol, piceatannol, genistein kaempferol, coumestrol, quercetin, catechin, camptothecin, guggulsterone, silymarin, capsaicin, vincristine, taxol, colchicine, digitonin, etoposide, anethol, eugenol, hypericin, sulforapane, forskolin, curcumin, vinblastine, butein, fisetin, isoliquiritigenin, silibinin and the PXR well-known agonist rifampicin were screened by luciferase assay in PXR promoter-reporter cell lines Hepx-1096/+43 and Hepx-497/+43 to check their effect on PXR-promoter activity and seeking PXR-promoter modulators (activators/inhibitors).

For screening the PXR-promoter modulating herbal drugs, Hepx-1096/+43 and Hepx-497/+43 stable cell lines were seeded into 48 well culture plates in complete DMEM medium and incubated in CO_2 incubator. Following the day, cells were treated separately with all the different herbal drugs as shown in the **Figure 23** for 24 h and luciferase activities were determined. Active herbal drugs treated Hepx-1096/+43 and Hepx-497/+43 cells were compared with their respective cells treated with vehicle alone (DMSO:ethanol in 1:1 ratio) and referred as control. For comparison purposes control cells were allotted a value of 100% for the basal luciferase activity present in the Hepx-1096/+43 and Hepx-497/+43 cells. **Figure 24** demonstrates the responses of all the tested drugs on PXR-promoter activity in Hepx-1096/+43 and Hepx-497/+43 cells. The Hepx-1096/+43 showed high increase in PXR promoter activity with herbal ingredients acacetin, resveratrol, piceatannol, genistein and kaempferol. Other herbal ingredients show moderate or no or low promoter activity (**Figure 24A**). Acacetin, resveratrol, piceatannol, genistein, kaempferol, coumestrol, camptothecin, quercetin, fisetin, isoliquiritigenin and eugenol significantly increased the PXR-promoter activity in Hepx-1096/+43 cells nearly by 250%, 276%, 206%, 228%, 235%, 130%, 153%, 156%, 126%, 140% and 124% respectively (**Figure 24A**). However, other drugs including sulforaphane, hypericin, forskolin, guggulsterone,

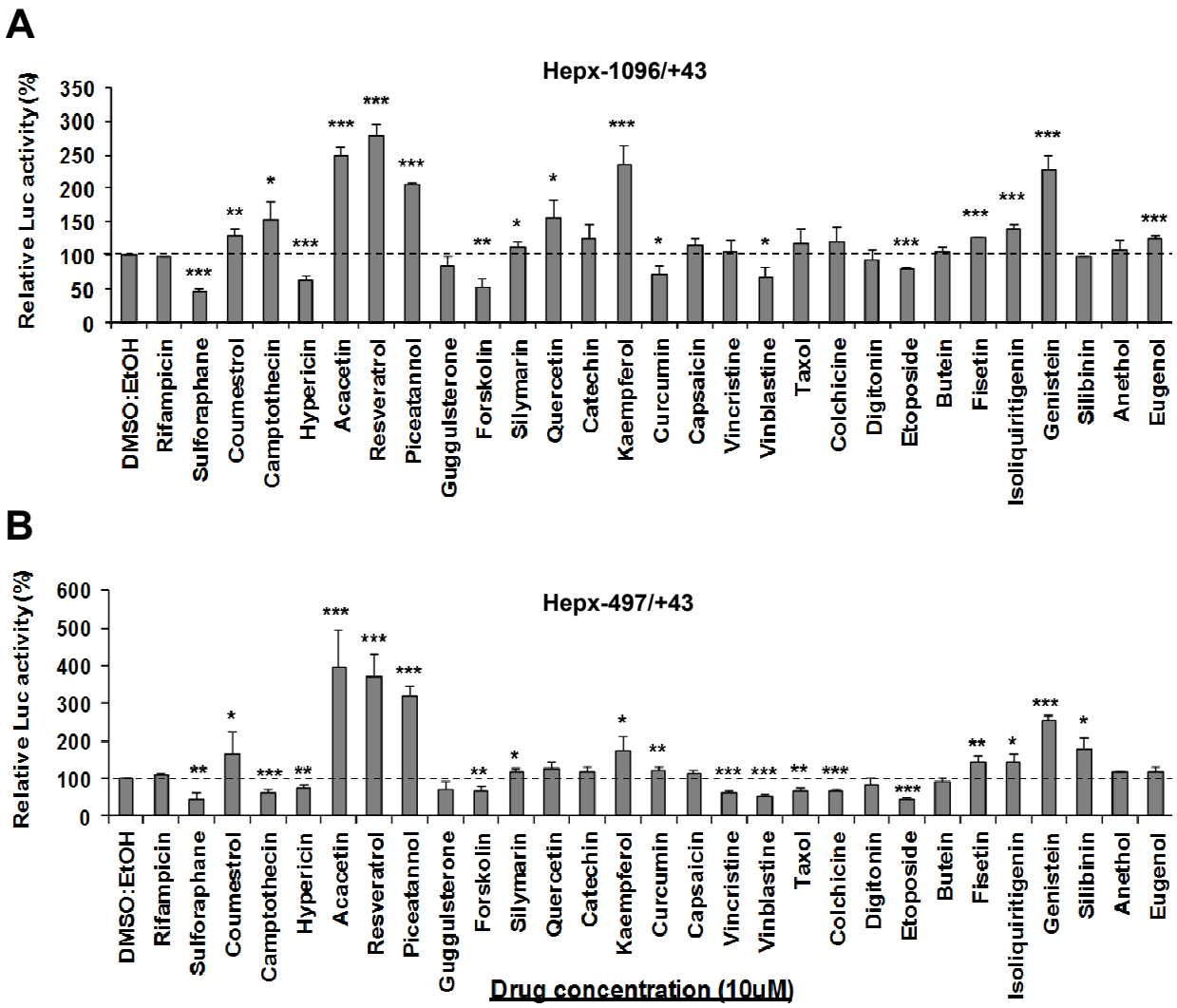

Figure 24: Evaluation of PXR-promoter reporter activity by herbal drugs in luciferase assay. *Graphical representation of relative promoter activity of Hepx-1096/+43 cells treated with different herbal drugs **(A)**. Plot showing relative promoter activity of Hepx-497/+43 cells treated with indicated herbal drugs **(B)**. Approximately 2.3X10^4 cells of either Hepx-1096/+43 or Hepx-497/+43 were seeded in 48-well culture plates. On reaching ~60% confluency, they were either vehicle treated (control) or treated with 10 μM of different active herbal ingredients and further incubated in a CO_2 incubator. After 24 h of incubation period cell lysates were prepared and luciferase activities were determined as described in 'Material and Methods' under 'optimization protocol in 48-well culture plate'. Luciferase values are expressed as percentage of the activity of control cells. Data represent the mean ± S.D. 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).*

Table IX: Effect of potential anti-cancer herbal drugs on PXR-promoter activity in Hepx-1096/+43 and Hepx-497/+43 cell line.

Serial No.	Effect of drugs on PXR-promoter	Hepx-1096/+43	Hepx-497/+43
1.	High activation	Acacetin, Genistein, Kaempferol, Piceatannol and Resveratrol	Acacetin, Genistein, Piceatannol and Resveratrol
2.	Moderate activation	Camptothecin, Coumestrol, Catechin, Fisetin, Isoliquiritigenin Quercetin and Silymarin	Coumestrol, Curcumin, Fisetin, Isoliquiritigenin, Kaempferol, Silibinin, and Silymarin
3.	No effect	Anethol, Butein Capsaicin, Colchicine, Digitonin, Eugenol, Rifampicin, Silibinin, Taxol and Vincristine	Anethol, Butein, Capsaicin, Catechin, Digitonin, Eugenol, Quercetin and Rifampicin
4.	Repression	Curcumin, Etoposide, Forskolin, Guggulsterone, Hypericin, Sulforapane and Vinblastine	Camptothecin, Colchicine, Etoposide, Forskolin, Guggulsterone, Hypericin, Sulforaphane, taxol, Vincristine, Vinblastine

curcumin, vinblastine and etoposide suppressed the PXR-promoter activity in Hepx-1096/+43 cell line nearly by 55%, 37%, 47%, 17%, 28%, 32%, 22% respectively (**Figure 24A**). Further, Hepx-497/+43 cell line showed high PXR promoter activity with herbal drugs; acacetin, resveratrol, piceatannol and genistein. The other herbal ingredients tested showed moderate to low promoter activity (**Figure 24B**). Acacetin, resveratrol, piceatannol, genistein, kaempferol, coumestrol, quercetin, fisetin, isoliquiritigenin, silibinin, curcumin and silymarin significantly increased the PXR-promoter activity in Hepx-497/+43 cells nearly by 395%, 371%, 317%, 255%, 171%, 162%, 126%, 144%, 143%, 177%, 122% and 117% respectively (**Figure 24B**). Sulforaphane,

camptothecin, hypericin, forskolin, guggulsterone, vincristine, vinblastine, taxol, colchicine and etoposide significantly inhibited the PXR-promoter activity in Hepx-497/+43 cells nearly by 55%, 37%, 26%, 34%, 31%, 40%, 49%, 35%, 34% and 56% respectively (**Figure 24B**). PXR well-known agonist rifampicin did not affect the PXR-promoter activity in both the cell lines (**Figure 24**). On the basis of the results obtained in these assays, herbal drugs responses were categorized into four category (i) high activation, (ii) moderate activation, (iii) no effect and (iv) repression on the basis of their modulatory effects on PXR-promoter activity i.e. up regulation (increase) or down regulation (decrease) and tabulated in simplified way **(Table IX)**. In general, Hepx-497/+43 cells showed high activation with herbal drugs as compared to Hepx-1096/+43 cells data. Camptothecin, curcumin, taxol and colchicine responded PXR-promoter inversely in both the cell lines (**Figure 24**). Camptothecin, taxol and colchicine increased PXR-promoter activity in Hepx-1096/+43 cells on the other hand they suppressed PXR-promoter activity in Hepx-497/+43 cells (**Figure 24**). Curcumin increased PXR-promoter activity in Hepx-497/+43 cell line while it inhibited PXR-promoter activity in Hepx-1096/+43 cells. Silibinin and vincristine did not alter PXR-promoter activity in Hepx-1096/+43 cell line however PXR-promoter activity was increased and suppressed by silibinin and vincristine respectively (**Figure 24**). The differential response of drugs could be contributed by different cis-regulatory elements in PXR proximal promoter.

Modulation of PXR-promoter activity by herbal drugs in dose dependent manner

Based on the available literature, 10 μM concentration of each drug was used to modulate the PXR-promoter activity in Hepx-1096/+43 and Hepx-497/+43 cell lines for the initial screening of their effect. The working concentration (10 μM) was routinely used in various laboratories and reported in the literature (Meijerman et al, 2008; Chang, 2009). However, the responsiveness of the PXR-promoter to different doses of herbal drugs cannot be overruled. Therefore, to rule out the possibilities of dose dependent effect of these herbal drugs on PXR-promoter activity, we used four different

concentrations of all the drugs (0.1, 1.0, 10 and 50 µM) along with control (no drug i.e. vehicle only) in all set of experiments in both the cell lines; Hepx-1096/+43 and Hepx-497/+43. **Figure 25** and **Figure 26** demonstrate dose-dependent effect of herbal drugs on PXR-promoter activity in Hepx-1096/+43 and Hepx-497/+43 cell lines respectively. As shown in **Figure 25A** and **26A**, acacetin, resveratrol, piceatannol and genistein were able to activate PXR-promoter at 1.0 µM of concentration also. However the higher activation by these drugs reached at 10 µM. Only genistein, quercetin and coumestrol were observed to increase the PXR-promoter activity at 50 µM concentration (**Figure 25B** and **26B**). Forskolin, sulforaphane, hypericin, etoposide and vinblastine also inhibited the PXR-promoter activity at lower concentrations than 10 µM in Hepx-1096/+43 cell line (**Figure 25B-D**). On the other hand, camptothecin, forskolin, sulforaphane, etoposide, colchicine, taxol, vincristine and vinblastine

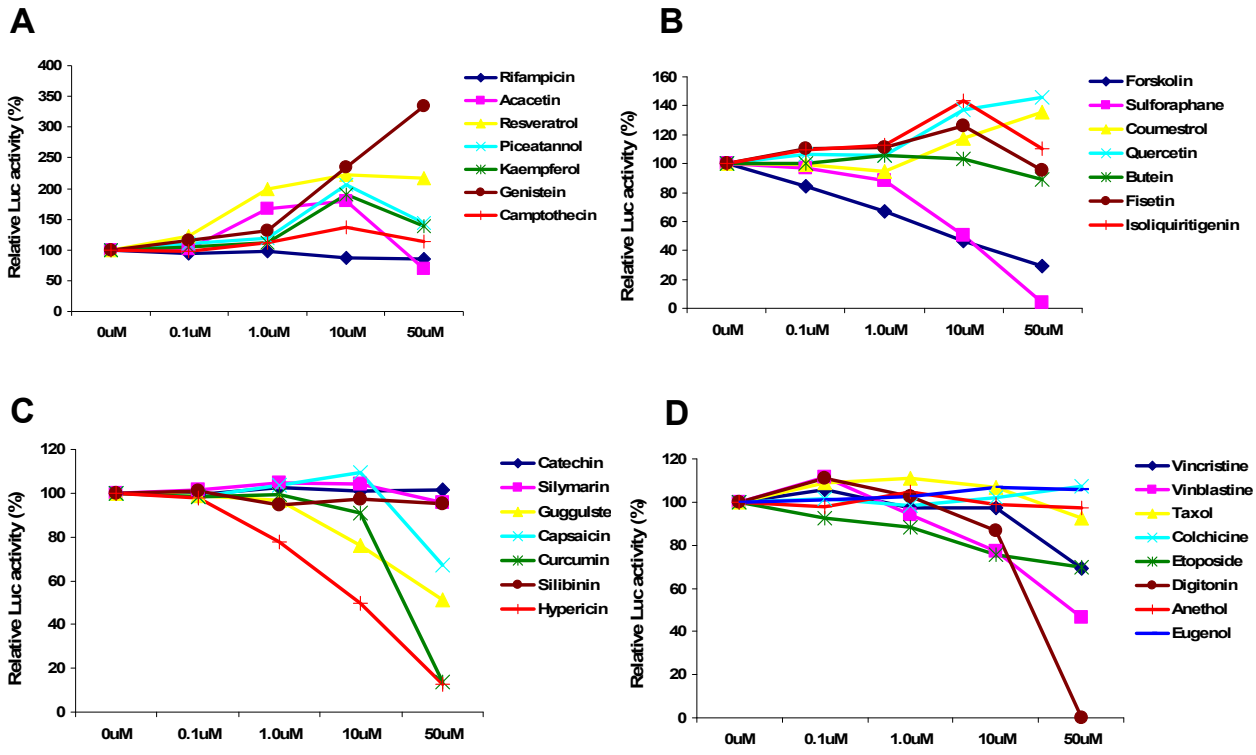

Figure 25: Dose-dependent effect of herbal drugs on PXR-promoter in Hepx-1096/+43 cell line. *Hepx-1096/+43 cells were seeded in 48-well culture plate and incubated for 24 h in the presence or absence of different concentration of herbal drug ranging from 0 to 50 µM. After 24* h of incubation period *luciferase assay was performed as described under 'optimization protocol*

in 48-well culture plate'. Relative Luc activity was calculated in comparison to DMSO:ethanol induced luciferase activity. Data represent the mean ± S.D. of three independent experiments. ***A*** *indicates rifampicin, acacetin, resveratrol, piceatannol, genistein, kaempferol and camptothecin,* ***B*** *represents forskolin,* sulforaphane, genistein, quercetin, butein, fisetin and *isoliquiritigenin.* ***C*** *demonstrates catechin, silymarin, guggulsterone, capsaicin, curcumin, silibinin, and hypericin.* ***D*** *depicts vincristine, vinblastine, taxol, colchicine, etoposide, digitonin, anethol and eugenol.*

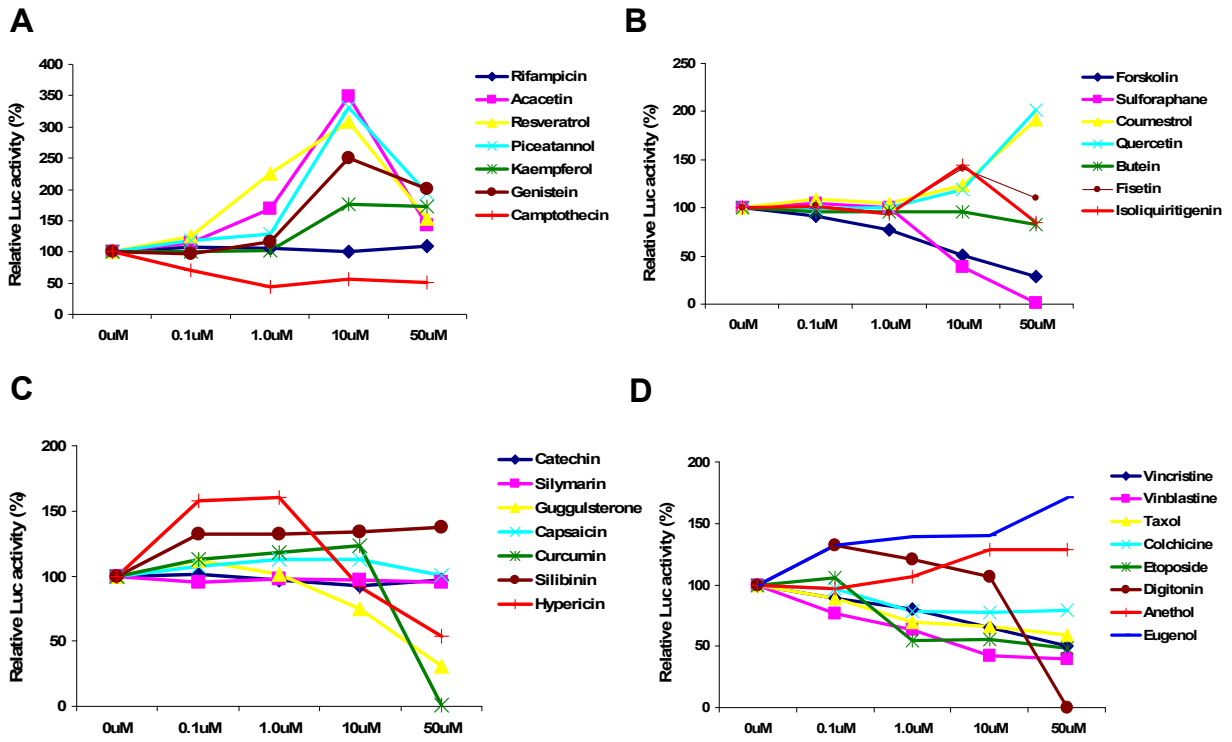

Figure 26: Dose-dependent effect of herbal drugs on PXR-promoter in Hepx-497/+43 cell line. *Hepx-497/+43 cells were seeded in 48-well culture plate and incubated for 24 h in the presence or absence of different concentration of herbal drug ranging from 0 to 50 µM. After 24 h of incubation period luciferase assay was done as described under 'optimization protocol in 48-well culture plate'. Relative Luc activity was calculated in comparison to DMSO:ethanol induced luciferase activity. Data represent the mean ± S.D. of three independent experiments.* ***A*** *indicates rifampicin, acacetin, resveratrol, piceatannol, genistein, kaempferol and camptothecin,* ***B*** *represents forskolin,* sulforaphane, genistein, quercetin, butein, fisetin and *isoliquiritigenin.* ***C*** *demonstrates catechin, silymarin, guggulsterone, capsaicin, curcumin, silibinin, and hypericin.* ***D*** *depicts vincristine, vinblastine, taxol, colchicine, etoposide, digitonin, anethol and eugenol.*

suppressed the PXR-promoter activity at lower concentrations than 10 μM in Hepx-497/+43 cell line (**Figure 26A-D**). Our results also showed that the inhibition of PXR-promoter activity increased with the increasing concentration of drugs. However, the higher inhibition at 50 μM concentration could be contributed by cell toxicity. Furthermore, as shown in **Figure 26**, only quercetin, coumestrol and eugenol increased the PXR-promoter activity at 50 μM concentration in Hepx-497/+43 cell line. In conclusion, overall results derived from the dose-dependent effects of herbal drugs also indicated that the 10 μM concentration is the optimum concentration for the sufficient modulation of PXR-promoter activity.

Forskolin inhibits both basal and induced PXR-promoter activity in Hepx-1096/+43 and Hepx-497/+43 cell lines.

Forskolin, an efficacious activator of adenylate cyclase increases the intracellular cAMP concentration and consequently activate PKA signalling pathway in cells (Seamon et al, 1981). Forskolin also increase PXR transcriptional activity and has been established as PXR agonist in our study (**Chapter-I**; **Figure 11**) as well as in other's studies (Dowless et al, 2005; Ding and Staudinger, 2005a). Surprisingly, in luciferase assays, forskolin inhibited the PXR-promoter activity in both the cell lines; Hepx-1096/+43 and Hepx-497/+43 (**Figure 24-26**). Further, we tested whether forskolin can inhibit the drugs-induced PXR-promoter activity in Hepx-1096/+43 and Hepx-497/+43 cell lines or it only affects basal activity. In this context, Hepx-1096/+43 and Hepx-497/+43 cells were propagated in 48-well culture plates and treated with control (DMSO:ethanol) or 10 μM concentrations of forskolin, acacetin, resveratrol, piceatannol, genistein, kaempferol, camptothecin, quercetin and coumestrol alone or these drugs in combination with 10 μM of forskolin. Following the drugs treatment, luciferase assay was performed. **Figure 27** exhibited the effect of forskolin on PXR-promoter activity. Our results showed that forskolin significantly inhibited the basal PXR-promoter activity in Hepx-1096/+43 and Hepx-497/+43 cells. Interestingly, forskolin also inhibited the acacetin, resveratrol, piceatannol, genistein, kaempferol, camptothecin, quercetin and coumestrol induced PXR-promoter activity significantly in both

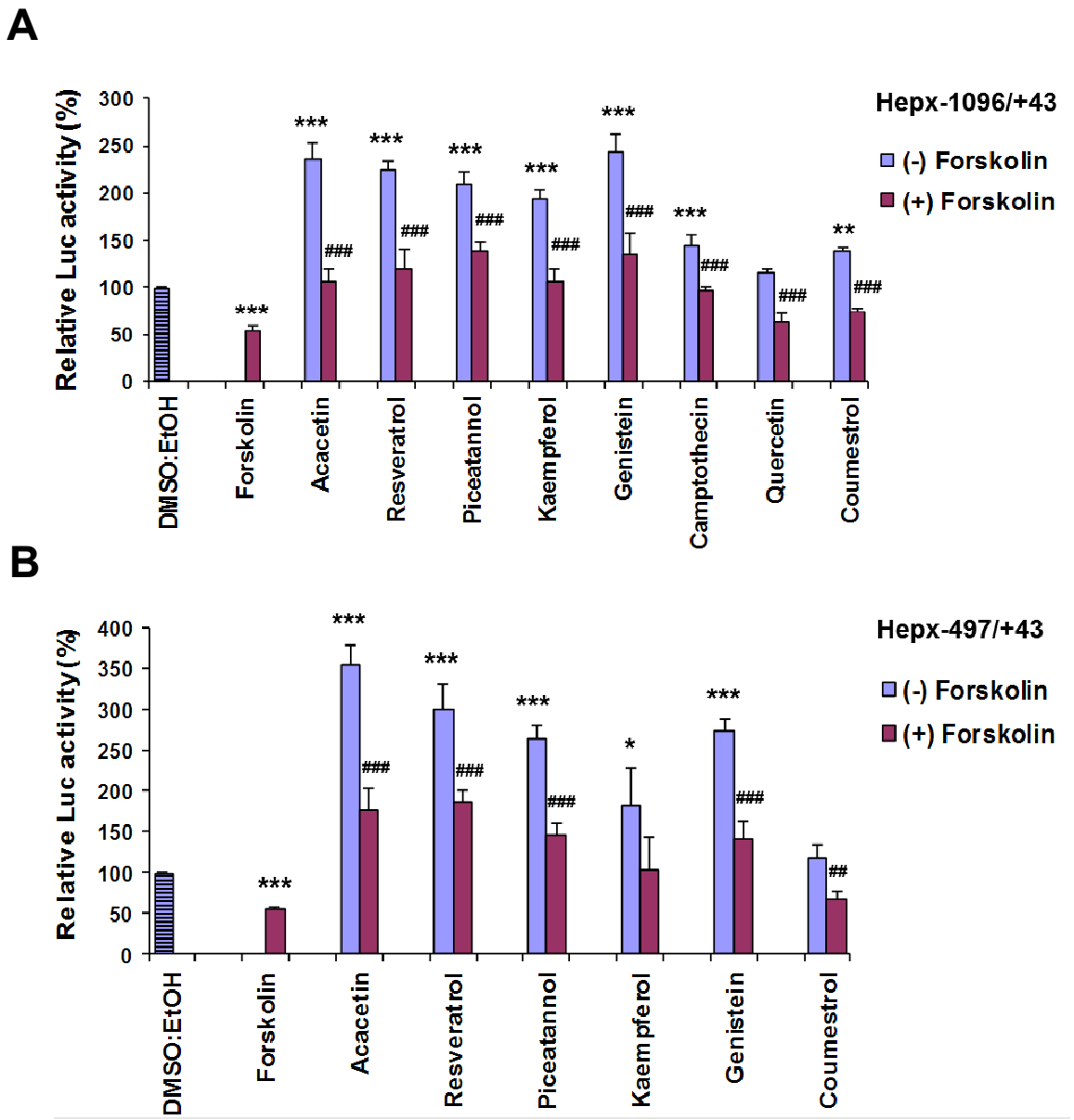

Figure 27: Forskolin inhibits both basal and induced PXR-promoter activity in Hepx-1096/+43 and Hepx-497/+43 cell lines. *Hepx-1096/+43 (**A**) and Hepx-497/+43 (**B**) cells were seeded in 48-well culture plates. On reaching ~60% confluency, cells were either vehicle treated (control) or treated with 10 μM of forskolin or 10 μM of indicated herbal drugs alone or forskolin in combination with indicated drugs and further incubated in a CO_2 incubator. After 24 h of incubation period, cells were lysed and luciferase activities were determined as described under 'optimized protocol in 48-well culture plate'. Luciferase values are expressed as relative luc activity of control cells. Data represent the mean ± S.D. of three independent experiments. Asterisks (*, ** and ***) signify luciferase values that differed significantly from the score of corresponding control (P<0.05, P<0.01 and P<0.001 respectively in Student's t-test). While the symbol (## and ###) signify luciferase values that differed significantly from the scores of different drugs alone (P<0.01 and P<0.001 respectively).*

the cell lines (**Figure 27A** and **27B**). For this experiment, camptothecin treatment was given only in Hepx-1096/+43 cell line since it repress the PXR-promoter activity in Hepx-497/+43 cell line. Collectively, these results indicated the dominancy of forskolin over other drugs to repress PXR-promoter activity.

Sulforaphane also inhibits both basal and induced PXR-promoter activity

Sulforaphane, a PXR antagonist reported recently also inhibited the PXR-promoter activity in Hepx-1096/+43 and Hepx-497/+43 cell lines (**Figure 24-26**). Further we tested whether sulforaphane can impede the drugs-induced PXR-promoter activity in Hepx-1096/+43 and Hepx-497/+43 cell lines or not. In this perspective, Hepx-1096/+43 and Hepx-497/+43 cells were propagated in 48-well culture plates and treated with control or 10 μM of sulforaphane, acacetin, resveratrol, piceatannol, genistein, kaempferol, camptothecin, quercetin and coumestrol alone or these drugs in combination with 10 μM of sulforaphane. Following the drugs treatment period, luciferase activities were taken. **Figure 28** demonstrates the effect of sulforaphane on PXR-promoter activity. Our results exhibited that sulforaphane significantly repressed the PXR-promoter activity in Hepx-1096/+43 and Hepx-497/+43 cells. Fascinatingly, sulforaphane also inhibited the acacetin, resveratrol, piceatannol, genistein, kaempferol, camptothecin, quercetin and coumestrol induced PXR-promoter activity significantly in both the cell lines (**Figure 28A** and **28B**). Collectively these results indicated the dominancy of sulforaphane over other drugs to repress PXR-promoter activity. These results indicated that sulforaphane not only inhibit the PXR transcriptional activity but also PXR-promoter activity.

Camptothecin differentially regulate the PXR promoter

Camptothecin is a known topoisomerase I inhibitor (Chen and Liu, 1994). Camptothecin also inhibited the PXR transcriptional activity and acted as the PXR antagonist in our study (**Chapter-I**; **Figure 17** and **21**) as well as in others study (Chen et al, 2010). In our initial experiments, camptothecin

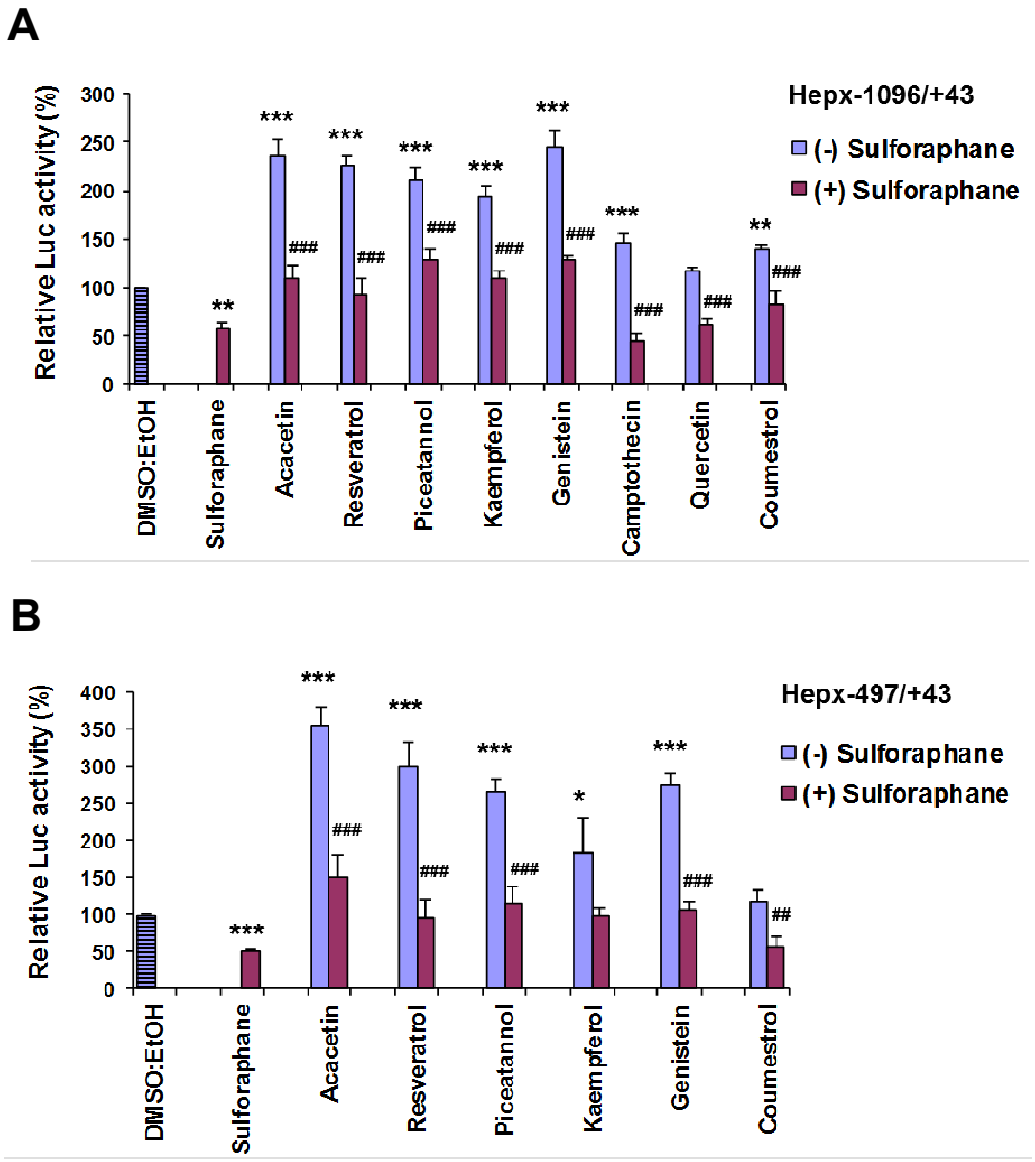

Figure 28: Sulforaphane inhibits both basal and induced PXR-promoter activity in Hepx-1096/+43 and Hepx-497/+43 cell lines. *Hepx-1096/+43 (**A**) and Hepx-497/+43 (**B**) cells were seeded in 48-well culture plates. On reaching ~60% confluency, cells were either vehicle treated (control) or treated with 10 μM of sulforaphane or 10 μM of indicated herbal drugs alone or sulforaphane in combination with indicated drugs and further incubated. After 24 h of incubation period, cells were lysed and luciferase activities were determined as described under 'optimized protocol in 48-well culture plate'. Luciferase values are expressed as relative luc activity of control cells. Data represent the mean ± S.D. of three independent experiments. Asterisks (*, ** and ***) signify luciferase values that differed significantly from the score of corresponding control (P<0.05, P<0.01 and P<0.001 respectively in Student's t-test). While the symbol (## and ###) signify luciferase values that differed significantly from the scores of different drugs alone corresponding to the treated (P<0.01 and P<0.001 respectively).*

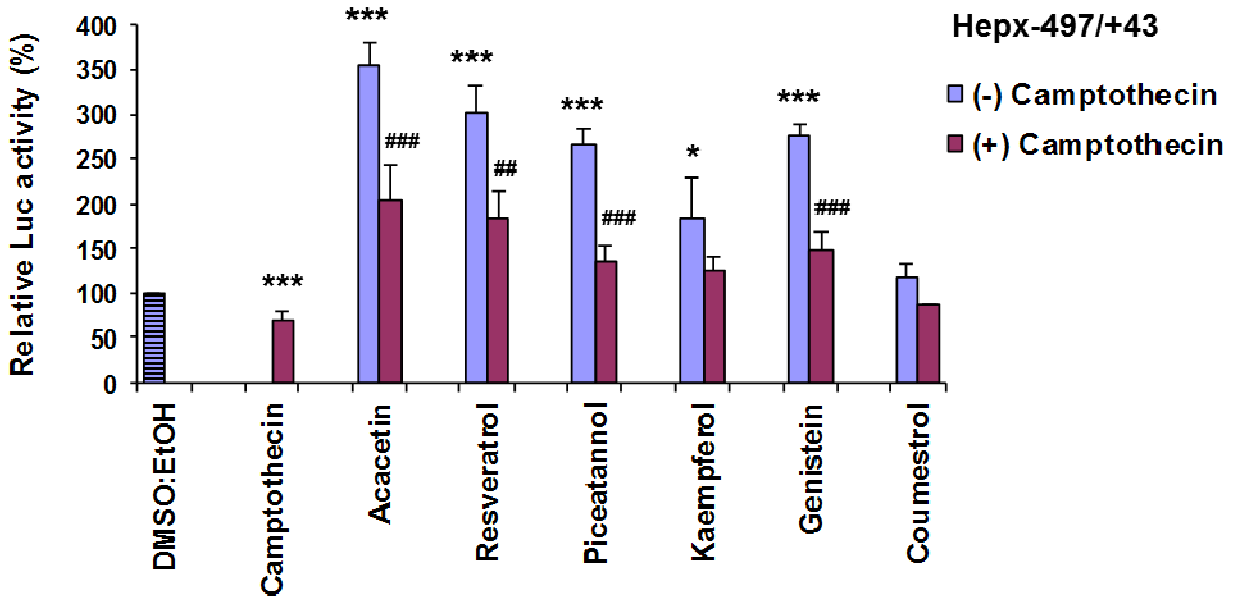

Figure 29: Camptothecin inhibits both basal and induced PXR-promoter activity in Hepx-497/+43 only. *Hepx-497/+43 cells were seeded in 48-well culture plates. On reaching ~60% confluency, cells were either treated with vehicle (control) or treated with 10 μM of camptothecin or 10 μM of indicated herbal drugs alone or camptothecin in combination with indicated drugs. After 24 h of drugs treatment, cells were lysed and luciferase assay were carried out as described under 'optimized protocol in 48-well culture plate'. Luciferase values are expressed as relative luc activity of control cells. Data represent the mean ± S.D. of three independent experiments. Asterisks (* and ***) signify luciferase values that differed significantly from the score of corresponding control (P<0.05 and P<0.001 respectively in Student's t-test). While the symbol (## and ###) signify luciferase values that differed significantly from the scores of different drugs alone corresponding to the treated one (P<0.01 and P<0.001 respectively).*

behaved oppositely in Hepx-1096/+43 and Hepx-497/+43 cell lines (**Figure 24-26**). Camptothecin activated PXR-promoter expression in Hepx-1096/+43 cell line (**Figure 24A**) while it inhibited the PXR-promoter activity in Hepx-497/+43 cell line (**Figure 24B**). The same results were obtained in dose-dependent experiments with both the cell lines (**Figure 25** and **26**). Further we tested whether camptothecin could inhibit the drugs-induced PXR-promoter activity in Hepx-497/+43 cell lines or not. In this context, Hepx-497/+43 cells were propagated in 48-well culture plates and treated with control or 10 μM of camptothecin, acacetin, resveratrol, piceatannol, genistein, kaempferol and coumestrol alone or these drugs in combination with 10 μM of camptothecin. Following the drugs incubation period, luciferase activities were determined.

Figure 29 exhibits the effect of camptothecin on PXR-promoter activity in Hepx-497/+43 cell line. Our results showed that camptothecin significantly inhibited the basal PXR-promoter activity. Additionally, camptothecin also inhibited the acacetin, resveratrol, piceatannol, genistein, kaempferol and coumestrol induced PXR-promoter activity significantly (**Figure 29**). The differential behavior of camptothecin in regulating PXR-promoter activity could be contributed by different cis regulatory elements in both cell lines and regulatory factors. Collectively these results indicated the dominancy of inhibitors over the activators to regulate PXR-promoter activity.

Effect of herbal drugs on the endogenous protein expression of PXR in HepG2 and LS180 cell lines

Herbal drugs regulated the PXR-promoter activity in Hepx-1096/+43 and Hepx-497/+43 stable cell lines (**Figure 24-26**). Further to see the effect of herbal drugs on endogenous PXR protein expression directly, we conducted our experiments in HepG2 and LS180 cell lines. HepG2 and LS180 cells were propagated in 100 mm culture plates and treated with vehicle (control) or different drugs at 10 μM concentration for 24 h. Following the incubation period, cell lysates were prepared and protein was quantified as described in 'Materials and Methods'. Equal protein was run on SDS-gel and western blotting was performed. **Figure 30** demonstrates the endogenous PXR protein expression profile. Western blotting analysis indicated that rifampicin, acacetin, resveratrol, piceatannol, kaempferol, genistein, forskolin induced the PXR protein expression in HepG2 and LS180 cells while sulforaphane attenuated PXR protein expression (**Figure 30**).

DISCUSSION

PXR is pivotal for the body's response to toxic xenobiotics and endogenous metabolites. As a ligand-activated transcription factor, PXR regulates all stages of xenobiotic metabolism and transport and is responsible for important inductive drug interactions. Now, screening assays for assessing PXR activation potential of new and existing drugs are becoming essential components of the drug discovery programs. PXR is also involved in the lipid

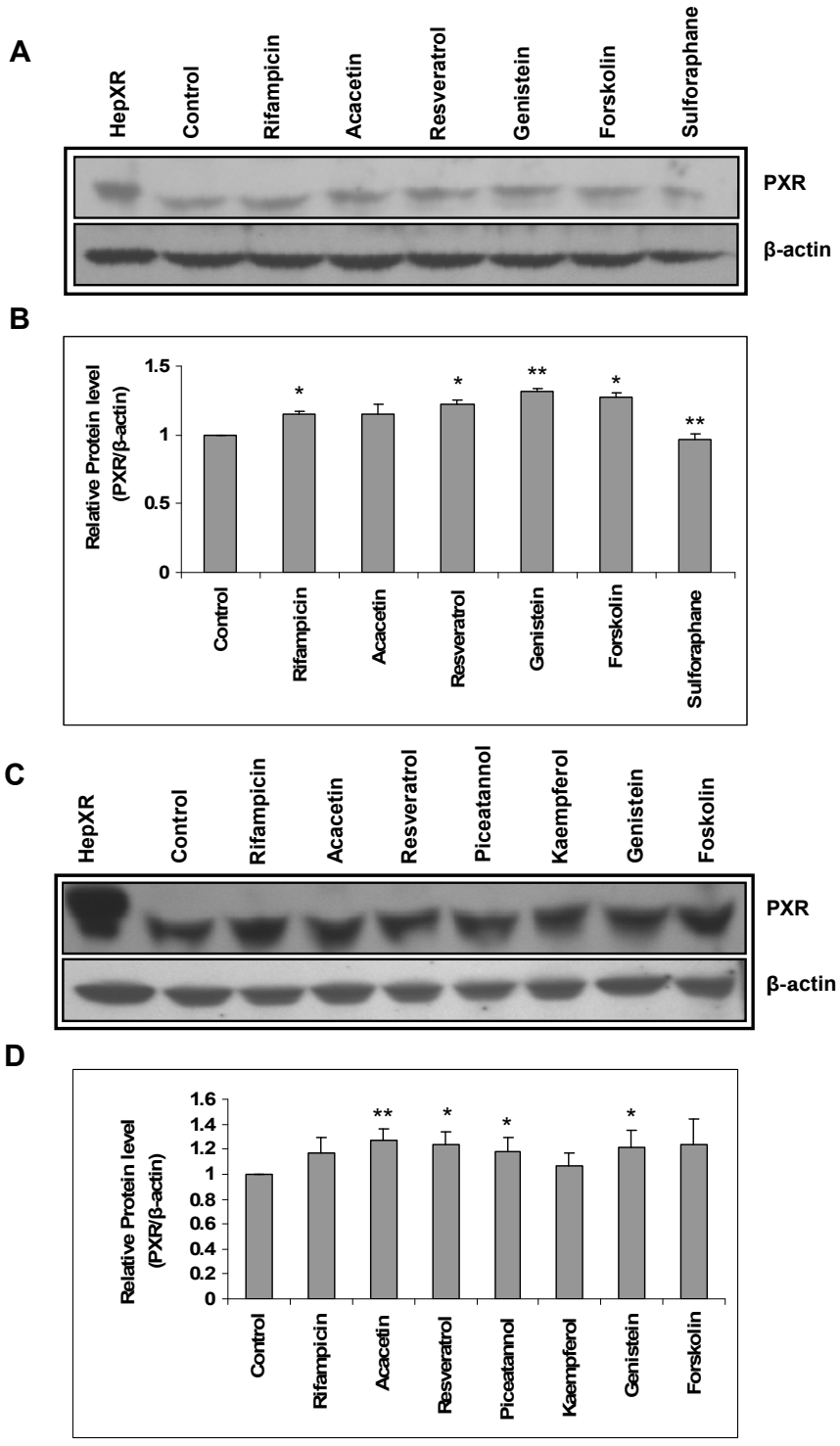

Figure 30: Effect of herbal drugs on protein expression of PXR in HepG2 and LS180 cell lines. *HepG2 (**A**) and LS180 cells (**C**) were grown in 100 mm culture plates and next day cells were treated with control (vehicle) or 10 μM of indicted drugs and allowed to incubate for 24 h. Following the treatment period, cell lysate were prepared and proteins were quantified. Equal amount of protein samples were run on SDS-gel. Proteins were transferred to PVDF membrane and western blotting was performed as described in 'Materials and Methods'. **Figure A** and **C** demonstrate the western blots of PXR in HepG2 and LS180 cells. The*

lysate of HepXR cells were used as positive control for PXR protein since PXR overexpressed in HepXR cells. β-actin was used as loading control. ***Figure B*** *and* ***D*** *depict the quantitation of bands of PXR protein by densitometry from HepG2 and LS180 cells respectively. Data represent the mean ± S.D of three independent experiments. Asterisks (* and **) signify values that differed significantly from the scores of corresponding controls (P<0.05 and P<0.01 respectively in Student's t-test).*

homeostasis providing opportunities for treatments based on PXR agonists for diseases involving aberrant cholesterol and bile acid levels. The expression of PXR in many other tissues besides liver and intestine suggest PXR may have additional protective functions in the body, which contribute to disease outcomes in diverse clinical situations with potential for novel therapeutic approaches (Matic et al, 2007). The regulation of drug-metabolizing enzymes by PXR may result in clinical drug-drug interactions, in which one drug accelerates the metabolism of a second medication and may change or cause adverse or beneficial results. Although there are various methods, which are available to assess the pharmacological properties by drug screening, but due to their high cost, lengthy experimental duration offers only limited use. In this context, we needed alternative methods, which provide faster, easier and more reproducible results.

For this purpose, in this section of our study, for the first time we have generated two PXR promoter cell lines Hepx-1096/+43 and Hepx-497/+43 by stably integrating two selected regions of proximal PXR promoter-reporter constructs in liver cell line HepG2. These cell lines will serve as a useful tool for studying the transcriptional regulation of PXR and also for the screening of drugs, xenobiotics and endobiotics. Additional advantages of these stable PXR-promoter-reporter cell lines would be in their ability to predict potential drug-drug interactions, to identify PXR regulatory proteins, and also to evaluate the modulatory effects of an experimental molecule on PXR promoter activity.

Further, we attempted to understand the regulation of PXR-promoter by herbal drugs and to find out PXR gene modulators using both the PXR-promoter cell lines Hepx-1096/+43 and Hepx-497/+43. By performing luciferase assays in both the cell lines, 28 anti-cancer herbal drugs were screened to check their effect on PXR-promoter regulation and seeking PXR-

promoter modulators. We have shown that acacetin, resveratrol, piceatannol, genistein and kaempferol strongly increased PXR-promoter activity (positive regulation) while forskolin, sulforaphane, etoposide, hypericin, vinblastine significantly suppressed PXR-promoter activity (negative regulation). Furthermore, forskolin and sulforaphane also inhibited the induced PXR-promoter activity. Moreover, camptothecin, curcumin, taxol and colchicine showed inverse response to PXR-promoter in both the cell lines. A well-known PXR agonist rifampicin did not affected the PXR-promoter activity.

On the basis of their modulatory effects on PXR-promoter activity i.e. up-regulation or down-regulation, herbal drugs responses were categorized into four category (i) high activation, (ii) moderate activation, (iii) no effect and (iv) repression. Acacetin, genistein, kaempferol, piceatannol and resveratrol highly activated PXR-promoter activity in our promoter cell lines. Further, western blotting analysis also showed the induction of PXR protein by these drugs in liver and intestinal cell lines. These drugs also transcriptionally activated PXR and induced the CYP3A4 and MDR1 expression (Chapter-I). Resvertrol, piceatannol, butein, fisetin, isoliqiuiritigenin, quercitin, and kaempferol also activate NAD-dependent deacetylase sirtuin 1 (SIRT1) activity (Howitz et al, 2003). Further, genistein and resveratrol also activated AMP-activated protein kinase (AMPK) indirectly (Hwang et al, 2005; Hou et al, 2008). Recently, phosphodiesterase (PDE) enzymes were identified as direct targets of resveratrol and resveratrol was shown to activates SIRT1 indirectly through a signalling cascade involving cAMP, Epac1 and AMPK (Park et al, 2012). All these reports indicated the multiple targets of these herbal drugs and their mechanisms of action are very complex process. In this regard, how these drugs regulated PXR-promoter activity become an important question and it needs further investigation to find out their mechanism of actions for PXR regulation.

Phytoestrogen coumestrol has been shown as naturally occurring antagonist of PXR in a report (Wang et al, 2008a). Our results demonstrated that coumestrol increased the PXR-promoter activity at 10μM and even at 50 μM concentrations. Wang et al have shown that coumestrol inhibited PXR transcriptional activity at 25 μM concentration. However, in their study,

coumestrol activated ERα and ERβ transcriptional activity (Wang et al, 2008a). These results indicated the possibilities for the involvement of ERα and ERβ in PXR-promoter regulation by coumestrol since ER has binding sites on PXR proximal promoter (Aouabdi et al, 2006). However, it needs further investigation.

Forskolin, an efficacious activator of adenylate cyclase increases the intracellular cAMP concentration and consequently activate PKA signalling pathway in cells (Seamon et al, 1981). Independent of cAMP signalling, forskolin also increases PXR transcriptional activity and induces CYP3A4 gene and it has been established as PXR agonist (Dowless et al, 2005; Ding and Staudinger, 2005a). Our results also supported that forskolin transactivate PXR. Unexpectedly, forskolin inhibited the PXR-promoter activity significantly in both the PXR-promoter stable cell lines; Hepx-1096/+43 and Hepx-497/+43. Additionally, forskolin also inhibited the acacetin, resveratrol, piceatannol, genistein, kaempferol, camptothecin, quercetin and coumestrol induced PXR-promoter activity significantly in both the cell lines. Forskolin transactivated PXR by binding with it and increasing the interaction of nuclear receptor coactivators (Dowless et al, 2005; Ding and Staudinger, 2005a). How forskolin suppressed PXR-promoter activity needs further investigation. It could be possible it involves the cAMP dependent PKA signalling pathways in PXR gene regulation. However, our western blotting experiments did not show the inhibitory effect of forskolin on PXR protein expression.

Sulforaphane, a known PXR antagonist also inhibited the basal and induced PXR-promoter activity in Hepx-1096/+43 and Hepx-497/+43 cell lines. These results indicated the possibilities that sulforaphane may impart the inhibition of PXR transcriptional activity by inhibiting the PXR gene expression.

Further, camptothecin, curcumin, taxol and colchicine showed inverse response to PXR-promoter in both the cell lines. Camptothecin, taxol and colchicine increased PXR-promoter activity in Hepx-1096/+43 cells on the other hand they suppressed PXR-promoter activity in Hepx-497/+43 cells. Curcumin increased PXR-promoter activity in Hepx-497/+43 cell line while it inhibited PXR-promoter activity in Hepx-1096/+43 cells. Silibinin and

vincristine did not affect PXR-promoter activity in Hepx-1096/+43 cell line however PXR-promoter activity was augmented and suppressed by silibinin and vincristine respectively. The differential responses of these drugs could be contributed by different cis-regulatory elements in PXR proximal promoter in Hepx-1096/+43 and Hepx-497/+43 cell lines.

In conclusion, both the PXR-promoter cell lines Hepx-1096/+43 and Hepx-497/+43 served as an important tool for PXR-promoter regulation and seeking PXR modulators. Our study provided the evidences for PXR-promoter regulation by anticancer herbal drugs. Our results also indicated that the regulation of PXR-promoter by herbal drugs is very complex process and there are possibilities of involvement of various signalling pathways in regulation of PXR-promoter imparting the effect of herbal drugs. Future investigation in this direction may unravel the mechanisms of action of these drugs and role of signalling pathways in regulation of PXR gene in normal and pathological conditions.

* * *

CHAPTER-III

Identification of herbal drugs responsive signalling events involved in the regulation of PXR

INTRODUCTION

A fundamental principle in the process of drug metabolism implies drug biotransformation and transport using coordinately expressed systems (i.e., drug metabolizing enzymes and drug transporters). A 'master regulator' Pregnane and Xenobiotic Receptor (PXR) regulates the set of events to execute the process of drug metabolism and elimination (Kliewer et al, 1998; Konno et al, 2008; Gao and Xie, 2012). In addition to drug metabolism, activation of PXR modulates several key biochemical pathways of hepatic energy metabolism, including gluconeogenesis, β-oxidation of fatty acids, fatty acid uptake, cholesterol homeostasis, and lipogenesis (Konno et al, 2008; Lichti-Kaiser et al, 2009; Gao and Xie, 2012). Xenobiotic-sensing pathways control energy metabolism, mutually energy homeostasis regulate drug metabolism (Konno et al, 2008; Gao and Xie, 2012). However, the mechanisms mediating the effects of energy homeostasis on drug metabolism are not well understood. Moreover, how energy sensing cellular factors regulates PXR function also remains unclear.

Because of its promiscuous nature, PXR is activated by various ligands including xenobiotics, endobiotics, herbal drugs, etc. through direct binding. The transactivation functions of activated PXR are mediated by the recruitment of nuclear receptor coactivators including peroxisome proliferator-activated receptor coactivator 1 alpha (PGC-1α) SRC1, SCR2, PBP, etc. Nevertheless, the transcriptional activity of PXR is regulated not only by direct ligand binding (Timsit and Negishi, 2007) but also by few cell signalling pathways (Pondugula et al, 2009), such as those mediated by protein kinase C (PKC) (Ding and Staudinger, 2005b), protein kinase A (PKA) (Ding and Staudinger, 2005a; Lichti-Kaiser et al, 2009), cyclin-dependent kinase 2 (Cdk2) (Lin et al, 2009), nuclear factor κB (NF-κB) (Zhou et al, 2006; Gu et al, 2006; Xie and Tian, 2006) and cyclin-dependent kinase 5 (Cdk5) (Dong et al, 2010). However, the regulation of PXR gene transcription remains unexplored by signalling pathways. A little is known regarding the signal transduction pathways that interface with the PXR protein expression and activity.

An intracellular second messenger, cAMP regulates a number of different cellular processes, such as cell growth and differentiation, ion channel

conductivity, synaptic release of neurotransmitters, and gene transcription. Mostly, intracellular cAMP signalling is mediated by two groups of effectors that bind to cAMP; protein kinase A (PKA) and the cAMP-regulated guanine nucleotide exchange factors (cAMPGEFs) Epac1 and Epac2 (de Rooij et al, 1998; Kawasaki et al, 1998; Gloerich and Bos, 2010). Epac1 increases the intracellular Ca^{2+} levels and activates the calcium/calmodulin-dependent kinase kinase β (CaMKKβ)-AMPK pathway via phospholipase C and the ryanodine receptor Ca^{2+}-release channel (Park et al, 2012, Chung et al, 2012). AMP-activated protein kinase (AMPK) activity requires by one of at least two AMPK kinases; LKB1 or calcium/calmodulin-dependent kinase kinase β (CaMKKβ), which phosphorylate T172 in the Tloop of AMPK (Hardie et al, 2012; Chung et al, 2012). Activation of AMPK via energy depletion is thought to be dependent on LKB1 and activation of AMPK via increased intracellular Ca^{2+} is dependent on CaMKKβ (Hardie et al, 2012; Chung et al, 2012). AMPK is emerging as a key regulator of whole-body metabolism and also has been shown to regulate PR, PPARα, PPARγ and LXR transcriptional activity and SREBP-1c and CYP4F2 gene expression (Hsu et al, 2011; Sozio et al, 2011; Wu et al, 2011b; Yap et al, 2011). However, involvement of AMPK in the regulation of PXR expression and its transcriptional functions is still not elucidated.

Coactivator PGC-1α is a crucial regulator of liver energy metabolism and activates fatty acid oxidation and gluconeogenesis (Finck et al, 2005; Rodgers et al, 2005). PGC-1α cannot bind to DNA itself but functions by interacting with a number of transcriptional factors such as PPARα, HNF4α or PXR. Coactivation by PGC-1α is further modulated by diverse posttranslational modifications, including phosphorylation and acetylation (Finck et al, 2005; Rodgers et al, 2005). Sirtuin 1 (SIRT1) is a NAD-dependent deacetylase which targets and deacetylates PGC-1α enhancing its ability to coactivate gluconeogenic genes (Rodgers et al, 2005). Besides PGC-1α, SIRT1 targets many proteins including several nuclear receptors (Holness et al, 2010). The deacetylation mediated by SIRT1 may have either positive or negative effect on transcription depending on the target transcription factor and even promoter context (Rodgers et al, 2008). Higher levels of NAD+ increase SIRT1 expression

and also directly affect SIRT1 enzyme activity (Rodgers et al, 2008). AMPK has been shown to increase NAD^+ levels and activate SIRT1 and PGC-1α (Canto et al, 2010; Park et al, 2012, Chung et al, 2012).

In the previous chapters, we have shown the transcriptional modulation of PXR gene and its transcriptional functions by herbal drugs. In general, these herbal drugs have multiple targets and their mechanisms of actions are very complex process. These drugs also involved in the regulation of various transcription factors and cascade of signalling proteins in normal physiology and disease (Ding and Staudinger, 2005a; Ding and Staudinger, 2005b; Dong et al, 2010; Gupta et al, 2011; Hsu et al, 2011; Whitlock and Baek, 2012). Therefore, how these drugs regulated PXR-promoter activity become an important question and it needs to be addressed for further investigation to find out their mechanism of actions for PXR regulation. Also, which signalling pathway(s) regulate the PXR gene transcription and its transcriptional functions is not well understood. Hence, in this section of the study, we elucidated the role of various signalling pathways in the transcriptional regulation of PXR-promoter and its transcriptional functions. Employing the inhibitors of various signalling pathways in combination with anti-cancer herbal drugs, we have shown the involvement of SIRT1, cyclic AMP-dependent PKA, AMPK, CaMKKβ, MAPK/ERK 1/2 and PI3K/Akt pathways in the regulation of PXR-promoter and its transcriptional functions in response to anti-cancer drugs. We also showed the role of PXR in cell growth inhibition, cell cycle arrest and apoptosis of HepG2 cells.

RESULTS

In the previous chapters, we have shown the transcriptional modulation of PXR gene and its transcriptional functions by herbal drugs using PXR proximal promoter stable cell lines (Hepx-1096/+43 and Hepx-497/+43) and HepXREM (HepG2 stably transfected with human PXR and XREM-Luc). However, which signalling pathways regulate the PXR gene and its transcriptional functions in response to anti-cancer herbal drugs is not clear. Therefore, regulation of PXR-promoter and receptor transcriptional activity by

herbal drug-responsive signalling events has been explored in this part of the study.

Deacetylase SIRT1 inhibitors nicotinamide and sirtinol inhibit both basal and induced PXR-promoter activity

In Chapter II of our study, to explore the transcriptional regulation of PXR gene by anti-cancer herbal drugs, we have developed two PXR proximal promoter liver cell lines Hepx-1096/+43 and Hepx-497/+43. Further, we have shown that that acacetin, resveratrol, piceatannol, genistein and kaempferol strongly increased the PXR-promoter activity while forskolin and sulforaphane repressed it significantly. Besides, these drugs also modulated the PXR-mediated transcriptional activity might be by binding to PXR-LBD (**Chapter-I**). Nonetheless how these drugs regulate the PXR gene promoter activity remains unanswered. Therefore, to find out the possible mechanism of action of PXR activator drugs, we tested the role of NAD-dependent deacetylase sirtuin 1 (SIRT1) by using its inhibitors nicotinamide and sirtinol since resveratrol, piceatannol, butein, fisetin, isoliqiuiritigenin, quercitin, kaempferol also shown to activate the SIRT1 protein (Howitz et al, 2003). So, there is possibility for the involvement of SIRT1 in PXR gene promoter regulation.

In this context, to study the role of SIRT1 in regulation of PXR-promoter activity, PXR proximal promoter cell lines Hepx-1096/+43 and Hepx-497/+43 were grown in 48-well culture plates and treated with vehicle (DMSO:ethanol) or 10 μM of acacetin, resveratrol, piceatannol, kaempferol, genistein, camptothecin, quercetin and coumestrol alone or in combination with 5 mM of nicotinamide and 50 μM of sirtinol. Nicotinamide and sirtinol treatment was given 30 min before the other drugs treatment. Following the drugs treatment period, luciferase activities were taken. **Figure 31** demonstrates the effect of nicotinamide and sirtinol on PXR-promoter activity. Interestingly, as shown in **Figure 31**, nicotinamide and sirtinol inhibited the basal PXR-promoter activity in both the cell lines significantly ($P<0.001$). Furthermore, nicotinamide and sirtinol also inhibited the acacetin, resveratrol, piceatannol, kaempferol, genistein, quercetin and coumestrol induced PXR-promoter activity significantly in both the PXR-promoter cell lines (**Figure 31**). Collectively these

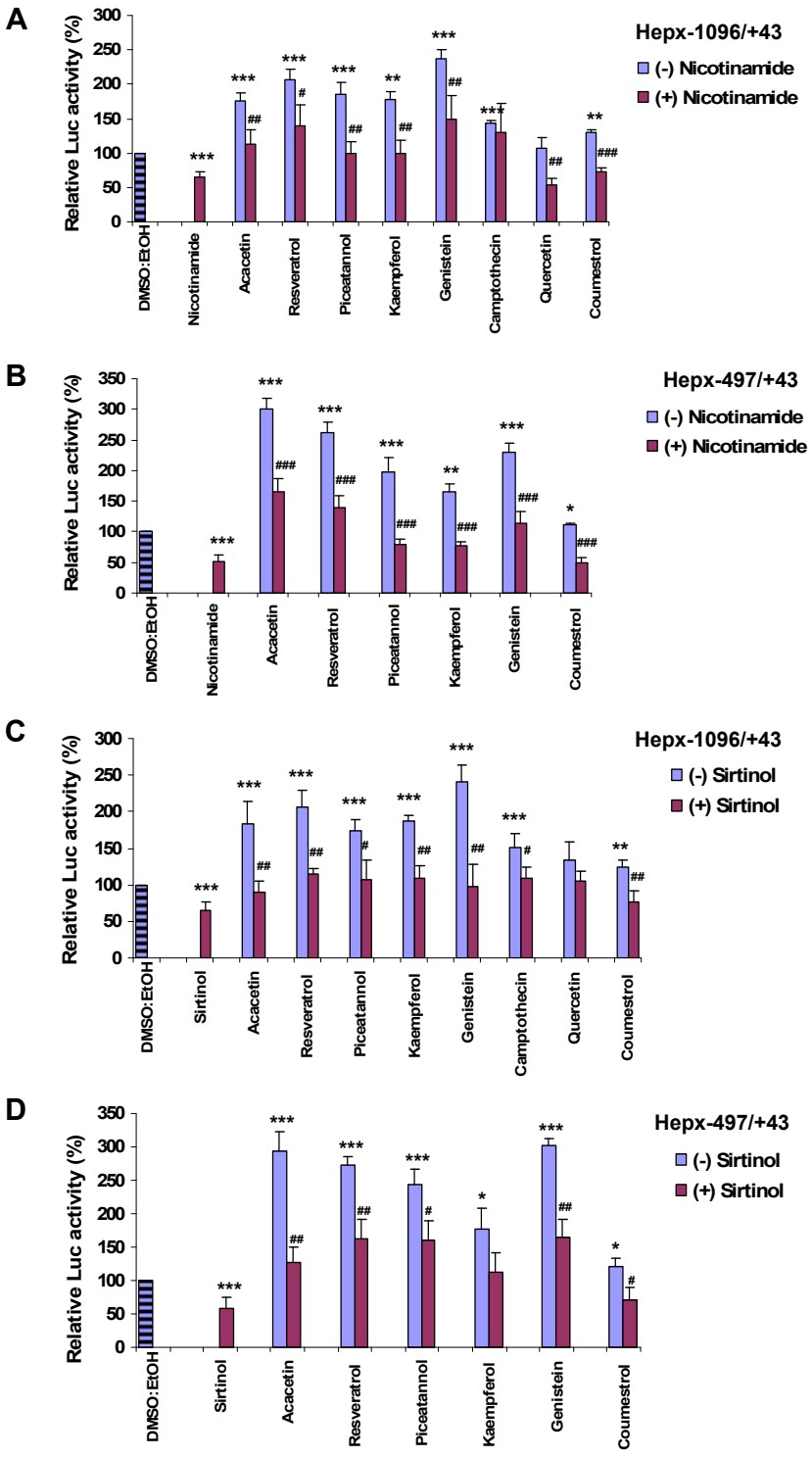

Figure 31: SIRT1 inhibitors nicotinamide and sirtinol inhibit both basal and activator drugs-induced PXR-promoter activity. *Hepx-1096/+43 and Hepx-497/+43 cells were propagated in 48-well culture plates. On reaching ~60% confluency, cells were either vehicle treated (control) or treated with 10 μM of indicated drugs without or with SIRT1 inhibitors, nicotinamide (5.0 mM) in Hepx-1096/+43 and Hepx-497/+43 cells (**A** and **B**) and sirtinol (50 μM) in Hepx-1096/+43 and Hepx-497/+43 cells (**C** and **D**) and further incubated in a CO_2*

incubator. After 24 h of incubation period, cells were lysed and luciferase activities were determined as described under 'optimized protocol in 48-well culture plate'. Luciferase values are expressed as relative Luc activity of control cells. Data represent the mean ± S.D. of three independent experiments. Asterisks (, ** and ***) signify luciferase values that differed significantly from the scores of DMSO:EtOH (P<0.05, P<0.01 and P<0.001 respectively in Student's t-test). While the symbol (#, ## and ###) signify luciferase values that differed significantly from the scores of corresponding (-) nicotinamide or (-) sirtinol (P<0.05, P<0.01 and P<0.001 respectively).*

results indicated that inhibition of SIRT1 activity by its inhibitors block the activation of PXR-promoter by herbal drugs. These results also demonstrate that nicotinamide and sirtinol not only attenuate the PXR-mediated transcriptional activity (**Chapter-I**, **Figure 21**) but also PXR-promoter activity. In conclusion, these results potentiate the role of SIRT1 in regulation of PXR gene promoter.

Cyclic AMP signalling regulates PXR promoter and receptor transcriptional functions

The intracellular levels of cyclic AMP (cAMP) are determined by the activities of the adenylyl cyclases (ACs) and cyclic nucleotide phosphodiesterases (PDEs). The adenylyl cyclase synthesizes cAMP from ATP, which is hydrolyzed to AMP by phosphodiesterases. We hypothesized if cAMP signalling has a role in the regulation of PXR gene or its transcriptional activity by herbal drugs, blocking the cAMP synthesis will affect the PXR gene promoter and transcriptional activity. Therefore, to check the role of cAMP signalling in the regulation of PXR gene and its transcriptional activity by herbal drugs, we first employed the adenylyl cyclase (AC) inhibitor MDL-12,330A to block the cAMP synthesis in the cells.

In this context to execute this, PXR proximal promoter cell lines Hepx-1096/+43 and Hepx-497/+43 were cultured in 48-well culture plates. Next day, 10 μM of resveratrol, piceatannol, acacetin, kaempferol and genistein was added to the untreated cells or in 30 min pretreated cells with 10 μM of adenylyl cyclase inhibitor MDL-12,330A. After 24 h of the drugs treatments period, luciferase activities were determined. Interestingly, as shown in **Figure 32** and **33**, adenylyl cyclase inhibitor MDL-12,330A inhibited the basal PXR-

promoter activity in both the cell lines Hepx-1096/+43 ($P<0.01$) and Hepx-497/+43 ($P<0.05$) significantly. Furthermore, cyclase inhibitor MDL-12,330A also inhibited the resveratrol, piceatannol, acacetin, kaempferol and genistein induced PXR-promoter activity extremely significantly in Hepx-1096/+43 (**Figure 32**). However, MDL-12,330A only inhibited the acacetin induced PXR-promoter activity in Hepx-497/+43 cell line (**Figure 33**). These results indicated that inhibition of adenylyl cyclase by MDL-12,330A prevented the activation of PXR-promoter by inhibiting cAMP synthesis. These results suggested that intracellular cAMP levels positively regulate basal and drug-induced PXR gene promoter activity.

Further, to test the role of cAMP signalling in the regulation of PXR-mediated transcriptional activity, HepXREM (stably transfecting with human PXR and XREM-Luc) cells were grown in 48-well culture dishes and treated with 10 μM of rifampicin, acacetin, resveratrol, piceatannol, kaempferol, genistein and forskolin alone or in combination with 10 μM of MDL-12330A (adenylyl cyclase inhibitor). MDL-12330A treatment inhibited the basal PXR transcriptional activity significantly ($P<0.05$) in HepXREM cells as compared to control (**Figure 34**). Additionally, pretreatment of MDL-12330A in HepXREM cells significantly diminished the rifampicin, acacetin, resveratrol, piceatannol, kaempferol, genistein and forskolin augmented PXR transcriptional activity (**Figure 34**). The inhibition of induced PXR transcriptional activity by MDL-12330A was reached nearly up to basal levels in case of rifampicin ($P<0.01$), acacetin ($P<0.01$), kaempferol ($P<0.001$), forskolin ($P<0.01$). These results also demonstrate that adenylyl cyclase inhibitor MDL-12,330A not only attenuate the PXR-promoter activity but also inhibit PXR-mediated transcriptional activity. In conclusion, these results potentiate the role of cAMP signalling in regulation of basal and drug-induced PXR gene promoter activity and PXR-mediated transcriptional activity.

AMPK regulates PXR promoter and receptor transcriptional functions

AMP-activated protein kinase (AMPK) is a well-known serine/threonine kinase that functions as an intracellular energy sensor and has been

implicated in the modulation of glucose and fatty acid metabolism (Shaw et al, 2005; Zang, 2004). AMPK is activated by physiological stimuli including exercise, muscle contraction, and hormones such as adiponectin and leptin, as well as by physiological stresses, glucose deprivation, hypoxia, oxidative stress, and osmotic shock conditions (Kemp et al, 2003; Carling, 2004; Park et al, 2012, Chung et al, 2012). AMPK, which is emerging as a key regulator of whole-body metabolism, has been shown to increase NAD^+ levels and activate SIRT1 and PGC-1α (Canto et al, 2010; Park et al, 2012, Chung et al, 2012). AMPK also has been shown to regulate PR, PPARα, PPARγ and LXR transcriptional activity and SREBP-1c and CYP4F2 gene expression (Hsu et al, 2011; Sozio et al, 2011; Wu et al, 2011b; Yap et al, 2011). Since AMPK has been shown to be activated by resveratrol and genistein indirectly (Hwang et al, 2005; Dasgupta et al, 2007; Hou et al, 2008) and also, AMPK is activated by elevated cAMP levels through upstream CaMKKβ and LKB1 pathways. Therefore, we speculated the involvement of AMPK in regulation of PXR gene promoter and PXR-mediated transcriptional activity.

To determine if AMPK is involved in regulation of basal or drugs-induced PXR-promoter and its transcriptional activity, we employed well known AMPK inhibitor dorsomorphin (compound C) and indirect activator AICAR (5-aminoimidazole-4-carboxamide-1-D-ribofuranoside). AICAR, a compound widely used to activate AMPK (Fogarty and Hardie, 2010) is converted by adenosine kinase to ZMP, which activates AMPK by mimicking the actions of AMP as an allosteric activator of the enzyme (Henin et al, 1995; Hsu et al, 2011). Therefore, to perform this, PXR proximal promoter cell lines Hepx-1096/+43 and Hepx-497/+43 were grown in 48 well plates. Following the day, 10 μM of resveratrol, piceatannol, acacetin, kaempferol and genistein was added to the untreated cells or in pretreated cells either with 10 μM of dorsomorphin (AMPK inhibitor) or 500 μM of AICAR (AMPK activator). After 24 h of the drugs treatments, luciferase activities were determined. Treatment of AMPK inhibitor dorsomorphin did not affect the basal PXR-promoter activity in Hepx-1096/+43 cell line (**Figure 32**). However, basal PXR-promoter activity was increased in Hepx-497/+43 cell line significantly (**Figure 33**). Interestingly, pretreatment of AMPK inhibitor dorsomorphin diminished the

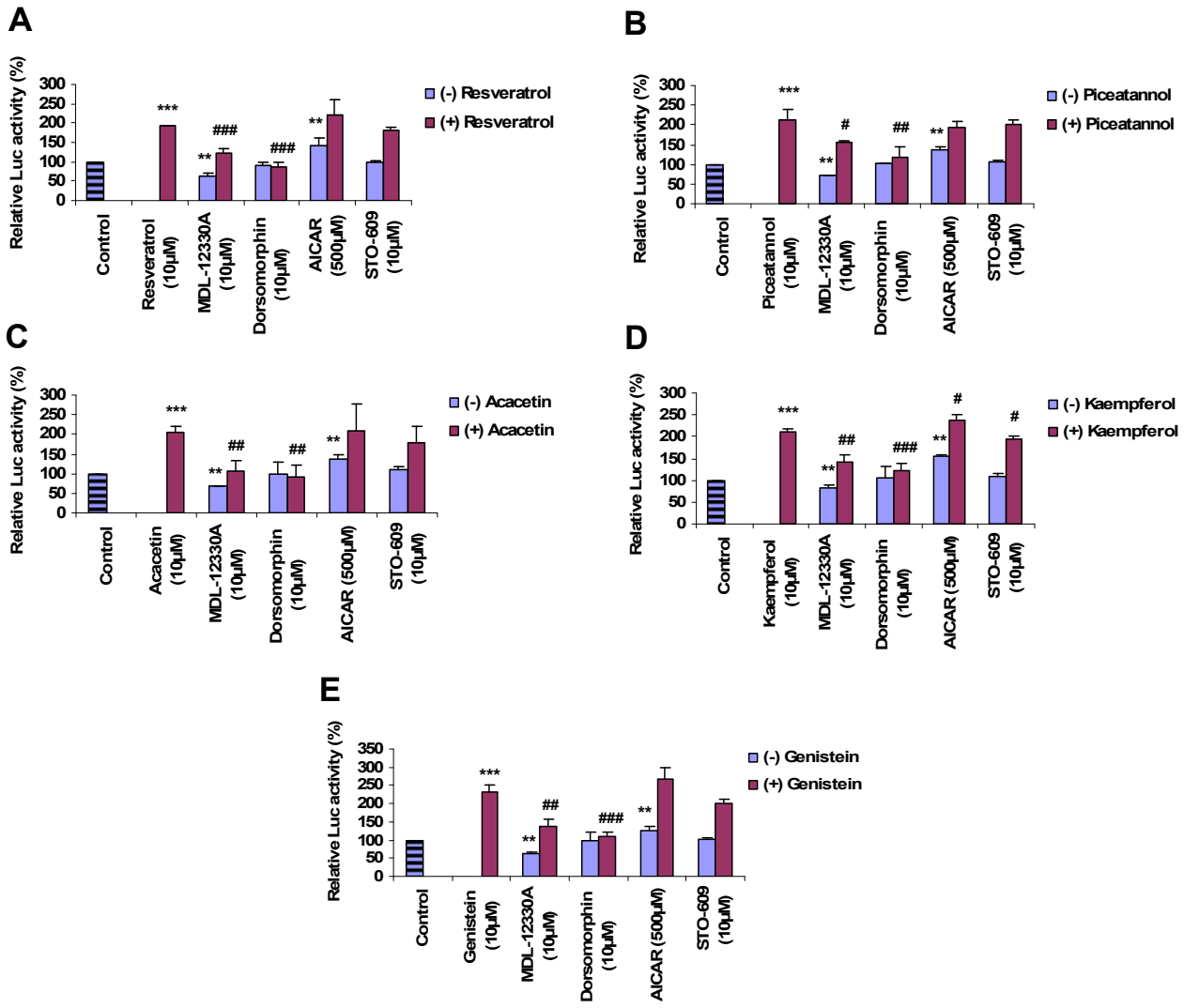

Figure 32: Effects of adenylyl cyclase inhibitor, AMPK inhibitor and activator and CaMKKβ inhibitor on PXR-promoter activity in Hepx-1096/+43 cell line. *Hepx-1096/+43 cells were grown in 48-well culture plates. On reaching ~60% confluency, cells were either treated with vehicle (control) or with 10 μM of resveratrol (**A**), piceatannol (**B**), acacetin (**C**), kaempferol (**D**) and genistein (**E**) alone or in combination with 10 μM of MDL-12330A (adenylyl cyclase inhibitor), 10 μM of dorsomorphin (AMPK inhibitor), 500 μM of AICAR (AMPK activator) and 10 μM of STO-609 (CaMKKβ inhibitor) pretreated cells. After 24 h of incubation period of drugs treatments, cells were lysed and luciferase activities were determined as described under 'Materials and Methods'. Luciferase values are expressed as relative Luc activity of control cells. Data represent the mean ± S.D. 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). While the symbol (#, ## and ###) signify luciferase values that differed significantly from the scores of resveratrol, piceatannol, acacetin, kaempferol and genistein alone (P<0.05, P<0.01 and P<0.001 respectively).*

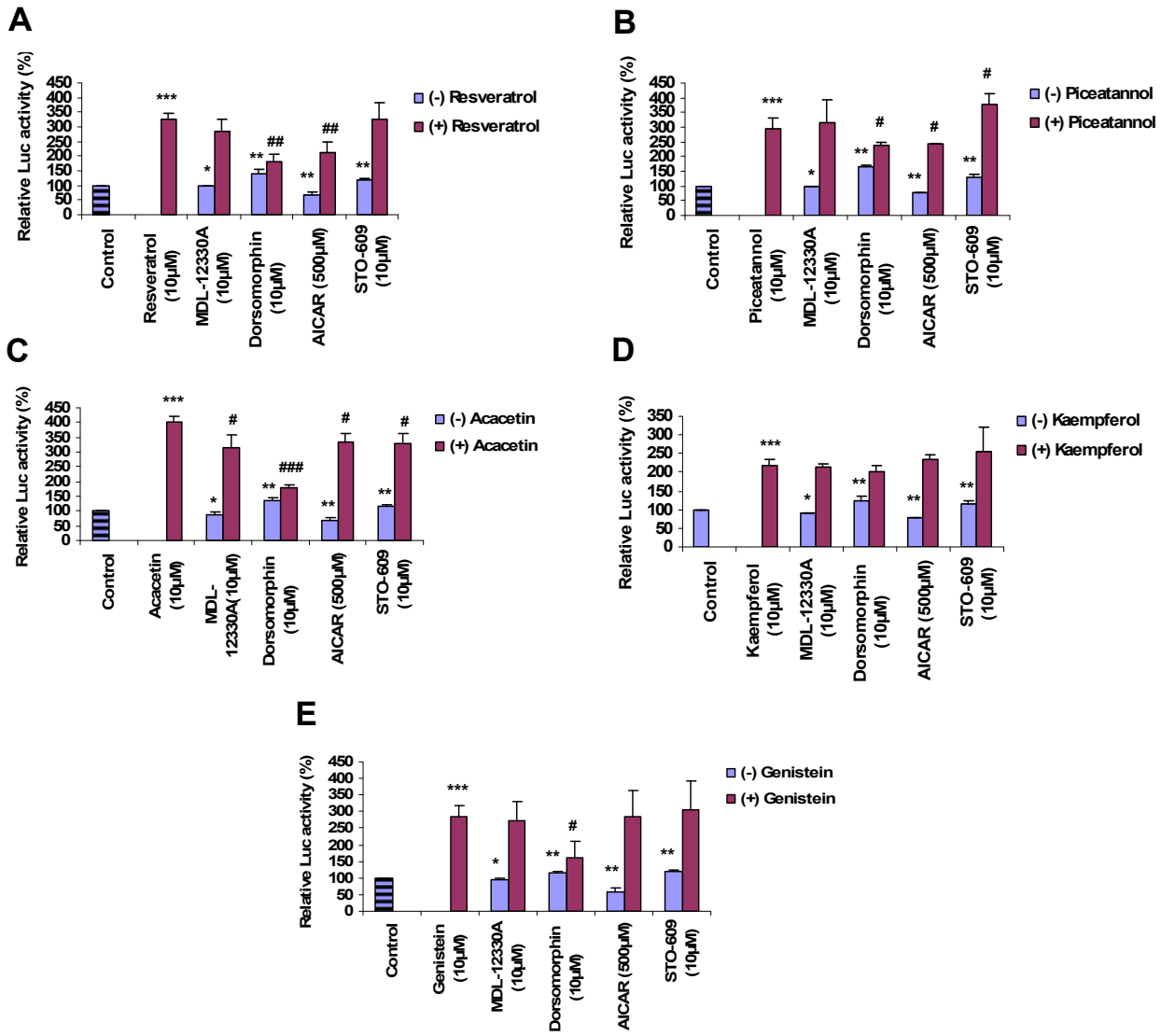

Figure 33: Effects of adenylyl cyclase inhibitor, AMPK inhibitor and activator and CaMKKβ inhibitor on PXR-promoter activity in Hepx-497/+43 cell line. *Hepx-497/+43 cells were propagated in 48-well culture plates. On reaching ~60% confluency, cells were either treated with vehicle (control) or with 10 μM of resveratrol (**A**), piceatannol (**B**), acacetin (**C**), kaempferol (**D**) and genistein (**E**) alone or in combination with 10 μM of MDL-12330A (adenylyl cyclase inhibitor), 10 μM of dorsomorphin (AMPK inhibitor), 500 μM of AICAR (AMPK activator) and 10 μM of STO-609 (CaMKKβ inhibitor) pretreated cells. After 24 h of incubation period of drugs treatments, cells were lysed and luciferase activities were determined as described under 'Materials and Methods'. Luciferase values are expressed as relative Luc activity of control cells. Data represent the mean ± S.D. 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). While the symbol (#, ## and ###) signify luciferase values that differed significantly from the scores of resveratrol, piceatannol, acacetin, kaempferol and genistein alone (P<0.05, P<0.01 and P<0.001 respectively).*

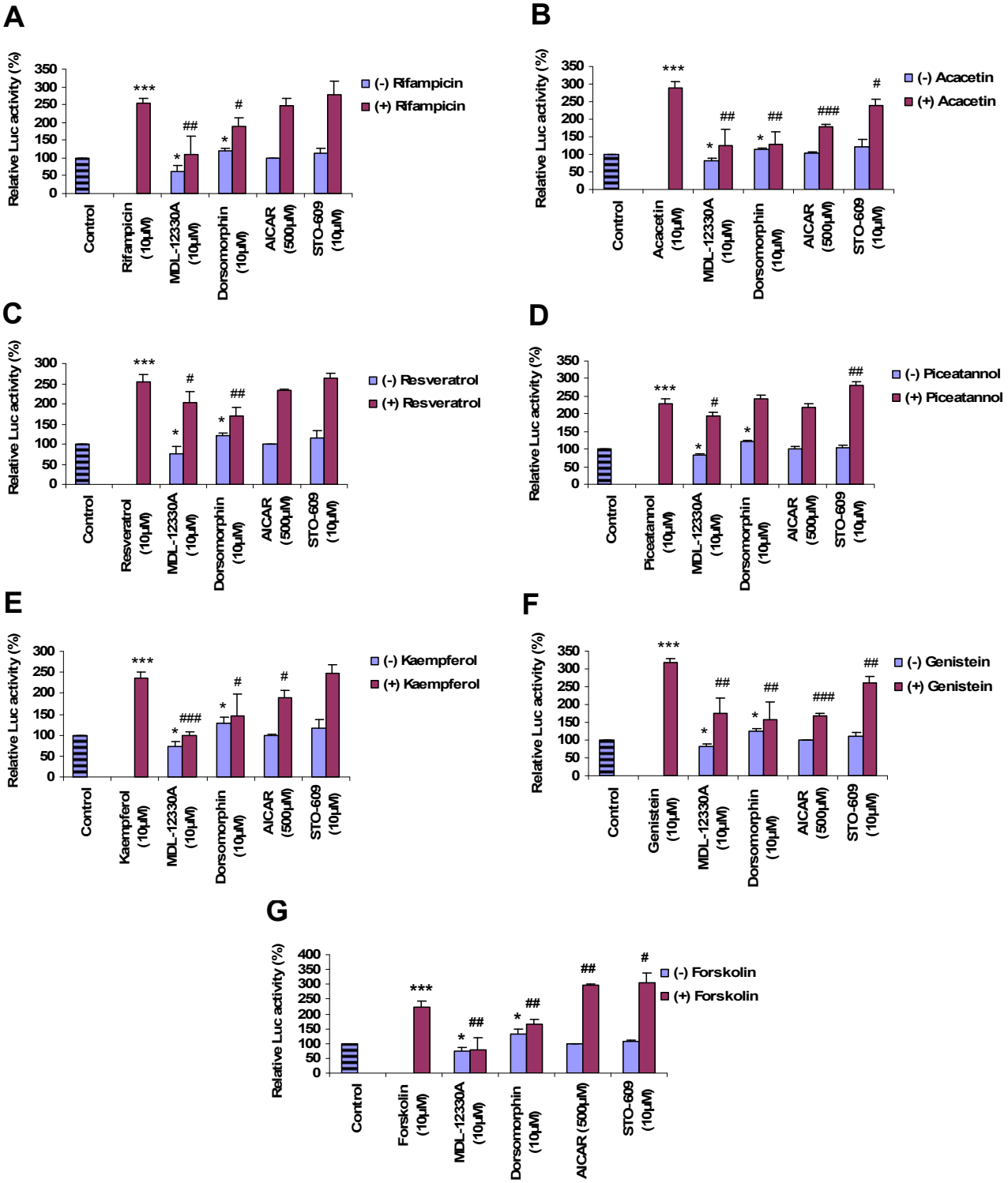

Figure 34: Effects of adenylyl cyclase inhibitor, AMPK inhibitor and activator and CAMKKβ inhibitor on PXR-mediated transcriptional activity in HepXREM cell line. *HepXREM cells were grown in 48-well culture plates. On reaching ~60% confluency, cells were either treated with vehicle (control) or with 10 μM of rifampicin (**A**), acacetin (**B**), resveratrol (**C**), piceatannol (**D**), kaempferol (**E**) and genistein (**F**) alone or in combination with 10 μM of MDL-12330A (adenylyl cyclase inhibitor), 10 μM of dorsomorphin (AMPK inhibitor),*

500 μM of AICAR (AMPK activator) and 10 μM of STO-609 (CaMKKβ inhibitor) pretreated cells. After 24 h of drugs treatments, cells were lysed and luciferase activities were determined as described under 'Materials and Methods'. Luciferase values are expressed as relative Luc activity of control cells. Data represent the mean ± S.D. 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). While the symbol (#, ## and ###) signify luciferase values that differed significantly from the scores of rifampicin, acacetin, resveratrol, piceatannol, kaempferol and genistein alone (P<0.05, P<0.01 and P<0.001 respectively).*

resveratrol, piceatannol, acacetin, kaempferol and genistein increased PXR-promoter activity nearly up to the basal levels in Hepx-1096/+43 cell line (**Figure 32**). The inhibition of resveratrol, piceatannol, acacetin kaempferol and genistein induced PXR-promoter activity by dorsomorphin is statistically extremely significantly (P<0.001). Further, pretreatment of dorsomorphin also inhibited the resveratrol, piceatannol, acacetin and genistein increased PXR-promoter activity in Hepx-497/+43 cell line (**Figure 33**). However, kaempferol induced PXR-promoter activity was not altered by dorsomorphin in Hepx-497/+43 cell line (**Figure 33**). Furthermore, the treatment of AMPK activator AICAR increased the basal PXR-promoter activity significantly (P<0.01) in Hepx-1096/+43 cell line (**Figure 32**). Conflictingly, AICAR significantly repressed the basal PXR-promoter activity in Hepx-497/+43 cell line (**Figure 33**). AICAR synergistically augmented the kaempferol induced PXR-promoter activity significantly (P<0.05) in Hepx-1096/+43 cell line (**Figure 32**). AICAR also increased the resveratrol and genistein induced PXR-promoter activity but non-significantly in Hepx-1096/+43 cell line (**Figure 32**). On the other hand, pretreatment of AICAR inhibited the resveratrol, piceatannol and acacetin induced PXR-promoter activity in Hepx-497/+43 cell line (**Figure 33**). Though, genistein and kaempferol increased PXR-promoter activity was not affected by AICAR pretreatment (**Figure 33**).

These results indicated the differential regulation of PXR-promoter activity by AMPK in Hepx-1096/+43 and Hepx-497/+43 cell lines. It appears that AMPK positively regulates PXR-promoter and increased the PXR-promoter activity in Hepx-1096/+43 cell line while AMPK negatively regulates PXR-promoter and attenuated the PXR-promoter activity in Hepx-497/+43 cell line.

It also appears that the induction of PXR-promoter activity by resveratrol, piceatannol, acacetin, kaempferol and genistein is also mediated through AMPK activation. The discrepancy between the results derived from the Hepx-1096/+43 and Hepx-497/+43 cell lines could be because of the cis-regulatory elements and dominancy of trans-regulatory factors which may be regulated by AMPK activation or inhibition.

Further, to find out the role of AMPK in the regulation of PXR transcriptional activity, HepXREM cells were propagated in 48-well culture dishes and treated with 10 μM of rifampicin, acacetin, resveratrol, piceatannol, kaempferol, genistein and forskolin alone or in combination with 10 μM of AMPK inhibitor dorsomorphin or 500 μM of AICAR (AMPK activator). After 24 h of the drugs treatments, luciferase activities were determined. Rifampicin, acacetin, resveratrol, piceatannol, kaempferol, genistein and forskolin increased the PXR transcriptional activity extremely significantly ($P<0.001$) (**Figure 34**). Treatment of AMPK inhibitor dorsomorphin augmented the basal PXR transcriptinal activity significantly ($P<0.05$) in HepXREM cell line (**Figure 34**). However, pretreatment of AMPK inhibitor dorsomorphin reduced the rifampicin, acacetin, resveratrol, kaempferol, genistein and forskolin increased PXR transcriptional activity significantly (**Figure 34**). Further, AMPK activator AICAR did not affect the PXR transcriptional activity (**Figure 34**). Furthermore, AICAR impeded the acacetin, kaempferol and genistein induced PXR transcriptional activity significantly p value less than 0.001, 0.05 and 0.001 respectively (**Figure 34**). Remarkably, forskolin induced PXR transcriptional activity was synergistically augmented by AICAR pretreatment significantly ($P<0.01$) in HepXREM cells (**Figure 34**).

Regulation of PXR promoter and receptor transcriptional functions by calcium/calmodulin-dependent kinase kinase β (CaMKKβ)

AMP-activated protein kinase (AMPK) activity requires one of at least two AMPK kinases; calcium/calmodulin-dependent kinase kinase β (CamKKβ) or LKB1 which activate it by phosphorylating T172 in the Tloop of AMPK (Chung et al, 2012; Hardie et al, 2012). Since, genistein has been recently reported to activate CamKKβ (Hsu et al, 2011). Therefore, we speculated if CaMKKβ is

involved in regulation of PXR gene promoter and its transcriptional activation. Hence, we tested the role of AMPK upstream kinase CaMKKβ in regulation of PXR-promoter activity and PXR transcriptional activity by using STO-609, a well-known inhibitor of CaMKKβ. STO-609 treatment did not change the basal PXR-promoter activity in Hepx-1096/+43 cell line (**Figure 32**). However, the basal PXR-promoter activity was elevated significantly ($P<0.01$) by STO-609 treatment in Hepx-497/+43 cell line (**Figure 33**). Pretreatment of STO-609 only inhibited the kaempferol increased PXR-promoter activity significantly ($P<0.05$) in Hepx-1096/+43 cells (**Figure 32**). On the other hand, only acacetin induced PXR-promoter activity was diminished by STO-609 pretreatment in Hepx-497/+43 cell line (**Figure 33**). Also, STO-609 synergistically augmented the piceatannol induced PXR-promoter activity in Hepx-497/+43 cells (**Figure 33**). STO-609 pretreatment did not affect the resveratrol, kaempferol and genistein induced PXR-promoter activity in Hepx-497/+43 cells (**Figure 33**). These results indicated that PXR-promoter activity is partially regulated in dependent and independent of CaMKKβ in Hepx-497/+43 and Hepx-1096/+43 cell lines. Further, these finding also indicated that regulation of PXR-promoter by AMPK may involve the LKB1 pathway, another AMPK upstream kinase.

Furthermore, we determined the involvement of CaMKKβ in regulation of PXR-mediated transcriptional activity in HepXREM cell line. STO-609 did not affect the PXR transcriptional activity in HepXREM cell line (**Figure 34**). Pretreatment of STO-609 in HepXREM cells inhibited the acacetin and genistein increased PXR transcriptional activity significantly. However, piceatannol and forskolin elevated PXR transcriptional was further augmented by STO-609 pretreatment in HepXREM cells (**Figure 34**). In STO-609 pretreated HepXREM cells, rifampicin, resveratrol and kaempferol increased PXR transcriptional activity was not affected (**Figure 34**).

Protein Kinase A (PKA) regulates PXR promoter and receptor transcriptional activity

Mostly, intracellular cAMP signalling is mediated by two groups of effectors that bind to cAMP; protein kinase A (PKA) and the cAMP-regulated guanine nucleotide exchange factors (cAMPGEFs) Epac1 and Epac2 (de Rooij

et al, 1998; Kawasaki et al, 1998; Gloerich and Bos, 2010). However, the principal intracellular target for cAMP is cAMP-dependent protein kinase (PKA). PKA signal transduction pathway is also involved in the phosphorylation of target proteins through indirect interaction with the classical mitogen-activated protein kinase signalling pathway. PKA activation phosphorylates the cAMP-response element binding (CREB) protein, a transcription factor that modulates genes containing cAMP-response elements (CRE) (Dumaz and Marais, 2005). Additionally, studies using mouse models indicate that PKA signalling interfaces with constitutive androstane receptor (CAR) activity by modulating CAR-protein cofactor interactions and also by increasing the expression of the Car gene itself (Ding et al, 2006). Recently, modulation of PXR transcriptional activity has been shown by protein kinase C (PKC) and PKA (Ding and Staudinger, 2005a; 2005b; Lichti-Kaiser et al, 2009). Recently, PKA has been shown to regulate PXR transcriptional activity in species specific manner (Lichti-Kaiser et al, 2009). Though, there are few reports on modulation of PXR transcriptional activity but the role of PKA in regulation of PXR gene transcription is not investigated. Moreover, it is also not clear that PKA is involved in herbal drugs induced PXR gene promoter and PXR transcriptional activation. Therefore, we investigated the role of PKA in regulation of PXR gene promoter and its transcriptional activity.

To determine the involvement of PKA in the control of PXR gene transcription, we used H89 dihydrochloride (H89), a well-known PKA inhibitor in the transcriptional assays done in PXR-promoter cell lines Hepx-1096/+43 and Hepx-497/+43. As shown in **Figure 35** and **36**, treatment of resveratrol, piceatannol, acacetin, kaempferol and genistein increased the PXR-promoter activity extremely significantly ($P<0.001$) in Hepx-1096/+43, Hepx-497/+43. Treatment of PKA inhibitor H89 suppressed the basal PXR-promoter activity in Hepx-1096/+43 cell line significantly ($P<0.01$) (**Figure 35**). However, in Hepx-497/+43 cell line PXR-promoter basal activity was not affected by H89 treatment (**Figure 36**). Further, pretreatment of H89 in Hepx-1096/+43 cells significantly diminished the resveratrol, piceatannol, acacetin, kaempferol and genistein increased PXR-promoter activity as compared to the activities of the corresponding drugs alone (**Figure 35**). On the other hand, only resveratrol

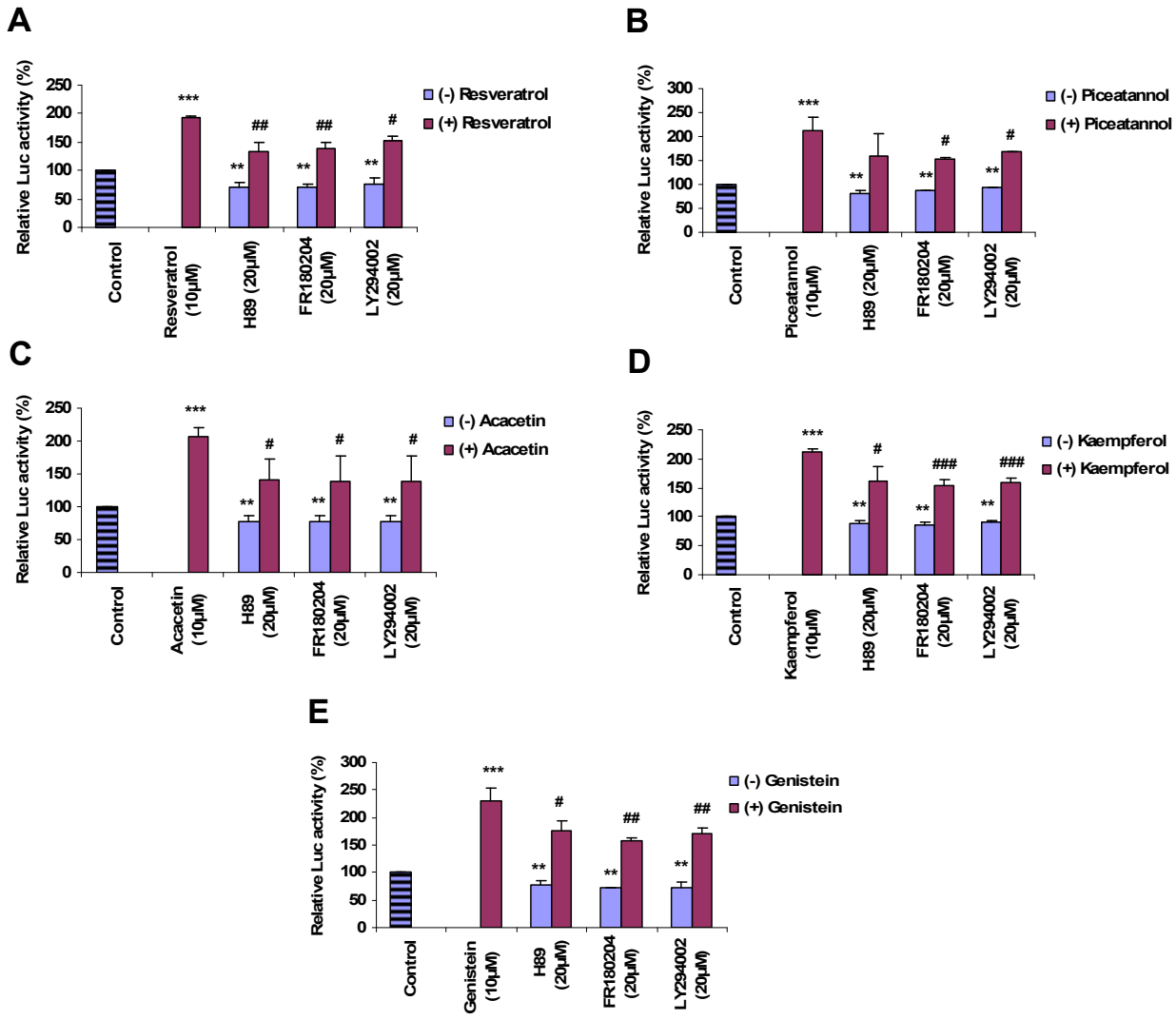

Figure 35: Effects of PKA, MAPK/ERK-1/2 and PI3 kinase/AKT pathway inhibitors on PXR-promoter activity in Hepx-1096/+43 cell line. *Hepx-1096/+43 cells were cultured in 48-well culture plates. On reaching ~60% confluency, cells were either treated with vehicle (control) or with 10 μM of resveratrol (**A**), piceatannol (**B**), acacetin (**C**), kaempferol (**D**) and genistein (**E**) alone or in combination with 20 μM of H89 (PKA inhibitor), FR180204 (MAPK/ERK-1/2 inhibitor), LY294002 (PI3 kinase/AKT inhibitor) pretreated cells. After 24 h of drugs treatments, cells were lysed and luciferase activities were determined as described under 'Materials and Methods'. Luciferase values are expressed as relative Luc activity of control cells. Data represent the mean ± S.D. of three independent experiments. Asterisks (** and ***) signify luciferase values that differed significantly from the scores of corresponding controls (P<0.01 and P<0.001 respectively in Student's t-test). Whereas the symbol (#, ## and ###) signify luciferase values that differed significantly from the scores of resveratrol, piceatannol, acacetin, kaempferol and genistein alone (P<0.05, P<0.01 and P<0.001 respectively).*

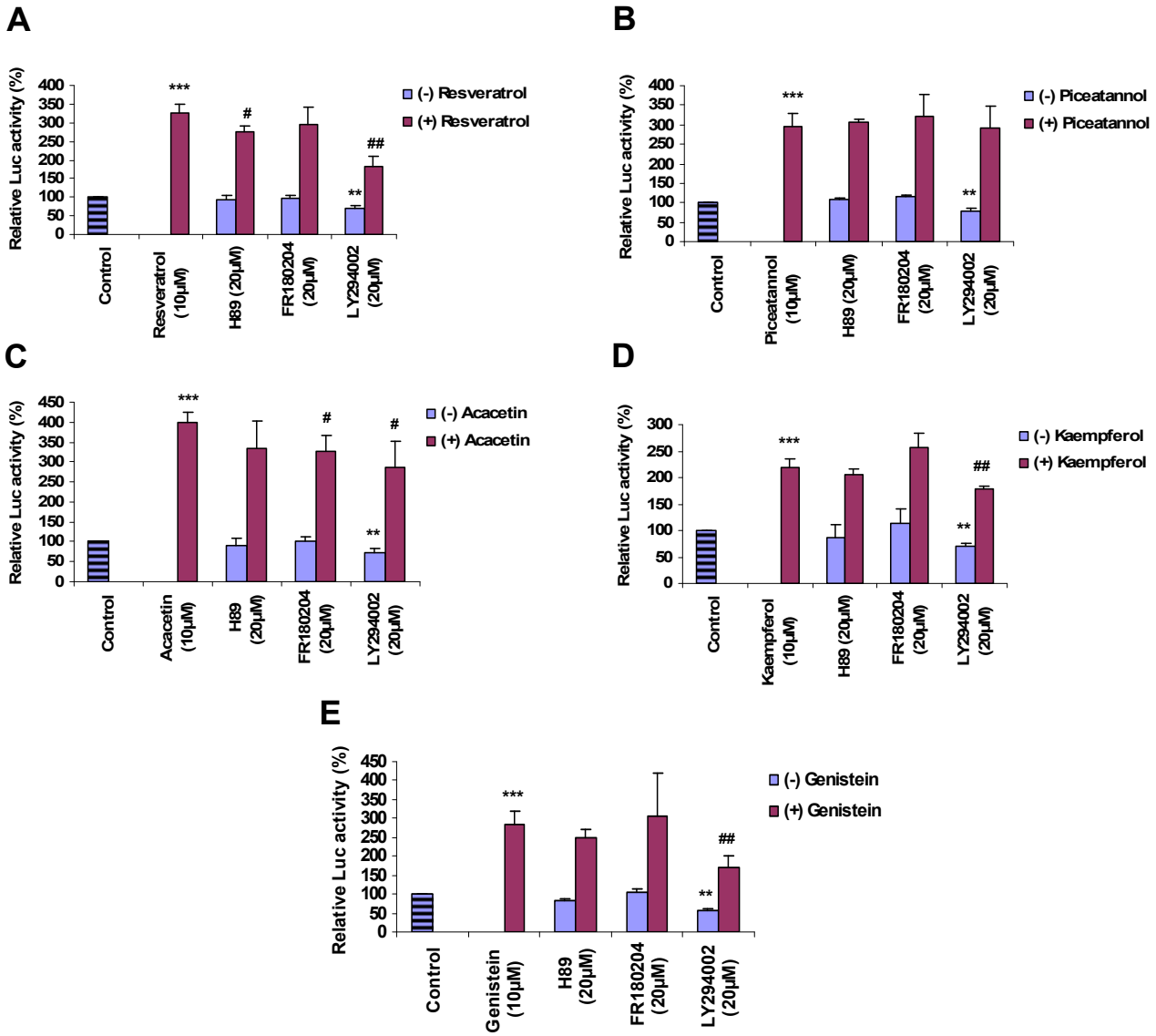

Figure 36: Effects of PKA, MAPK/ERK-1/2 and PI3 kinase/AKT pathway inhibitors on PXR-promoter activity in Hepx-497/+43 cell line. *Hepx-497/+43 cells were grown in 48-well culture plates. On reaching ~60% confluency, cells were either treated with vehicle (control) or with 10 µM of resveratrol (**A**), piceatannol (**B**), acacetin (**C**), kaempferol (**D**) and genistein (**E**) alone or in combination with 20 µM of H89 (PKA inhibitor), FR180204 (MAPK/ERK-1/2 inhibitor), LY294002 (PI3 kinase/AKT inhibitor) pretreated cells. After 24 h of drugs treatments, cells were lysed and luciferase activities were determined as described under 'Materials and Methods'. Luciferase values are expressed as relative Luc activity of control cells. Data represent the mean ± S.D. of three independent experiments. Asterisks (** and ***) signify luciferase values that differed significantly from the scores of corresponding controls (P<0.01 and P<0.001 respectively in Student's t-test). While the symbol (# and ##) signify luciferase values that differed significantly from the scores of resveratrol, piceatannol, acacetin, kaempferol and genistein alone (P<0.05 and P<0.01 respectively).*

increased PXR-promoter activity was significantly inhibited by pretreatment of H89 in Hepx-497/+43 cells (**Figure 36**). Though, H89 also inhibited the acacetin, kaempferol and genistein increased PXR-promoter activity in Hepx-497/+43 cells but non-significantly (**Figure 36**).

Further, to investigate the role of PKA in regulation of PXR-mediated transcriptional activation by resveratrol, piceatannol, acacetin, kaempferol, genistein and forskolin, HepXREM cell line was treated with these drugs and H89 (PKA inhibitor) alone or in combination. As shown in **Figure 37**, treatments of rifampicin, acacetin, resveratrol, piceatannol, kaempferol, genistein and forskolin increased the PXR transcriptional activity extremely significantly ($P<0.001$) in HepXREM cell line. However, treatment of H89 did not show any significant effect on basal PXR transcriptional activity though it appeared to be inhibited (**Figure 37**). Surprisingly, rifampicin, acacetin, resveratrol, piceatannol, kaempferol, genistein and forskolin augmented PXR transcriptional activity was diminished significantly by pretreatment of H89 in HepXREM cell line as compared to the corresponding drugs treated alone (**Figure 37**). The inhibition of forskolin induced PXR transcriptional activity by H89 reached to the below basal levels and extremely significantly ($P<0.001$).

MAPK/ERK-1/2 pathways regulate PXR-promoter and receptor transcriptional function

Mitogen-activated protein kinases (MAPKs) are a family of protein kinases that play central role in the signalling pathways of cell proliferation, survival, and apoptosis, are essential nodes in many cellular regulatory circuits including those that take place on DNA (Fan et al, 2007; Klein et al, 2013). Most members of the four MAPK subgroups that exist in canonical three kinase cascades extracellular signal-regulated kinases 1 and 2 (ERK1/2), ERK5, c-Jun N-terminal kinases (JNK1-3), and p38 MAPK have been shown to perform regulatory functions on chromatin (Fan et al, 2007; Klein et al, 2013). ERK1/2, serine/threonine protein kinase is activated by mitogens and growth factors through a Ras/Raf/MEK signalling cascade leading to cell growth and survival. SAPK/JNK1/2 and p38 MAPK are preferentially activated by proinflammatory cytokines and oxidative stress, resulting in cell differentiation

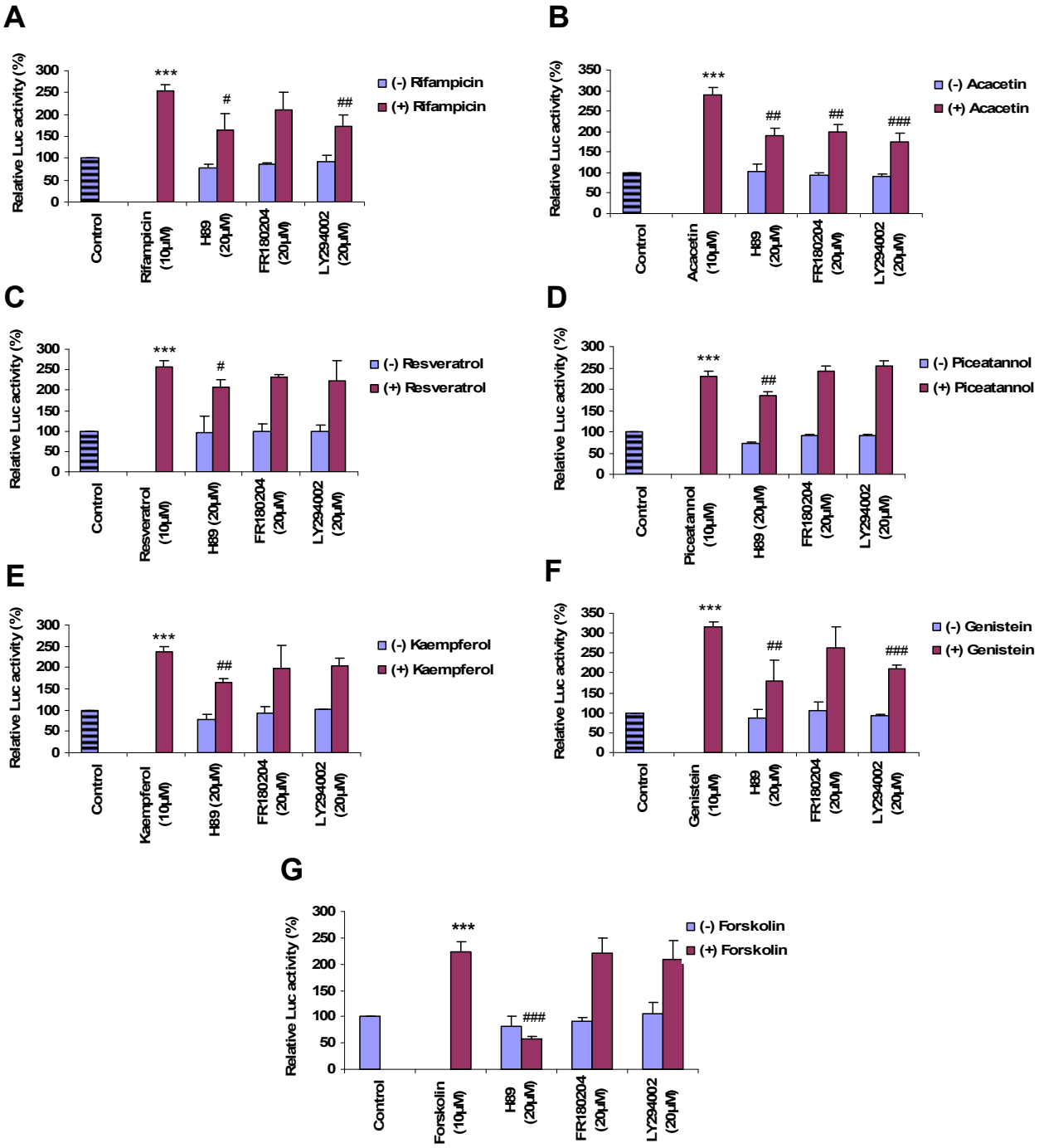

Figure 37: Effects of PKA, MAPK/ERK-1/2 and PI3 kinase/AKT pathway inhibitors on PXR-mediated transcriptional activity in HepXREM cell line. *HepXREM cells were propagated in 48-well culture plates. Next day cells were either treated with vehicle (control) or with 10 μM of rifampicin (**A**), acacetin (**B**), resveratrol (**C**), piceatannol (**D**) kaempferol (**E**) and genistein (**F**) alone or in combination with 20 μM of H89 (PKA inhibitor), FR180204 (MAPK/ERK-1/2 inhibitor), LY294002 (PI3 kinase/AKT inhibitor) pretreated cells. After 24 h of drugs treatments, cells were lysed and luciferase activities were determined as described under 'Materials and Methods'. Luciferase values are expressed as relative Luc activity of control cells. Data represent the mean ± S.D. of three*

independent experiments. Asterisks (***) signify luciferase values that differed significantly from the scores of corresponding controls (P<0.001 in Student's t-test). While the symbol (#, ## and ##) signify luciferase values that differed significantly from the scores of rifampicin, acacetin, resveratrol, piceatannol, kaempferol and genistein alone (P<0.05, P<0.01 and P<0.001 respectively).

and apoptosis (Chen et al, 1996; Minden and Karin, 1997; Raingeaud et al, 1995). MEK/ERK-1/2 pathway also regulates the transcriptional activity of nuclear receptors (Zassadowski et al, 2012).

To determine if MAPK/ERK-1/2 signalling is involved in the regulation of PXR gene transcription, FR180204 (5-(2-phenylpyrazolo[1,5-a]pyridin-3-yl)-1H-pyrazolo[3,4-c]pyridazin-3-amine) a selective ERK inhibitor was used in the transcriptional assays experiments carried out in PXR-promoter cell lines Hepx-1096/+43 and Hepx-497/+43. As shown in **Figure 35** and **36**, resveratrol, piceatannol, acacetin, kaempferol and genistein increased the PXR-promoter activity extremely significantly (P<0.001) in Hepx-1096/+43 and Hepx-497/+43 cells. Treatment of ERK 1/2 selective inhibitor FR180204 repressed the basal PXR-promoter activity in Hepx-1096/+43 cell line significantly (P<0.01) (**Figure 35**). However, in Hepx-497/+43 cells, PXR-promoter basal activity was not affected by FR180204 treatment (**Figure 36**). Further, pretreatment of ERK1/2 selective inhibitor FR180204 in Hepx-1096/+43 cells significantly inhibited the resveratrol, piceatannol, acacetin, kaempferol and genistein increased PXR-promoter activity as compared to the activities of the corresponding drugs only (**Figure 35**). On the other hand, only acacetin increased PXR-promoter activity was significantly inhibited by pretreatment of FR180204 in Hepx-497/+43 cell line (**Figure 36**). However, FR180204 also inhibited the resveratrol increased PXR-promoter activity in Hepx-497/+43 cells but not significant (**Figure 36**). Unexpectedly, pretreatment of FR180204 synergistically elevated the kaempferol increased PXR-promoter activity in Hepx-497/+43 cells but the synergistic induction was not significant (**Figure 36**). These results indicated the differential regulation of PXR-promoter activity in both the cell lines by MAPK/ERK-1/2. Nonetheless, results from Hepx-1096/+43 cell line which was integrated with big fragment of PXR proximal promoter indicated clearly that MAPK/ERK-1/2 positively

regulate PXR gene transcription possibly by phosphorylating the trans regulatory factors of PXR-promoter. Besides, these results indicated that resveratrol, piceatannol, acacetin, kaempferol and genistein mediated PXR-promoter activation is also attributed by MAPK/ERK 1/2.

Further, to examine the role of MAPK/ERK-1/2 in the regulation of PXR-mediated transcriptional function, HepXREM cell line was treated with rifampicin, acacetin, resveratrol, piceatannol, kaempferol, genistein and forskolin and ERK1/2 selective inhibitor FR180204 alone or in combination. As shown in **Figure 37**, treatments of rifampicin, acacetin, resveratrol, piceatannol, kaempferol, genistein and forskolin increased the PXR transcriptional activity extremely significantly ($P<0.001$) in HepXREM cell line. However, treatment of ERK1/2 selective inhibitor FR180204 did not show any significant effect on basal PXR transcriptional activity (**Figure 37**). However, acacetin increased PXR transcriptional activity was inhibited significantly ($P<0.01$) by FR180204 pretreatment in HepXREM cells (**Figure 37**). Rifampicin, resveratrol, kaempferol and genistein increased PXR transcriptional activity was also inhibited by FR180204 however it was not statistically significant (**Figure 37**).

Phosphatidylinositide-3-OH kinase (PI3K)/Akt pathway regulates PXR promoter and receptor transcriptional functions

The PI3Ks (phosphatidylinositol-3-OH-kinases) regulate cellular signalling networks that are involved in processes linked to the survival, growth, proliferation, metabolism and specialized differentiated functions of cells (Gharbi et al, 2007; Martini et al, 2013). The subversion of this network is common in cancer and has also been linked to disorders of inflammation (Gharbi et al, 2007; Martini et al, 2013).

To investigate the involvement of PI3K/Akt pathway in the regulation of PXR gene transcription, LY294002 (a well-known selective PI3K inhibitor) was employed in the transcriptional assays experiments carried out in PXR-promoter cell lines Hepx-1096/+43 and Hepx-497/+43. LY294002 is cell permeable that acts as a competitor inhibitor of the ATP binding site of PI3K. As shown in **Figure 35** and **36**, resveratrol, piceatannol, acacetin, kaempferol

and genistein increased the PXR-promoter activity extremely significantly (P<0.001) in Hepx-1096/+43 and Hepx-497/+43 cells. Treatment of PI3K selective inhibitor LY294002 inhibited the basal PXR-promoter activity in both the promoter cell lines significantly (P<0.01) (**Figure 35** and **36**). Further, pretreatment of PI3K selective inhibitor LY294002 in both the cell lines; Hepx-1096/+43 and Hepx-1096/+43 significantly diminished the resveratrol, piceatannol, acacetin, kaempferol and genistein increased PXR-promoter activity as compared to the activities of the corresponding drugs only treatment (**Figure 35** and **36**). These results indicated the PI3K/Akt pathway positively regulates the PXR gene transcription.

Further, to explore the role of PI3K/Akt pathway in the regulation of PXR-mediated transcriptional functions, HepXREM cell line was treated with rifampicin, acacetin, resveratrol, piceatannol, kaempferol, genistein and forskolin and PI3K selective inhibitor LY294002 alone or in combination. As shown in **Figure 37**, treatments of rifampicin, acacetin, resveratrol, piceatannol, kaempferol, genistein and forskolin increased the PXR transcriptional activity extremely significantly (P<0.001) in HepXREM cell line. Treatment of PI3K selective inhibitor LY294002 did not show any effect on basal PXR transcriptional activity (**Figure 37**). However, rifampicin, acacetin and genistein increased PXR transcriptional activity was diminished extremely significantly (P<0.01, P<0.001 and P<0.001 respectively) by LY294002 in HepXREM cells as compared to the activities of these drugs treated alone (**Figure 37**). Though, resveratrol, kaempferol and piceatannol increased PXR transcriptional activity was affected significantly by LY294002 (**Figure 37**).

Regulation of PXR promoter and receptor transcriptional functions by histone acetyltransferases (CPB/P300) inhibitors

Histone acetyltransferases (CPB/P300) regulate the gene transcription process through acetylating either histone or non-histone proteins. Since resveratrol, genistein, kaempferol also has been shown to activate the histone acetyltransferases (CBP/P300). Therefore we speculated that the effect of herbal drugs on PXR-promoter activity and its transcriptional

functions may be imparted partially through histone acetyltransferases (CBP/P300) activation by these drugs.

In this context, to investigate the involvement of histone acetyltransferases (CPB/P300) in the regulation of PXR gene transcription, anacardic acid and C646 [histone acetyltransferases (CPB/P300) inhibitors] was used in the transcriptional assays experiments done in PXR-promoter cell lines Hepx-1096/+43 and Hepx-497/+43. Anacardic acid (2-Hydroxy-6-pentadecylbenzoic acid, 6-Pentadecylsalicylic acid) is a cell permeable salicylic acid analog that acts as a potent, non-competitive inhibitor of p300 and PCAF (p300/CBP-associated factor) histone acetyltransferase (HAT) activities. C646 (4-[4-[[5-(4, 5-Dimethyl-2-nitropheny-l)-2-furanyl]methylene]-4, 5-dihydro-3-methyl-5-oxo--1H-pyrazol-1-yl]benzoic acid) is a competitive histone acetyltransferase (HAT) p300/CBP inhibitor (p300/CBP Inhibitor IV, HAT Inhibitor V) and is selective versus other acetyltransferases. As shown in **Figure 38** and **39**, resveratrol, piceatannol, acacetin, kaempferol and genistein increased the PXR-promoter activity extremely significantly ($P<0.001$) in both the promoter cell lines. Treatment of histone acetyltransferases (CPB/P300) inhibitors anacardic acid and C646 did not affected the basal PXR-promoter activity in Hepx-1096/+43 cell line (**Figure 38**). On the other hand, in Hepx-497/+43 cells, PXR-promoter basal activity was increased significantly only by C646 (**Figure 39**). Further, pretreatment of histone acetyltransferases (CPB/P300) inhibitors anacardic acid and C646 in Hepx-1096/+43 cells significantly inhibited the kaempferol and genistein increased PXR-promoter activity (**Figure 38**). Resveratrol, piceatannol and acacetin induced PXR-promoter activity was not significantly affected by anacardic acid and C646 (**Figure 38**). On the other hand, pretreatment of anacardic acid and C646 in Hepx-497/+43 cells did not show any significant effect on the resveratrol, piceatannol, acacetin, kaempferol and genistein elevated PXR-promoter activity (**Figure 39**).

Further, to examine the role of histone acetyltransferases (CPB/P300) in the regulation of PXR-mediated transcriptional function, HepXREM cell line was treated with rifampicin, acacetin, resveratrol, piceatannol, kaempferol, genistein and forskolin and histone acetyltransferases (CPB/P300) inhibitors anacardic acid and C646 alone or in combination. As shown in **Figure 40**,

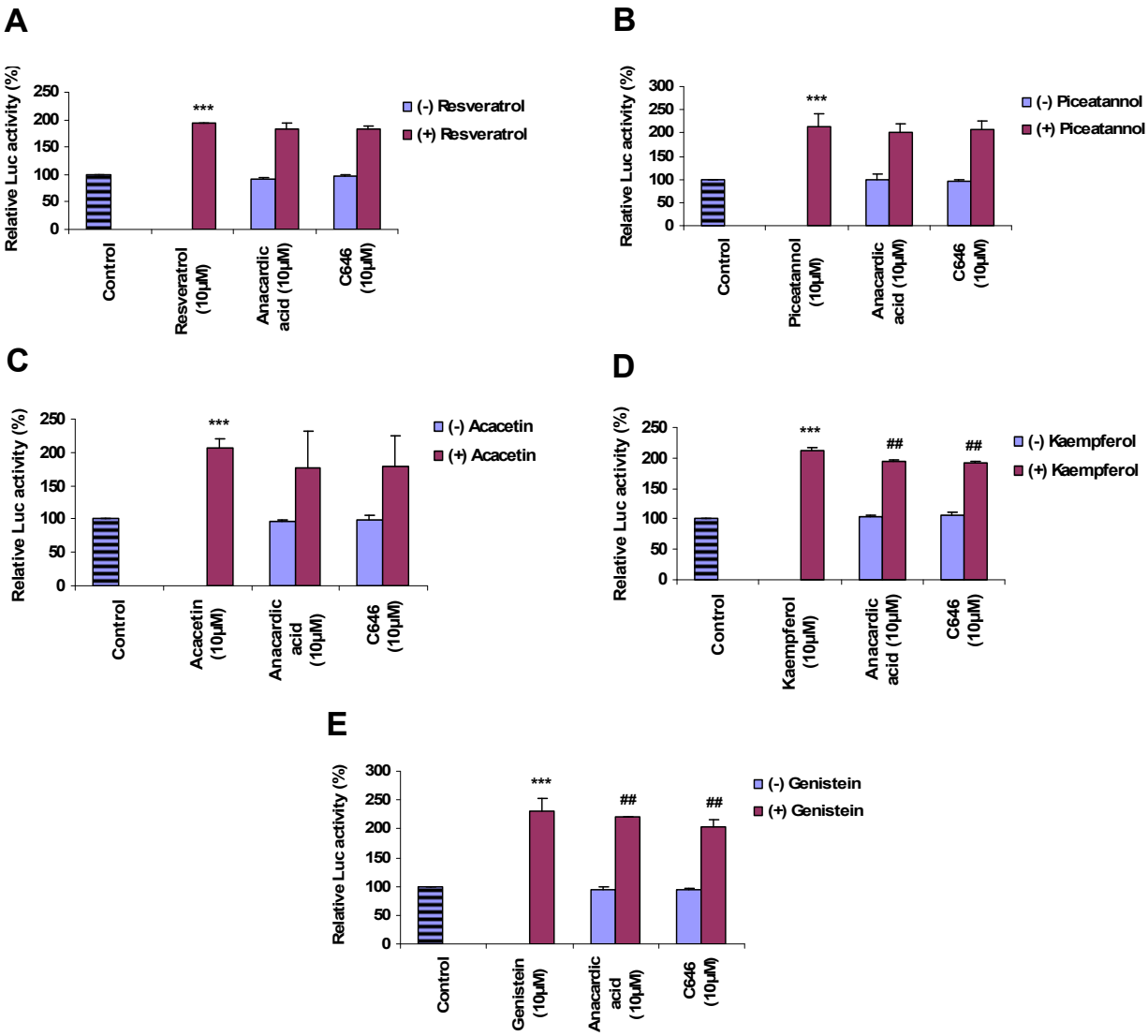

Figure 38: Effects of histone acetyltransferases (CPB/P300) inhibitors on PXR-promoter activity in Hepx-1096/+43 cell line. *Hepx-1096/+43 cells were cultured in 48-well culture plates. On reaching ~60% confluency, cells were either treated with vehicle (control) or with 10 µM of resveratrol (**A**), piceatannol (**B**), acacetin (**C**), kaempferol (**D**) and genistein (**E**) alone or in combination with 10 µM of anacardic acid and C646 [histone acetyltransferases (CPB/P300) inhibitors] pretreated cells. After 24 h of drugs treatments, cells were lysed and luciferase activities were determined as described under 'Materials and Methods'. Luciferase values are expressed as relative Luc activity of control cells. Data represent the mean ± S.D. of three independent experiments. Asterisks (***) signify luciferase values that differed significantly from the scores of corresponding controls (P<0.001 in Student's t-test). While the symbol (##) signify luciferase values that differed significantly from the scores of kaempferol and genistein alone (P<0.01).*

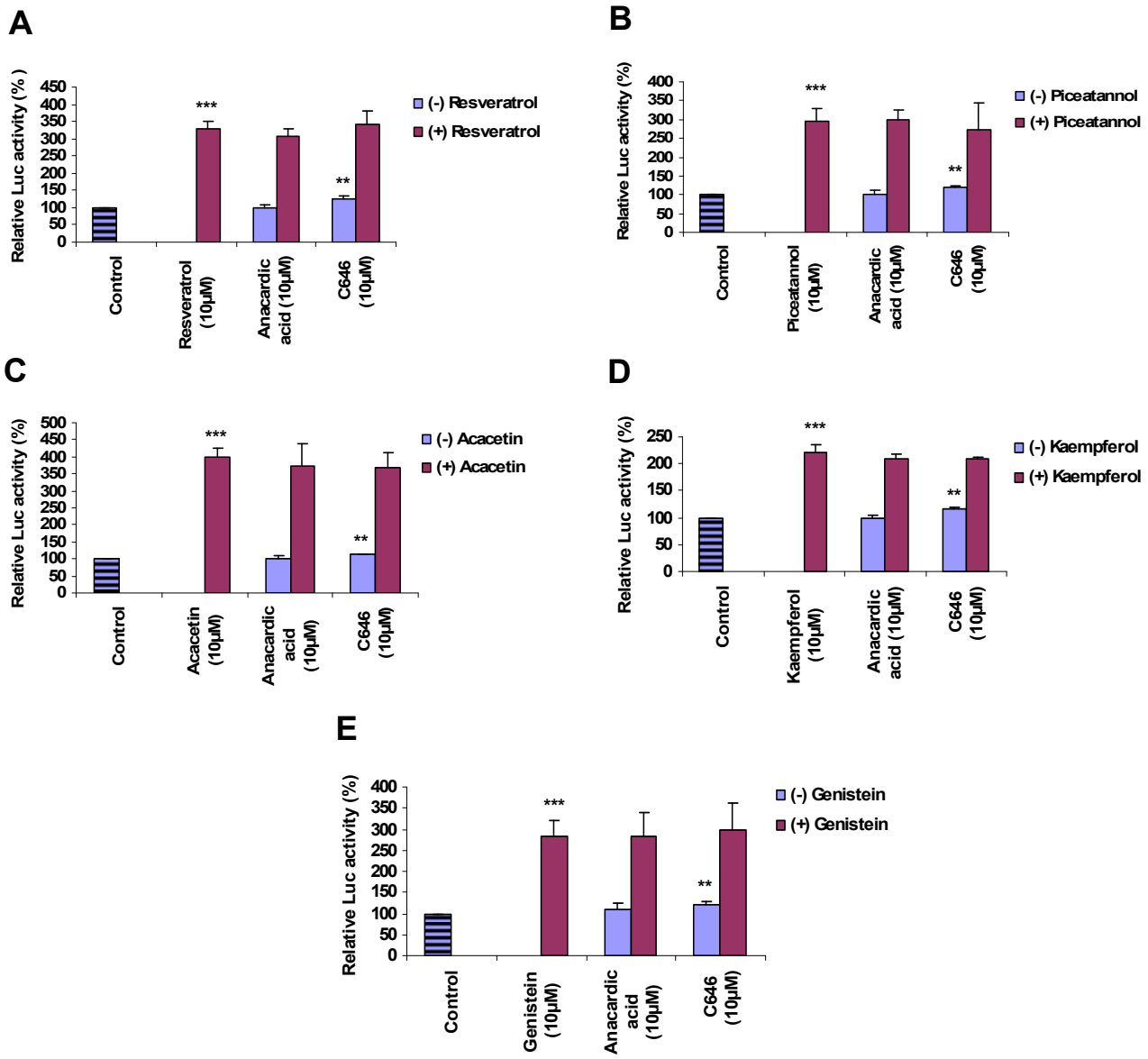

Figure 39: Effects of histone acetyltransferases (CPB/P300) inhibitors on PXR-promoter activity in Hepx-497/+43 cell line. *Hepx-497/+43 cells were grown in 48-well culture plates. Next day, cells were either treated with vehicle (control) or with 10 µM of resveratrol (**A**), piceatannol (**B**), acacetin (**C**), kaempferol (**D**) and genistein (**E**) alone or in combination with 10 µM of anacardic acid and C646 [histone acetyltransferases (CPB/P300) inhibitors] pretreated cells. After 24 h of drugs treatments, cells were lysed and luciferase activities were determined as described under 'Materials and Methods'. Luciferase values are expressed as relative Luc activity of control cells. Data represent the mean ± S.D. of three independent experiments. Asterisks (** and ***) signify luciferase values that differed significantly from the scores of corresponding controls (P<0.01 and P<0.001 in Student's t-test respectively).*

treatments of rifampicin, acacetin, resveratrol, piceatannol, kaempferol, genistein and forskolin increased the PXR transcriptional activity extremely significantly (P<0.001) in HepXREM cell line. Only treatment of C646 in HepXREM cell line augmented the basal PXR transcriptional activity significantly (**Figure 40**). However, anacardic acid did not show any significant effect on basal PXR transcriptional activity (**Figure 40**). Though, anacardic acid pretreatment repressed the acacetin and genistein increased PXR transcriptional activity significantly (P<0.01) (**Figure 40B** and **40F**). Anacardic acid did not show any significant effect on rifampicin, resveratrol, piceatannol, kaempferol and forskolin elevated PXR transcriptional activity (**Figure 40**). Pretreatment of C646 inhibited the only genistein increased PXR transcriptional activity significantly (P<0.01) (**Figure 40F**). C646 did not show any significant effect on rifampicin, acacetin, resveratrol, kaempferol and forskolin elevated PXR transcriptional activity (**Figure 40**). Surprisingly, C646 synergistically augmented the piceatannol increased PXR transcriptional activity significantly (P<0.01) (**Figure 40D**).

Inhibition of PXR-promoter response by forskolin is not mediated by activation of cAMP-dependent PKA pathway

Forskolin is a well-known adenylyl cyclases (ACs) activator and elevates cAMP levels. Most of the functions of forskolin are mediated by cAMP-dependent PKA pathway. Forskolin also activates PXR transcriptional functions. In our experiments, forskolin suppressed the PXR-promoter basal as well as activator drugs-induced activity (**Chapter-II**). Nonetheless, the question remains unanswered how forskolin inhibited the PXR-promoter activity and needs to be address. Is the suppression of PXR-promoter by forskolin involves the cAMP-dependent PKA pathway or it deviates to other signalling pathway(s). Therefore, to investigate the mechanism of forskolin mediated inhibition of PXR-promoter activity; we employed the inhibitors of various signalling pathways including cAMP-dependent PKA, AMPK, CaMKKβ, MAPK/ERK-1/2 and PI3K/Akt pathways in PXR-promoter cell lines Hepx-1096/+43 and Hepx-497/+43. As shown in the **Figure 41**, forskolin inhibited the PXR-promoter activity extremely significantly (P<0.001) nearly 68% as compared to control in

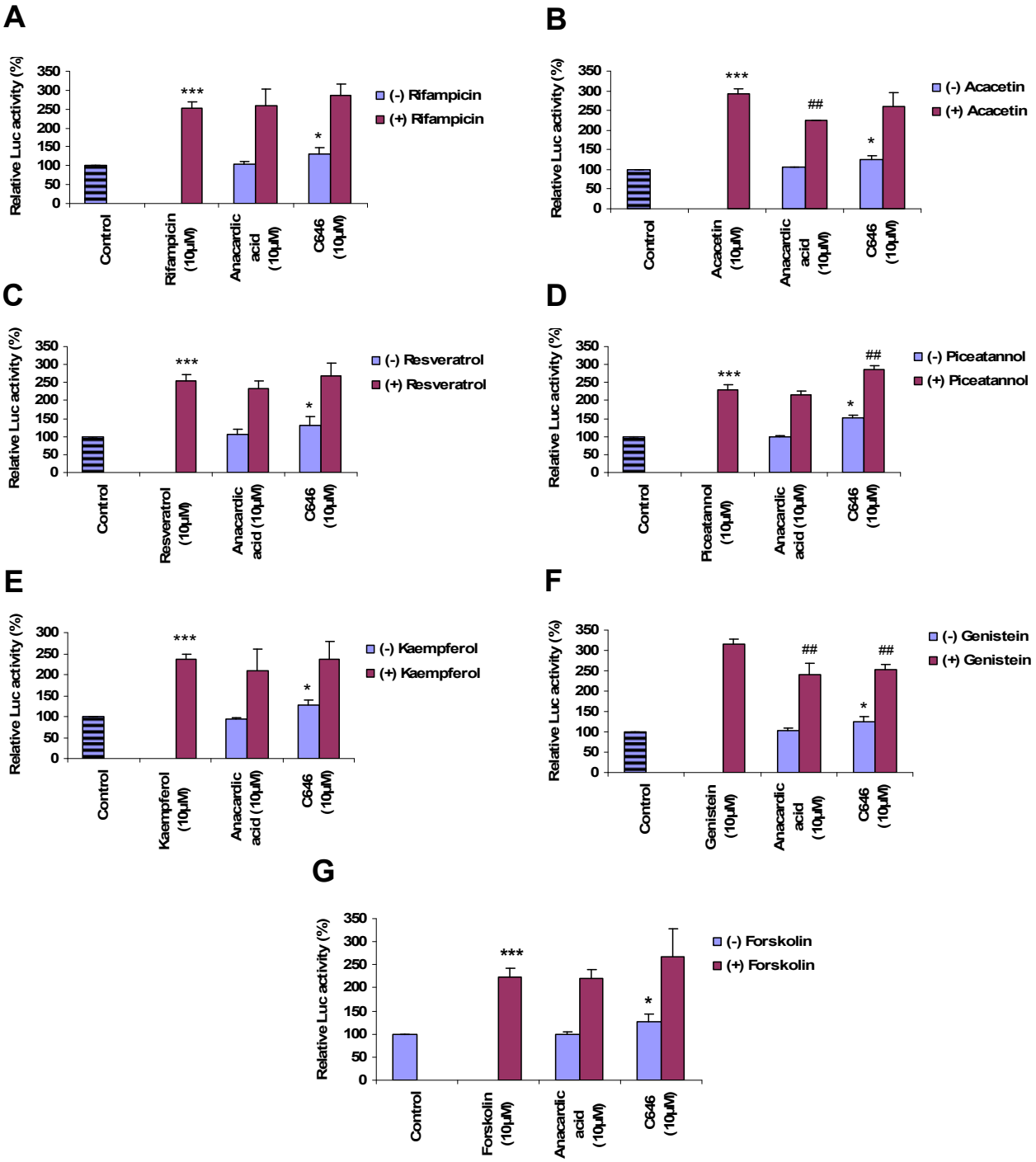

Figure 40: Effects of histone acetyltransferases (CPB/P300) inhibitors on PXR-mediated transcriptional activity in HepXREM cell line. *HepXREM cells were cultured in 48-well culture plates. Next day, cells were either treated with vehicle (control) or with 10 μM of rifampicin (**A**), acacetin (**B**), resveratrol (**C**), piceatannol (**D**), kaempferol (**E**) and genistein (**F**) alone or in combination with 10 μM of anacardic acid and C646 [histone acetyltransferases (CPB/P300) inhibitors] pretreated cells. After 24 h of drugs treatments, cells were lysed and luciferase activities were determined as described under 'Materials and Methods'. Luciferase values are expressed as relative Luc activity of control*

cells. Data represent the mean ± S.D. 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 in Student's t-test respectively). While the symbol (##) signify luciferase values that differed significantly from the scores of acacetin, piceatannol and genistein alone (P<0.01).*

both the promoter cell lines Hepx-1096/+43 and Hepx-497/+43. Treatment of MDL-12,330A (adenylyl cyclase inhibitor) and H89 (PKA inhibitor) in Hepx-1096/+43 inhibited PXR-promoter significantly nearly up to 40% and 28% respectively (**Figure 41A**). On the other hand, MDL-12,330A inhibited PXR-promoter activity significantly nearly 13% in Hepx-1096/+43 and H89 did not show any significant effect on it (Figure 41B). Forskolin Non-PKA activating analog 1, 9-dideoxy forskolin did not show any effect on PXR-promoter activity in both the cell lines (Figure 41A and 41B). Pretreatment of MDL-12,330A increased the forskolin mediated inhibition significantly nearly 15% and 32% in Hepx-1096/+43 and Hepx-497/+43 cells respectively (**Figure 41A** and **41B**). H89 and 1, 9-dideoxy forskolin did not show any effect on forskolin suppressed PXR-promoter activity in both the cell lines (**Figure 41A** and **41B**). Further, dorsomorphin (AMPK inhibitor) did not affect the PXR-promoter activity in Hepx-1096/+43 cell line however, PXR-promoter activity was significantly increased nearly 154% in Hepx-497/+43 cells significantly (**Figure 41C** and **41D**). Interestingly, pretreatment of dorsomorphin significantly increased the forskolin suppressed PXR-promoter activity in both the promoter cell lines nearly up to 56% from 38% and 60% from 39% respectively. Furthermore, AICAR (AMPK activator) and STO-609 (CaMKKβ inhibitor) did not show any significant effect on the forskolin mediated inhibition of PXR-promoter activity (**Figure 41C** and **41D**). Moreover, LY294002 (PI3K selective inhibitor) inhibited the basal PXR-promoter activity in both the promoter cell lines significantly (P<0.01) nearly 64% (**Figure 41E** and **41F**). Further, pretreatment of LY294002 in Hepx-497/+43 cells additively augmented the forskolin suppressed PXR-promoter activity significantly nearly 47% (**Figure 41F**). In Hepx-1096/+43 cell line, LY294002 increased the forskolin suppressed PXR-promoter activity a little but not significantly (**Figure 41F**). FR180204

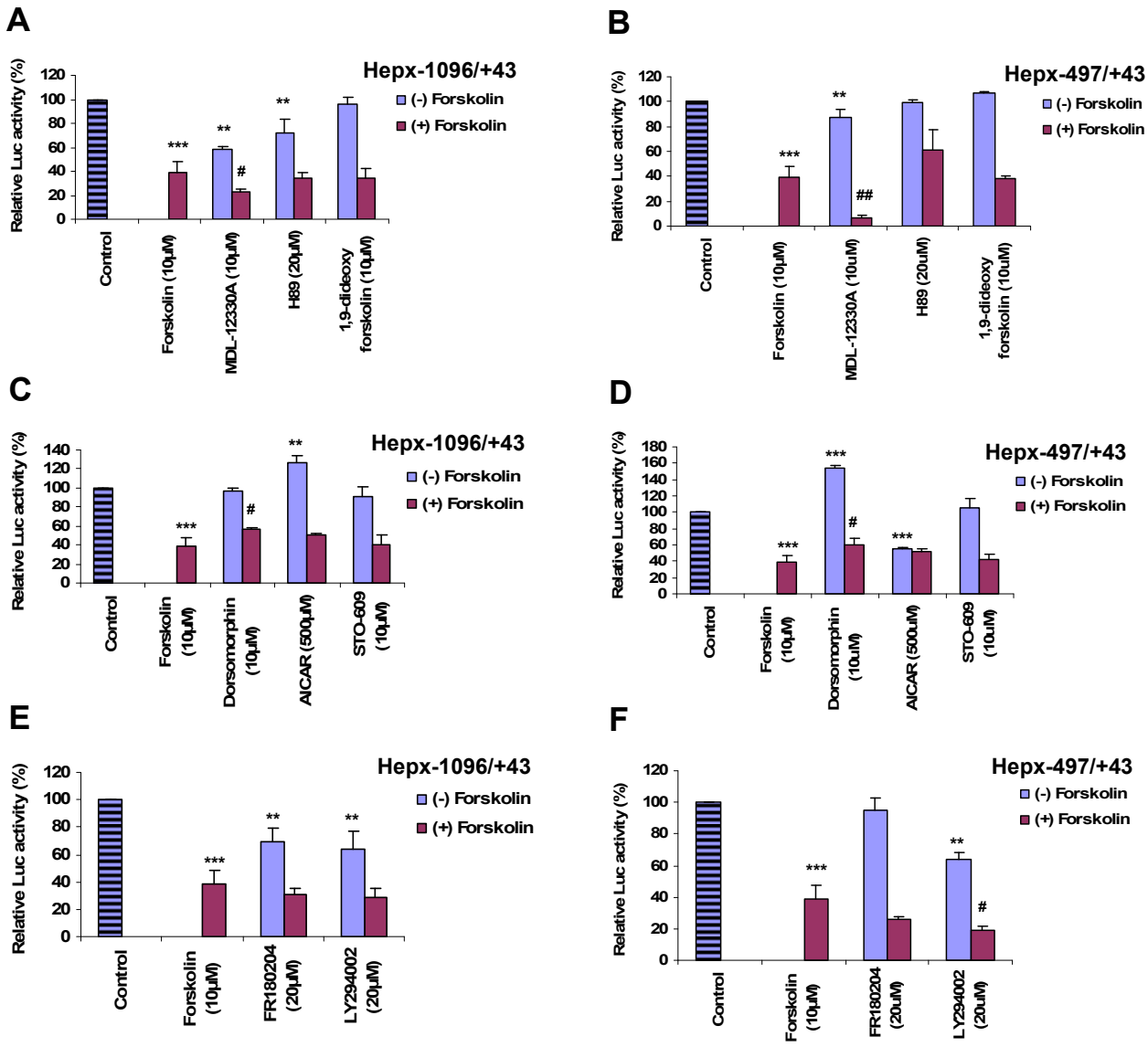

Figure 41: Effects of inhibitors/activators of various signalling pathways on forskolin suppressed PXR-promoter activity in Hepx-1096/+43 and Hepx-497/+43 cell lines. *Hepx-1096/+43 and Hepx-497/+43 cells were cultured in 48-well culture plates. Following the day, cells were either treated with vehicle (control) or with 10 μM of forskolin alone or in combination with pretreated cells with indicated inhibitors or activators of different signalling pathways. After 24 h of drugs treatments, cells were lysed and luciferase activities were determined as described under 'Materials and Methods'. Luciferase values are expressed as relative Luc activity of control cells. Data represent the mean ± S.D. of three independent experiments. Asterisks (** and ***) signify luciferase values that differed significantly from the scores of corresponding controls (P<0.01 and P<0.001 respectively in Student's t-test). While the symbol (# and ##) signify luciferase values that differed significantly from the scores of forskolin alone (P<0.05 and P<0.01 respectively).*

(MAPK/ERK-1/2 inhibitor) also did not show any significant effect on the forskolin repressed PXR-promoter activity (**Figure 41E** and **41F**). These results indicated that forskolin classical cAMP-dependent PKA pathway is not involved in the forskolin suppressed PXR-promoter-activity.

Role of PXR in proliferation of human liver cell line, HepG2

The role of PXR in cancer cell growth is controversial. Therefore, to know the role of PXR in HepG2 cells growth, we compared the cell viability of HepG2 and HepXR (Stably expressing human PXR in HepG2) cell lines in presence of rifampicin, acacetin, resveratrol, piceatannol, kaempferol, genistein and forskolin in dose and time dependent manner by MTT assays. **Figure 42** exhibits the cell viability comparison of HepG2 and HepXR cell lines. Our results showed that with the increasing time from 24 h to 72 h incubation with the drugs, the cell proliferation is more in HepG2 cells as compared to the HePXR cells (**Figure 42**). This indicated that over expression of PXR in HepXR cells reduced the cell proliferation.

Effect of PXR over expression and modulation on cell cycle progression

The effect of over PXR and its ligands on cell cycle progression was determined by flow cytometry and comparison was made between HepG2 cell line and human PXR overexpressing cell line HepXR in time dependent manner. Both the cell lines HepG2 and HepXR were propagated and treated with vehicle or 10 μM of rifampicin, acacetin, resveratrol, genistein, forskolin, sulforaphane, and camptothecin and allowed to incubate for 24 h and 48 h. Following the incubation period, cells were processed and analyzed by flow cytometry. **Figure 43** shows the comparative effect of PXR over expression and modulation on cell cycle progression in HepG2 and HepXR cell lines. The comparison of results between the HepG2 and HepXR cells after 24 h and 48 h drug treatment were made and summarized in **Table X**. As shown in **Figure 43** and **Table X**, flow cytometry analysis of drugs-treated HepG2 and HepXR cells staining with propidium iodide revealed that PXR arrested cell cycle in G0/G1 phase in 24 h incubation with most of the drugs. However, cell cycle

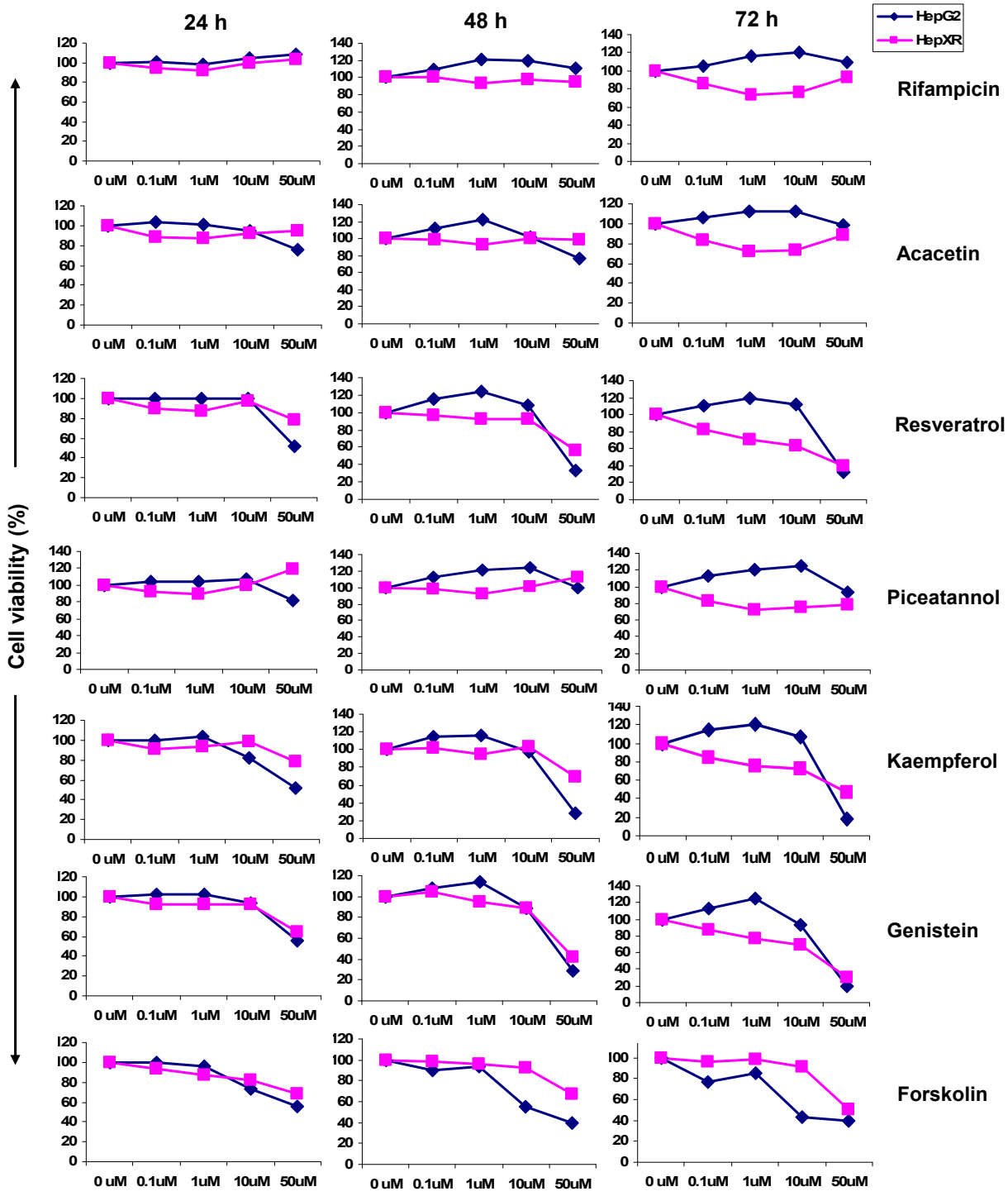

Figure 42: Cell viability comparative analysis of HepG2 and HepXR cell lines by MTT assay in dose- and time-dependent manner. *Equal number of HepG2 and HepXR cells were cultured in 96 well culture plate and allowed to proliferate up to ~60% confluency. Then cells were treated with either vehicle or four different concentrations (0.1, 1, 10 and 50 μM) of rifampicin, acacetin, resveratrol, piceatannol, kaempferol, genistein and forskolin. Treated cells were incubated for 24 h, 48 h and 72 h. Following the incubation period, cell viability was determined by MTT assay as described in 'Materials and Methods'. Data are expressed as the mean of two independent experiments.*

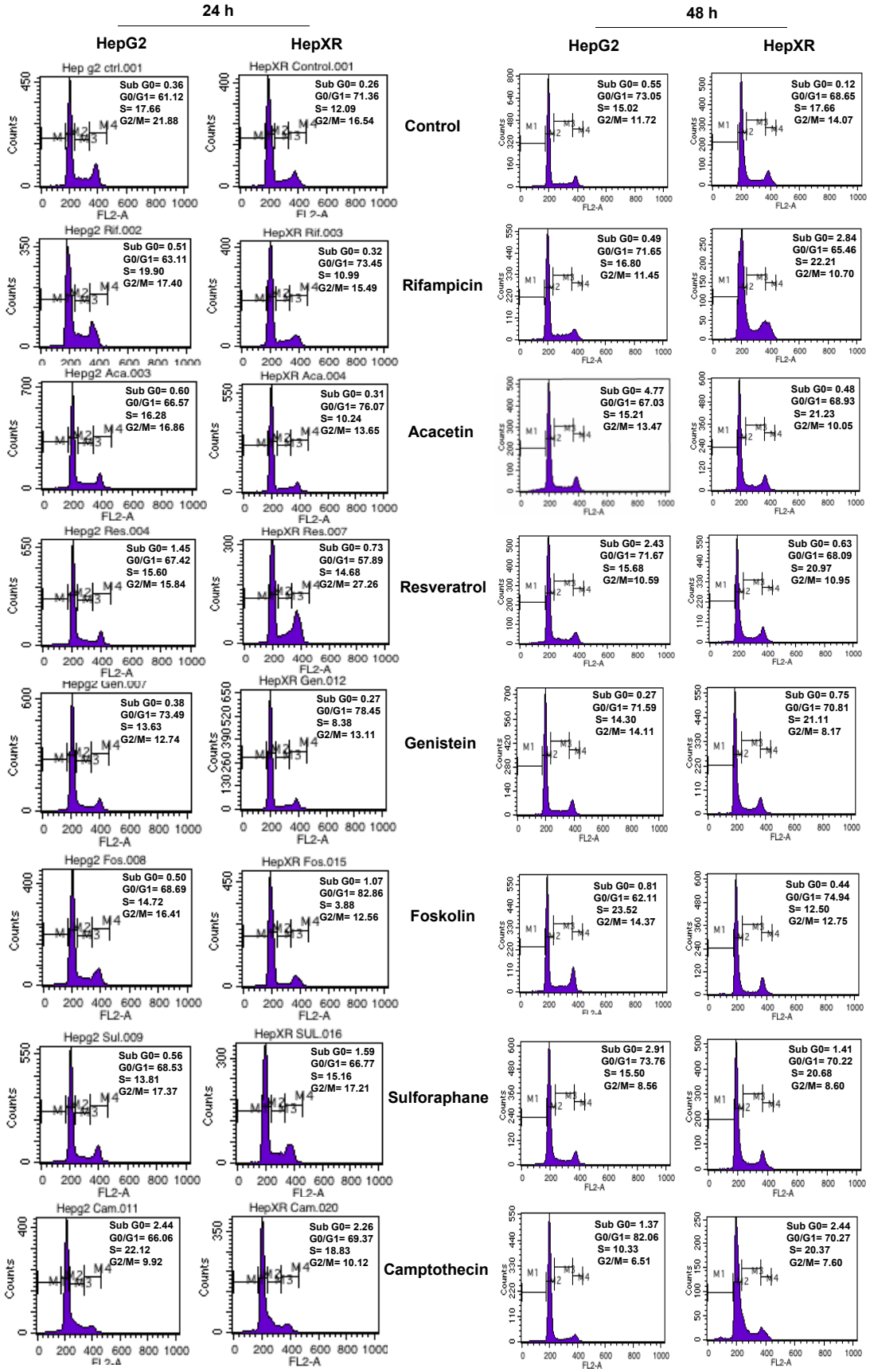

Figure 43: Effect of PXR over expression and modulation on cell cycle progression in HepG2 and HepXR cell lines. *HepG2 and HepXR cells were*

cultured in 6 well culture plate (1x10^6 cells /well) and allowed to proliferate up to ~60% confluency. Then cells were treated with either vehicle or 10 µM of rifampicin, acacetin, resveratrol, piceatannol, kaempferol, genistein, forskolin, sulforaphane, coumestrol and camptothecin. Treated cells were incubated for 24 h and 48 h. Following the incubation period, cells were fixed with ethanol and stained with propidium iodide and then cell cycle distribution was analyzed by flow cytometry as described in 'Materials and Methods'. The percentage of G0/G1, S and G2/M phases were calculated using Cell Quest software and indicated on the right upper side of each histogram. M1 signify Sub Go phase; M1 signify Go/G1 phase; M3 signify S phase and M4 signify G2/M phase of cell cycle.

Table X: A comparative analysis on cell cycle progression in HepG2 and HepXR cell line after drug treatment by FACS.

S. No.	Drugs (Conc. 10 µM)	Cell line	Cell cycle analysis (24 h)				Cell cycle analysis (48 h)			
			Sub G_0	G_0/G_1	S	G_2/M	Sub G_0	G_0/G_1	S	G_2/M
1.	Control	HepG2	0.36	61.12	17.66	21.18	0.55	73.05	15.02	11.72
		HepXR	0.26	71.36	12.09	16.54	0.12	68.65	17.66	14.07
2.	Rifamipicin	HepG2	0.51	63.11	19.90	17.04	0.49	71.66	16.80	11.45
		HepXR	0.32	73.45	10.99	15.49	2.84	65.46	22.21	10.70
3.	Acacetin	HepG2	0.60	66.57	16.28	16.86	4.77	67.03	15.21	13.47
		HepXR	0.31	76.07	10.24	13.65	0.48	68.93	21.23	10.05
4.	Resveratrol	HepG2	1.45	67.42	15.60	15.84	0.33	73.73	13.69	12.50
		HepXR	0.73	57.89	14.68	27.26	0.45	68.07	21.99	10.19
5.	Genistein	HepG2	0.38	73.49	13.63	12.74	0.27	71.59	14.30	14.11
		HepXR	0.27	78.45	8.38	13.11	0.75	70.81	21.11	8.17
6.	Forskolin	HepG2	0.50	68.69	14.72	16.41	0.81	62.11	23.52	14.37
		HepXR	1.07	82.86	3.88	12.56	0.44	74.94	12.50	12.75
7.	Sulforaphane	HepG2	0.56	68.53	13.81	17.37	2.91	73.76	15.50	8.56
		HepXR	1.59	66.77	15.16	17.21	1.41	70.22	20.68	8.60
8.	Camptothecin	HepG2	2.44	66.06	22.12	9.92	1.37	82.06	10.33	6.51
		HepXR	2.26	69.37	18.83	10.12	2.44	70.27	20.37	7.60

was arrested in the S phase in 48 h incubation with most of the drugs (**Figure 43** and **Table X**). As depicted in the results, over expression of PXR increased the G0/G1 and S phase arrest of HepXR cells in 24 h and 48 h of drugs treatments respectively.

Over expression of PXR induces apoptosis in HepG2 cell line

In this context, we investigated if PXR has an apoptotic or anti-apoptotic role in HepG2 cells. For this reason, HepG2 and HepXR cells were grown in 6 well culture plates and treated with either vehicle or indicated concentrations

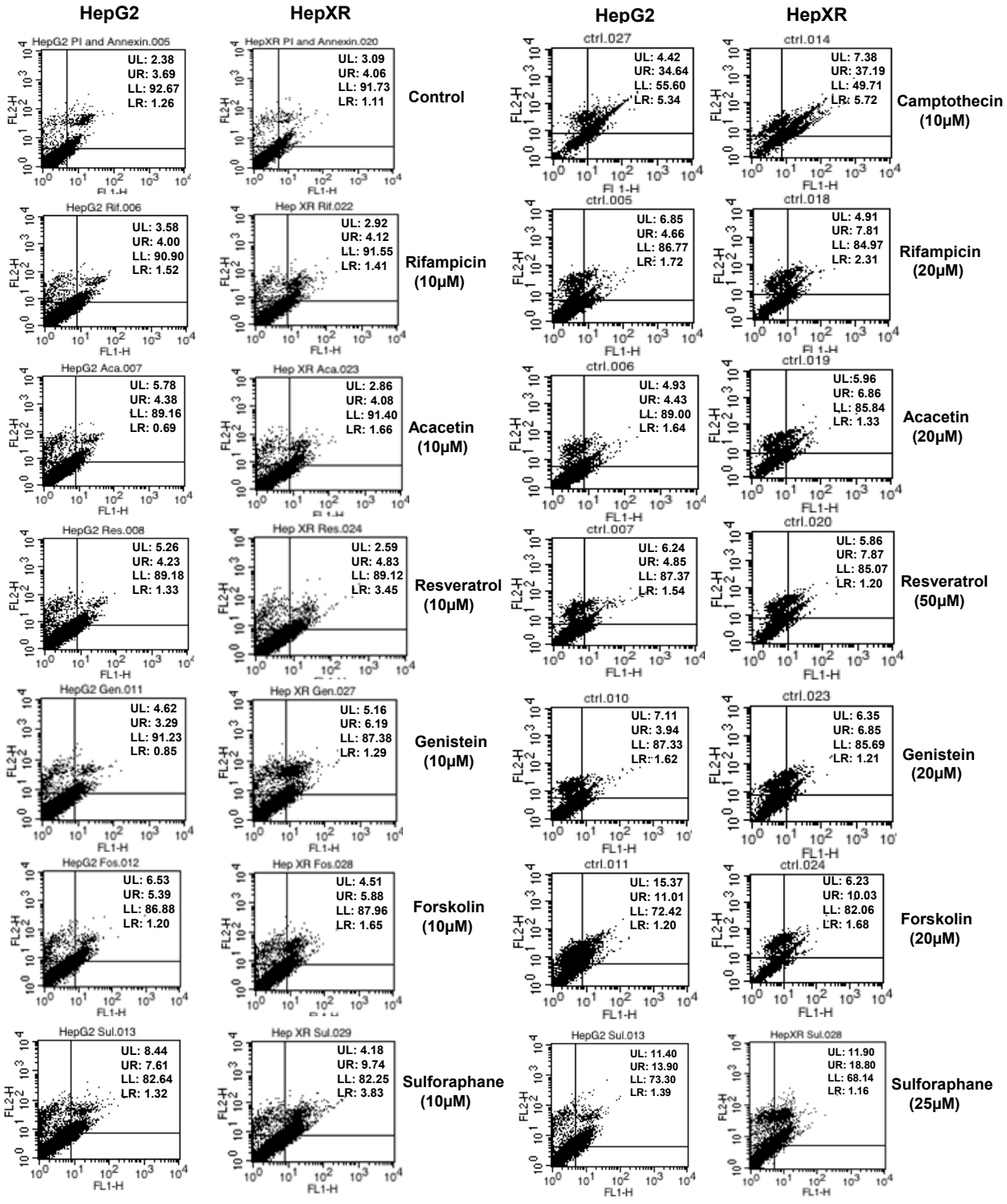

Figure 44: Cell apoptosis analysis in HepG2 and HepXR cell lines by FACS. *HepG2 and HepXR cells were cultured in 6 well culture plate (1x10^6 cells /well) and allowed to proliferate up to ~60% confluency. Then cells were treated with either vehicle or indicated drugs. Camptothecin was used as positive control for apoptosis. Treated cells were incubated for 24 h and following the incubation period cells were processed as described in 'Materials and Methods' using Annexin V FITC Apoptosis Detection kit. Processed samples were analyzed by flow cytometry. A minimum of 20,000 events were recorded for each sample.*

Apoptotic counts were calculated using Cell Quest software and indicated on the right upper side of each histogram. UL denotes upper left that signify dead cells, UR denotes upper right signify late apoptotic cells, LL denotes lower left signify viable cells and LR denotes lower right signify early apoptotic cells.

Table XI: A comparative analysis of cell apoptosis in HepG2 and HepXR cell line after 24h drug treatment by FACS.

S. No.	Drugs	Conc.	Cell line	Apoptosis				Conc.	Apoptosis			
				UL	UR	LL	LR		UL	UR	LL	LR
1.	Control		HepG2	2.38	3.69	92.67	1.26		2.38	3.69	92.67	1.26
			HepXR	3.09	4.06	91.73	1.11		3.09	4.06	91.73	1.11
2.	Rifamipicin	10 µM	HepG2	3.58	4.00	90.90	1.52	20 µM	6.85	4.66	86.77	1.72
			HepXR	2.92	4.12	91.55	1.41		4.91	7.81	84.97	2.31
3.	Acacetin	10 µM	HepG2	5.78	4.38	89.16	0.69	20 µM	4.93	4.43	89.00	1.64
			HepXR	2.86	4.08	91.40	1.66		5.96	6.87	85.84	1.33
4.	Resveratrol	10 µM	HepG2	5.26	4.23	89.18	1.33	50 µM	6.24	4.85	87.37	1.54
			HepXR	2.59	4.83	89.12	3.45		5.86	7.87	85.07	1.20
5.	Genistein	10 µM	HepG2	4.62	3.29	91.23	0.85	20 µM	7.11	3.94	87.33	1.62
			HepXR	5.16	6.19	87.38	1.29		6.35	6.85	85.69	1.21
6.	Forskolin	10 µM	HepG2	6.53	5.39	86.88	1.20	20 µM	15.37	11.01	72.42	1.20
			HepXR	4.51	5.88	87.96	1.65		6.23	10.03	82.06	1.68
7.	Sulforaphane	10 µM	HepG2	8.44	7.61	82.64	1.32	25 µM	11.40	13.90	73.30	1.39
			HepXR	4.18	9.74	82.25	3.83		11.90	18.80	68.14	1.16
8.	Camptothecin	10 µM	HepG2	4.42	34.64	55.60	5.94	10 µM	4.42	34.64	55.60	5.94
			HepXR	7.38	37.19	49.71	5.72		7.38	37.19	49.71	5.72

UL denotes upper left that signify dead cells, UR denotes upper right signify to late apoptotic cells, LL denotes lower left signify to viable cells and LR denotes lower right to signify early apoptotic cells.

of rifampicin, acacetin, resveratrol, genistein, forskolin, sulforaphane and camptothecin. Camptothecin was used as positive control for apoptosis. Treated cells were incubated for 24 h and following the incubation period cells were processed as described in 'Materials and Methods' using Annexin V FITC Apoptosis Detection kit. Processed samples were analyzed by flow cytometry. **Figure 44** demonstrates the apoptosis analysis in HepG2 and HepXR cell lines in response to herbal drugs. As shown in **Figure 44**, 10 µM of rifampicin, acacetin, resveratrol and genistein did not show apoptosis in 24 h incubation in both the cell lines (**Figure 44** and **Table XI**). However, higher concentrations of rifampicin, acacetin, resveratrol and genistein showed more

apoptosis in HepXR cells as compared to HepG2 cells although, the percentage of apoptosis is low (**Figure 44** and **Table XI**). Forskolin induced the apoptosis but did not showed any difference between the two cell lines. Both the concentration of sulforaphane also showed more apoptosis in HepXR cells as compared to HepG2 cells. Camptothecin, a positive control of apoptosis also did more apoptosis in HepXR cells as cpmpared to HepG2 cells (**Figure 44** and **Table XI**). These results indicated that over expression of PXR could be the reason of more apoptosis in HepXR cell line as compared to HepG2 cell line implying that PXR has a apoptotic role in HepG2 cell line.

DISCUSSION

Research during the recent few years has revealed several unsuspected roles of xenobiotic master regulator PXR beyond drug metabolism and transport. Its role in hepatic steatosis, vitamin D homeostasis, bile acids homeostasis, steroid hormones homeostasis, inflammatory bowel diseases, cancer, etc. has been reported (Zhou et al, 2009; Ihunnah et al, 2011; Gao and Xie, 2012; Pondugula and Mani, 2013). Although the primary event governing activation of nuclear receptors is ligand binding, increasing evidences suggest that cell signalling pathways and modulation of nuclear receptor-cofactor-phosphorylation status also determines overall responsiveness to environmental stimuli (Rochette-Egly, 2003; Staudinger and Lichti-Kaiser, 2008). Post-translational modifications like acetylation, deacetylation, phosphorylation, dephosphorylation, somoylation has been implicated in gene transcription regulation of many transcription factors including nuclear receptors. A few of the recent reports indicate that some metabolic signal transduction pathways interface with PXR (Pondugula et al, 2009). However, the regulation of PXR gene transcription remains unexplored by signalling pathways. Herbal drugs have multiple targets and also involved in the regulation of various transcription factors and cascade of signalling proteins in normal physiology and disease (Ding and Staudinger, 2005a; 2005b; Dong et al, 2010; Gupta et al, 2011; Hsu et al, 2011; Whitlock and Baek, 2012).

In the present study, we have elucidated the role of various signalling pathways in the transcriptional regulation of PXR gene and its transcriptional

functions. We also provide evidences for the regulation of PXR-promoter and its transcriptional functions by SIRT1, cyclic AMP-dependent PKA, AMPK, CaMKKβ, MAPK/ERK-1/2, and PI3K/Akt pathways in response to anti-cancer herbal drugs using their specific inhibitors in luciferase assays.

We have shown that NAD-dependent deacetylase SIRT1 positively regulates PXR gene promoter. Our study demonstrated that SIRT1 well known inhibitors, nicotinamide and sirtinol repressed the basal PXR-promoter activity in both the promoter cell lines Hepx-1096/+43 and Hepx-497/+43. Furthermore, nicotinamide and sirtinol also significantly inhibited the acacetin, resveratrol, piceatannol, kaempferol, genistein, quercetin and coumestrol induced PXR-promoter activity in both the PXR-promoter cell lines. Collectively, these findings indicated that inhibition of SIRT1 activity by its inhibitors attenuated the PXR-promoter basal as well as herbal drugs-induced activity. Our results also demonstrated that nicotinamide and sirtinol not only attenuate the PXR-mediated transcriptional activity (Chapter-I) but also PXR-promoter activity. In conclusion, these findings suggested that SIRT1 positively regulates the PXR gene transcription and also involved in herbal drugs mediated PXR-promoter activation. A recent study by Buler et al (2011) also demonstrated energy sensing factors PGC-1α and SIRT1 modulate PXR expression and function in rodents. They have suggested that deacetylation of PGC-1α by SIRT1 may regulate PXR expression and transcriptional function.

Further, we investigated the role of cAMP signalling in regulation of PXR-promoter and its transcriptional functions. Our results indicated that inhibition of adenylyl cyclases by MDL-12,330A prevented the activation of PXR-promoter. Since adenylyl cyclases are responsible for cAMP synthesis therefore the inhibitory effect of MDL-12,330A on PXR-promoter could be mediated by reduced cAMP levels. Thus, our results suggested that intracellular cAMP levels positively regulate basal and resveratrol, piceatannol, acacetin, kaempferol and genistein induced PXR gene promoter activity. These findings also suggested that resveratrol, piceatannol, acacetin, kaempferol and genistein may elevate the cAMP levels. At least, resveratrol has been reported to increase the intracellular levels of cAMP (El-Mowafy and Alkhalaf, 2003).

Further, we have also shown that cAMP signalling positively regulate PXR-mediated transcriptional activity in HepXREM cell line since MDL-12330A inhibited the basal PXR transcriptional activity. Additionally, our results also showed that rifampicin, acacetin, resveratrol, piceatannol, kaempferol, genistein and forskolin augmented PXR transcriptional activity also dependent on cAMP levels. Taking together, our findings also demonstrated that reduced cAMP levels not only attenuate the PXR-promoter activity but also inhibit PXR-mediated transcriptional activity. In conclusion, these results suggest the positive role of cAMP signalling in regulation of basal and drug-induced PXR gene promoter activity and PXR-mediated transcriptional function.

AMPK is emerging as a key regulator of whole-body metabolism and has been shown to increase NAD^+ levels and activate SIRT1 and PGC-1α (Canto et al, 2009, 2010; Fulco et al, 2008; Um et al, 2010, Park et al, 2012, Chung et al, 2012). Further, we investigated if AMPK has a role in regulation of PXR-promoter expression. Our results indicated the differential regulation of PXR-promoter by AMPK in Hepx-1096/+43 and Hepx-497/+43 cell lines. It appears that AMPK positively regulates PXR-promoter and increased the PXR-promoter activity in Hepx-1096/+43 cell line while AMPK negatively regulates PXR-promoter and attenuated the PXR-promoter activity in Hepx-497/+43 cell line. It also appears that the induction of PXR-promoter activity by resveratrol, piceatannol, acacetin, kaempferol and genistein may also mediated through AMPK activation. The discrepancy between the results derived from the Hepx-1096/+43 and Hepx-497/+43 cell lines could be because of the cis-regulatory elements and dominancy of unknown trans-regulatory factors which may be regulated by AMPK activation or inhibition.

Further, the role of AMPK was investigated in the regulation of PXR transcriptional function. Our results indicated that AMPK inhibition by dorsomorphin exhibited the positive effect on the basal PXR transcriptional activity in HepXREM cell line. Nonetheless, our results also indicated that inhibition of AMPK activity by dorsomorphin reduced the rifampicin, acacetin, resveratrol, kaempferol, genistein and forskolin increased PXR transcriptional activity. These findings suggested that AMPK activation could be involved in ligand-mediated PXR transcriptional activation. However, AMPK indirect

activator AICAR did not affect the PXR transcriptional activity. Furthermore, AICAR impeded the acacetin, kaempferol and genistein induced PXR transcriptional activity. Remarkably, forskolin induced PXR transcriptional activity was synergistically augmented by AICAR. Rifampicin mediated PXR transcriptional activation was not altered by AICAR. Dorsomorphin and AICAR results appeared to be interesting but contradictory to each other in drug dependent manner. Further to established the phenomenon more appropriately one needs to do specific experiments by overexpressing or knocking down the AMPK gene and also simultaneously checking the AMPK activity in conjugation with pharmacological inhibitor or direct activator of AMPK. AICAR, a compound widely used to activate AMPK (Fogarty and Hardie, 2010) is converted by adenosine kinase to ZMP, which activates AMPK by mimicking the actions of AMP as an allosteric activator of the enzyme (Henin et al, 1995; Hsu et al, 2011). Recently, AMPK also has been shown to regulate PR, PPARα, PPARγ and LXR transcriptional activity and SREBP-1c, and CYP4F2 gene expression (Hsu et al, 2011; Sozio et al, 2011; Wu et al, 2011b; Yap et al, 2011).

Moreover, we have examined if the AMPK upstream kinase CaMKKβ is involved in regulation of PXR-promoter. Our results indicated that PXR-promoter activity is differentially regulated in CaMKKβ dependent and independent manner in Hepx-497/+43 and Hepx-1096/+43 cell lines respectively. Partial inhibition of the kaempferol and acacetin augmented PXR-promoter activity in Hepx-1096/+43 and Hepx-497/+43 cells respectively by STO-609 indicated that involvement of CaMKKβ in the kaempferol and acacetin mediated PXR-promoter activation. However, the synergistic effect of STO-609 on piceatannol induced PXR-promoter activity in Hepx-497/+43 cells indicated that CaMKKβ inhibit the PXR-promoter activation by piceatannol. Further, these findings also indicated that regulation of PXR-promoter by AMPK may involve the LKB1 pathway, another AMPK upstream kinase.

Furthermore, we determined role of CaMKKβ in regulation of PXR-mediated transcriptional functions in HepXREM cell line. Our results indicated that CaMKKβ is not involved in the control of basal PXR transcriptional activity

since STO-609, a CaMKKβ inhibitor did not affected this process. However, CaMKKβ partially regulated the acacetin and genistein mediated PXR transcriptional activity positively because STO-609 treatment attenuated this process. Genistein has been recently shown to activate CamKKβ (Hsu et al, 2011). Nevertheless, piceatannol and forskolin mediated PXR transcriptional activation was appeared to be regulated negatively by CaMKKβ as its inhibitor STO-609 treatment synergistically elevated the piceatannol and forskolin induced PXR transcriptional activity. Our findings also suggested the possibility of cross-talk between PKA and CaMKKβ signalling pathway in regulation of the drugs mediated PXR transcriptional activation. These results also indicated that the regulation of PXR transcriptional activity by AMPK may involves the LKB1 pathway.

Protein kinase (PKA) is the principal intracellular target for cAMP. Though, there are a few reports on modulation of PXR transcriptional activity (Ding and Staudinger, 2005a; 2005b; Lichti-Kaiser et al, 2009) but the role of PKA in regulation of PXR gene transcription is not investigated yet. Moreover, it is also not clear that PKA is involved in herbal drugs induced PXR gene promoter and PXR transcriptional activation. Therefore, we investigated if PKA signalling is involved in the regulation of PXR gene transcription. These results indicated the differential regulation of PXR-promoter activity in both the cell lines by PKA. Nonetheless, results from Hepx-1096/+43 cell line which was integrated with big fragment of PXR proximal promoter indicated clearly that PKA positively regulate PXR gene transcription might be by phosphorylating the trans regulatory factors of PXR-promoter. Additionally, our results indicated that PKA is also involved in resveratrol, piceatannol, acacetin, kaempferol and genistein mediated PXR-promoter activation. These findings also suggested that these drugs may be involved in PKA activation. In agreement of our findings, a study on another nuclear receptor CAR, indicate that PKA signalling interfaces with CAR activity by modulating CAR-protein cofactor interactions and also by increasing the expression of the Car gene itself in mouse models (Ding et al, 2006).

Furthermore, the role of PKA was investigated in the regulation of PXR mediated transcriptional functions. Our findings indicated that PKA do not

affect the PXR basal transcriptional activity. However, it positively regulates the rifampicin, acacetin, resveratrol, piceatannol, kaempferol, genistein and forskolin mediated PXR transcriptional functions since H89 pretreatment significantly diminished the induced PXR transcriptional activity in HepXREM cell line (Figure 37). Since, the entire induction of PXR transcriptional activity by forskolin was inhibited by H89. Therefore, these results also suggested that forskolin increased PXR transcriptional activity involved the PKA activation. However, forskolin has been shown to transactivate PXR independent of PKA activation (Ding and Staudinger, 2005a; Dowless et al, 2005). Collectively, our results also indicated that transactivation of PXR by rifampicin, acacetin, resveratrol, piceatannol, kaempferol and genistein also involved the activation of PKA. Recently, modulation of PXR transcriptional activity has been shown by protein kinase C (PKC) and PKA (Ding and Staudinger, 2005a; 2005b; Lichti-Kaiser et al, 2009). Also, in a recent report, PKA has been shown to regulate PXR transcriptional activity in species specific manner (Lichti-Kaiser et al, 2009).

Further, we investigated if MAPK/ERK-1/2 pathway is involved in the regulation of PXR gene transcription. Our results indicated the differential regulation of PXR-promoter in both the cell lines by MAPK/ERK-1/2. Nonetheless, results from Hepx-1096/+43 cell line which was integrated with big fragment of PXR proximal promoter indicated clearly that MAPK/ERK-1/2 positively regulate PXR gene transcription possibly by phosphorylating the trans regulatory factors of PXR-promoter. Besides, our results indicated that resveratrol, piceatannol, acacetin, kaempferol and genistein mediated PXR-promoter activation is also partially attributed by MAPK/ERK-1/2.

Further, the role of MAPK/ERK-1/2 was examined in the regulation of PXR-mediated transcriptional functions in HepXREM cell line. Our results indicated that MAPK/ERK-1/2 do not regulate the PXR basal transcriptional activity. Furthermore, our results also indicated that transactivation of PXR by acacetin also involved the activation of MAPK/ERK-1/2 since pretreatment of ERK-1/2 selective inhibitor FR180204 inhibited significantly acacetin induced PXR transcriptional activity.

Moreover, we have investigated the role of PI3K/Akt pathway in the regulation of PXR gene transcription. Our findings strongly showed that PI3K/Akt pathway positively regulates the PXR gene transcription since inhibition of PI3K activity by its selective inhibitor LY294002 significantly repressed the PXR-promoter activity in both the promoter cell lines. Besides, our results indicated clearly that PI3K/Akt pathway activation is also involved in resveratrol, piceatannol, acacetin, kaempferol and genistein mediated PXR-promoter activation since LY294002 pretreatment significantly diminished these drugs elevated PXR-promoter activity.

Further, the role of PI3K/Akt pathway was explored if it controls the human PXR-mediated transcriptional functions. Our results indicated that PI3K/Akt pathway do not regulate the PXR basal transcriptional activity. However, we have shown that rifampicin, acacetin and genistein mediated PXR transcriptional activation also involves the PI3K/Akt pathway activation since inhibition of PI3K/Akt pathway by LY294002 suppressed the PXR-mediated activity of rifampicin, acacetin and genistein. There are possibilities that PXR may be a direct target of PI3K/Akt pathway or it may be regulated indirectly by downstream factors in response of these ligands. Several transcription factors including nuclear receptors and coactivators also has been shown as the targets of PI3K/Akt pathway and their transcriptional functions either activated or inhibited in context dependent manner. Some includes, AR, ERα and ERβ, Nur77, PGC-1 α (Wen et al, 2000; Campbell et al, 2001, Lin et al, 2003; Lie et al, 2007). A report by Kodama et al (2004) has shown that Akt suppressed the transcriptional activity of mouse CAR and PXR, affecting the interaction with its coactivator forkhead in rhabdomyosarcoma (FKHR or FOXO1). Akt possibly accomplishes this negative regulation by phosphorylating and translocating the nuclear FKHR into the cytoplasm for proteasomal degradation (Tang et al, 1999), consequently minimizing the levels of nuclear FKHR available for interacting with and activating mouse CAR and PXR. The reason behind the discrepancy between our results and Kodama et al (2004) could be species specific differences. However, it is not known whether Akt phosphorylates mouse and human PXR and regulates its activity independently of FKHR. Recently, Pondugula et al (2009) showed that p70

S6K, a downstream kinase in the PI3K-Akt pathway, phosphorylates and negatively regulates the transcriptional activity of human PXR and this inhibition of PXR by p70 S6K was not caused by reduced protein levels of PXR (Pondugula et al, 2009).

Further, we investigated if the histone acetyltransferases (CPB/P300) are involved in the regulation of PXR gene transcription. Our results indicated the differential regulation of PXR-promoter activity in both the promoter cell lines by histone acetyltransferases (CPB/P300). Nevertheless, results from Hepx-497/+43 cell line indicated that histone acetyltransferases (CPB/P300) negatively regulate PXR gene transcription. Conversely, these results indicated that at least kaempferol and genistein mediated PXR-promoter activation is also attributed by histone acetyltransferases (CPB/P300) but to least extent.

Furthermore, we examined the role of histone acetyltransferases (CPB/P300) in the regulation of PXR-mediated transcriptional functions. Our results indicated that inhibition of histone acetyltransferases (CPB/P300) increased the PXR basal transcriptional activity. Further, our results also showed that transactivation of PXR by genistein and acacetin also involved the activation of histone acetyltransferases (CPB/P300). However, inhibition of histone acetyltransferases (CPB/P300) induces the synergistic effect on piceatannol induced PXR transactivation. In a recent report, Biswas et al (2011) also suggested that reduced acetylation is responsible for transcriptional activation of PXR in response to its well know agonist rifampicin.

Apart from the PXR-promoter activation by activators, we further investigated how forskolin inhibits the PXR-promoter activity. We have shown that the inhibition of PXR-promoter expression by forskolin is not mediated by activation of cAMP-dependent PKA pathway since pretreatment of PKA inhibitor (H89) did not showed any effect on this process. Moreover inhibition of PXR-promoter activity by H89 and MDL-12,330A (adenylyl cyclase inhibitor) is much less than forskolin implying that PKA activation is not required for forskolin mediated inhibition of PXR-promoter. Further, inhibition of adenylyl cyclase by MDL-12,330A added the suppressive effect of forskolin on PXR-promoter suggesting that elevated cAMP levels could be required for this action

of forskolin. But the downstream effect of forskolin on PXR-promoter is deviated from cAMP-dependent PKA pathway. Further, AMPK may be involved in this process since inhibition of AMPK by dorsomorphin reversed the forskolin effect on PXR-promoter. Though, the rescue effect by AMPK inhibition is small but significant. Furthermore, inhibition of PI3K/Akt pathway by LY294002 also added the inhibitory effect of forskolin on PXR-promoter.

Further, we investigated the role of PXR in HepG2 cell proliferation, cell cycle progression and apoptosis. Our results indicated that PXR over expression in HepG2 cell line, inhibited cell proliferation and arrested the cell cycle in G0/G1 phase (24 h) and S phase (48 h) in time dependent manner with drugs treatments. Further, our results also indicated that the over expression of PXR induced more apoptosis in HepXR cell line as compared to HepG2 cell line. In agreement, recently Zhuang et al (2011) also have shown that activation of PXR arrest cell cycle in G0/G1 phase in 24 h treatment of Rifampicin. However, they have not checked the effect for prolonged period.

In conclusion, the work presented here in contributes to the molecular understanding of the regulation of PXR-promoter and its transcriptional functions by novel agonists/antagonists, cAMP-dependent protein kinase A (PKA), AMP-activated Protein Kinase (AMPK), SIRT1, MAPK/ERK-1/2 and PI3K/Akt signalling. Taken together, the work presented highlights the understanding of interfaces existing between herbal drugs (ligands), signal transduction pathways and PXR-promoter regulation and transcriptional functions, which are critical for the development of safe and effective therapeutic strategies.

* * *

SUMMARY & CONCLUSIONS

Pregnane & Xenobiotic Receptor (PXR), an adopted orphan member of the nuclear receptor superfamily, is a key xenobiotic-sensing ligand-activated transcription factor. In response to xenobiotic exposure, PXR regulates genes involved in the xenobiotic metabolism and clearance to guard the body from their harmful effects (Wilson and Kliewer, 2002; Zhou et al, 2009; Ihunnah et al, 2011; Gao and Xie, 2012; Kodama and Negishi, 2013). Because of its highly promiscuous nature, PXR is activated by a wide array of structurally diverse chemicals including, xenobiotics, endobiotics, numerous clinical drugs, phytochemicals and dietary constituents through direct binding to regulate target genes (Willson and Kliewer, 2002; Ihunnah et al, 2011; Gao and Xie, 2012; Wang et al, 2013). Ligand-activated PXR executes its transcriptional regulatory functions with its heterodimeric partner Retinoic X Receptor (RXR) and PXR-RXR complex binds to specific DNA sequences for regulating gene expression by recruitment of coactivators like SRC1, SRC2, PGCα, PBP, etc. (Chen, 2008; Ihunnah et al, 2011; Tian, 2013; Wang et al, 2013). Research during the recent few years has revealed unanticipated wider roles of PXR beyond xenobiotic metabolism and elimination. These roles include its involvement in hepatic steatosis, vitamin D homeostasis, bile acids homeostasis, steroid hormones homeostasis, inflammatory bowel diseases, cancer, etc. (Zhou et al, 2009; Ihunnah et al, 2011; Gao and Xie, 2012; Pondugula and Mani, 2013). Although the primary event leading to activation of PXR is ligand binding, increasing amounts of evidences suggest that cell signalling pathways and modulation of PXR-cofactor-phosphorylation status also determines overall responsiveness to environmental stimuli (Rochette-Egly, 2003; Staudinger and Lichti-Kaiser, 2008). Post-translational modifications like acetylation, deacetylation, phosphorylation, dephosphorylation, sumoylation have also been implicated in gene transcription regulation of many nuclear receptors incuding PXR (Pondugula et al, 2009). A few recent reports indicate that some of the metabolic signal transduction pathways interface with PXR (Lichti-Kaiser et al, 2009; Pondugula et al, 2009). Nonetheless, the regulation of PXR gene transcription remains unexplored by signalling pathways. While PXR is known to be transcriptionally activate many genes, its own transcriptional mechanisms

remains inadequately explored. However, GR, PPARα, HNF4α, Sp1 and hnRNP K have been shown to bind to its proximal promoter sites and regulate PXR gene expression. (Pascussi et al, 2000; Aouabdi et al, 2006; Zhou et al, 2006; Gibson et al, 2006; Iwazaki et al, 2008, Saradhi, 2008).

A number of herbal drugs and dietary active constituents are known to regulate various transcription factors and cascade of signalling proteins in normal physiology and diseases (Ding and Staudinger, 2005a; 2005b; Dong et al, 2010; Gupta et al, 2011; Hsu et al, 2011; Whitlock and Baek, 2012). Several herbal drugs have been shown to regulate the transcriptional activity of nuclear receptors viz. ERα, PPARα, PPARγ, HNF4α, AR, GR, PR, PXR, CAR, LXR, FXR etc. (Brobst et al, 2004; Chang and Waxman, 2006; Chang, 2009; Harmsen et al, 2009, Cao et al, 2013). However, there are only a few nuclear receptors (ERα, CAR, etc.) exploited for their own transcriptional regulation (gene expression) by herbal drugs or dietary active constituents. Various herbal drugs have been shown to transactivate PXR and subsequently up-regulate the drug metabolism enzymes of Phase I, II and III (Meijerman et al, 2006; Negi et al, 2008; Satsu et al, 2008; Chang, 2009). However, not much is known about the transcriptional regulation of PXR gene by herbal drugs or other related ligands.

PXR is emerging as a potential therapeutic target for metabolic as well as inflammatory diseases including hepatic steatosis, bone disorders, inflammatory bowel disease and also cancer although it's abnormal activation may also have unfavorable effects on human health through adverse drug-drug or food-drug interactions. This points to a plausible concern in drug development and clinical therapy (Chang, 2009; Ihunnah et al, 2011; Gao and Xie, 2012; Kodama and Negishi, 2013). Therefore, characterizing natural molecules and herbal drugs in search for PXR novel agonist(s) or antagonist(s) may hold promise of developing suitable therapeutic molecules for the treatment of metabolic and inflammatory diseases. Since natural molecules and herbal drugs are known to regulate various transcription factors and cascade of signalling proteins in normal physiology and disease (Ding and Staudinger, 2005a; 2005b; Dong et al, 2010; Gupta et al, 2011; Hsu et al, 2011; Whitlock and Baek, 2012) then it is plausible to hypothesize that

natural molecules and herbal drugs may also influence the PXR gene expression along with its activation or inhibition. In this regard, it will be interesting to explore the novel insights underlying the transcriptional regulation of PXR and its transcriptional functions by herbal drugs for better understanding the roles of PXR in the human physiology and pathophysiology and finding the PXR novel transcriptional modulators holding therapeutic potential for metabolic diseases. For this purpose, we have examined the modulation of PXR functions and signalling by herbal anti-cancer drugs. The overall aim of the study described in the thesis was to gain better insight into the modulation of PXR functions and signalling by prospective anti-cancer herbal drugs and to search novel PXR agonist(s)/antagonist(s) and also PXR-promoter modulatory molecules. With this objective the first section of the study deals the modulation of PXR-mediated transactivation functions by prospective anti-cancer herbal drugs, and the second section deals the transcriptional regulation of PXR-promoter by prospective anti-cancer herbal drugs. The third section examines the regulation of PXR-promoter and its transcriptional functions via different signalling pathways involving prospective anti-cancer herbal drugs.

In the first part of the study, we investigated the potency of prospective anti-cancer herbal drugs in regulation of the PXR-mediated transcriptional functions and subsequently seeking the PXR agonist(s) or antagonist(s). To screen and evaluate the drugs with a reliable and reproducible cell based high-throughput assay, we first developed a promoter-reporter stable cell line and screened 28 anti-cancer drugs namely acacetin, resveratrol, piceatannol, genistein, kaempferol, coumestrol, quercetin, catechin, camptothecin, guggulsterone, silymarin, capsaicin, vincristine, taxol, colchicine, digitonin, etoposide, anethol, eugenol, hypericin, sulforapane, forskolin, curcumin, vinblastine, butein, fisetin, isoliquiritigenin and silibinin along with PXR well-known agonist rifampicin. The major findings from the first part of our studies are as given below:

- We have generated a stable cell line for PXR-mediated transcriptional assays in a human liver cell line HepG2 by stably integrating with human PXR and its target gene CYP3A4 promoter-reporter XREM-Luc. The cell

line was termed as 'HepXREM'. HepXREM cell line was characterized for PXR expression and its transcriptional functions by luciferase assay, RT-PCR, western blotting and immunocytochemistry. This cell line offers high-throughput *in vivo* analysis of PXR influencing factors. Among other applications, this cell line can be used to evaluate uncharacterized ligands, extracellular stimuli and upstream events in the PXR signalling pathway.

- Further, to search for novel PXR activators/antagonists we screened 28 structurally diverse anti-cancer herbal drugs with HepXREM stable cell line using promoter-reporter based luciferase assay. Interestingly, our results showed that acacetin, resveratrol, piceatannol, kaempferol, guggulsterone, forskolin, genistein, butein and isoliquiritigenin strongly transactivate PXR in HepXREM cell line as compared to PXR agonist rifampicin. Also, quercetin, vincristine, vinblastine and hypericin activated PXR moderately.
- By MTT assays and morphological assessment by phase contrast microscopy we observed that acacetin, resveratrol, kaempferol, guggulsterone, forskolin, genistein, butein, isoliquiritigenin, anethol and eugenol do not have any cytotoxic and morphological effects on HepG2 cells.
- Furthermore, we have shown that these selected drugs also transactivate MDR1 promoter activity in HepG2 cells transiently transfected with PXR and MDR1-tk-Luc promoter-reporter. These results suggested that these drugs regulate PXR transcriptional activity in promoter independent manner.
- Real-time PCR analysis of human CYP3A4 and MDR1 mRNA expression in HepG2 cells showed that acacetin and resveratrol induced the endogenous mRNA expression of CYP3A4 and MDR1 genes through activation of PXR.
- Mammalian two-hybrid experiments indicated that acacetin, resveratrol, piceatannol, kaempferol increased the PXR interaction with nuclear receptor coactivators SRC1 and PBP. These results imply that acacetin, resveratrol, piceatannol and kaempferol recruit nuclear receptor

coactivators to PXR and trigger the gene transcription of PXR regulated genes.

- ❖ Molecular docking analysis of herbal drugs with the ligand binding domain of PXR by AutoDock Tool. This indicated the possibility of direct interactions of these herbal drugs with LBD of PXR. The binding energies of different drugs are comparable with the binding energy of PXR agonist rifampicin.
- ❖ We have shown that anethol, etoposide, eugenol and camptothecin inhibit rifampicin-mediated PXR transcriptional activity in HepXREM cells and act as 'PXR novel antagonists' effective at low concentration.
- ❖ We report that deacetylase SIRT1 inhibitors nicotinamide and sirtinol inhibit basal and induced PXR transcriptional activity. These findings imply that SIRT1 inhibitors, nicotinamide and sirtinol act as a novel antagonist of the PXR and also suggest the possibility of a role of SIRT1 in PXR-mediated transactivation.

In brief, we conclude that the study provides a screening system and protocol that facilitates the identification of compounds with potential to activate or inhibit PXR transcriptional functions and signalling. The study also reports some potentially novel agonists and antagonists of PXR. Antagonists of PXR would be useful to study the molecular basis of receptor function. In addition, clinically they may prevent drug-drug interactions and adjust the efficacy of therapeutics that serve as PXR agonists.

In the second part of our study we have addressed the issues related to the transcriptional regulation of PXR-promoter by prospective anti-cancer herbal drugs. We have generated two PXR-promoter stable liver cell lines using two selected regions of proximal PXR promoter-reporter constructs (Saradhi, 2008). Both the cell lines were characterized and used for the screening of PXR-promoter modulators using diverse anti-cancer herbal drugs by luciferase assays. The major highlights from this part of our studies are outlined below:

- ❖ We have generated two PXR-promoter cell lines 'Hepx-1096/+43' and 'Hepx-497/+43' by stably integrating two selected regions of proximal PXR promoter-reporter constructs (p-1096/+43 Luc and p-497/+43 Luc

respectively) in liver cell line HepG2. Both the cell lines were characterized by PCR and luciferase assays. These cell lines serve as a useful tool for studying the transcriptional regulation of PXR and also for the screening of drugs, xenobiotics and endobiotics. Additional advantages of these stable PXR-promoter-reporter cell lines would be in their ability to predict potential drug-drug interactions, to identify PXR regulatory proteins, and also to evaluate the modulatory effects of an experimental molecule on PXR-promoter activity.

- We attempted to understand the regulation of PXR-promoter by herbal drugs and search for PXR gene modulators using both the PXR-promoter cell lines; Hepx-1096/+43 and Hepx-497/+43. By performing luciferase assays in both the cell lines, 28 anti-cancer herbal drugs were screened to examine their modulatory effect on PXR-promoter regulation and seeking PXR-promoter modulators.
- We have shown that acacetin, resveratrol, piceatannol, genistein and kaempferol strongly increased PXR-promoter activity (positive regulation) while forskolin, sulforaphane, etoposide, hypericin, vinblastine significantly suppressed PXR-promoter activity (negative regulation) in luciferase assays.
- Furthermore, we have shown that forskolin and sulforaphane also inhibited the acacetin, resveratrol, piceatannol, genistein, kaempferol, quercetin and coumestrol induced PXR-promoter activation significantly in both the promoter cell lines. These results indicated the possibilities that sulforaphane may impart the inhibition of PXR transcriptional activity by inhibiting the PXR gene expression. However inhibition of PXR-promoter by forskolin is unexpected since it activates PXR transcriptional functions. However, our western blotting experiments did not show the inhibitory effect of forskolin on PXR protein expression.
- Phytoestrogen coumestrol has been shown as naturally occurring antagonist of PXR. Our results demonstrated that coumestrol increased the PXR-promoter activity at 10μM and even at 50 μM concentrations. These results suggest the possibilities for the involvement of ERα and ERβ in PXR-

promoter regulation by coumestrol since ER has binding sites on PXR proximal promoter. However, it needs further investigation to confirm it.

- Further, camptothecin, curcumin, taxol and colchicine exhibited inverse response on PXR-promoter in both the cell lines. Camptothecin, taxol and colchicine increased PXR-promoter activity in Hepx-1096/+43 cells. Contrary these drugs suppressed PXR-promoter activity in Hepx-497/+43 cells. Curcumin increased PXR-promoter activity in Hepx-497/+43 cell line while it inhibited PXR-promoter activity in Hepx-1096/+43 cells. Silibinin and vincristine did not influence PXR-promoter activity in Hepx-1096/+43 cell line, however, PXR-promoter activity was augmented and suppressed by silibinin and vincristine respectively. The differential responses of these drugs could be attributed to different cis-regulatory elements in PXR proximal promoter (in Hepx-1096/+43 and Hepx-497/+43 cell lines).
- Western blotting analysis also showed an induction of PXR protein by these drugs in HepG2 (liver) and LS180 (intestinal) cell lines.

In brief, the second part of this study demonstrated that the two PXR-promoter cell lines Hepx-1096/+43 and Hepx-497/+43 serve as important tool for the study of PXR-promoter regulation and seeking PXR modulators. This study provided the evidences for PXR-promoter regulation by anti-cancer herbal drugs. The results also suggested that the regulation of PXR-promoter by herbal drugs is rather a complex process and may involve various signalling pathways in regulation of PXR-promoter for imparting the effect of herbal drugs.

In the third part of the study, we have elucidated the role of various signalling pathways involved in the transcriptional regulation of PXR-promoter and its transcriptional functions. Employing the inhibitors of various signalling pathways in combination with anti-cancer herbal drugs in luciferase assays, we have provided evidences for the regulation of PXR-promoter and its transcriptional functions by deacetylase SIRT1, cyclic AMP-dependent PKA, AMPK, CaMKKβ, MAPK/ERK-1/2 and PI3K/Akt pathways in response to anti-cancer herbal drugs. Also, we have shown the role of PXR in cell growth

inhibition, cell cycle arrest and apoptosis of HepG2 cells. The major findings of this part of our studies are highlighted below:

- To investigate the possible mechanism of action of PXR activator drugs, we first speculated if NAD-dependent deacetylase SIRT1 has a role in transcriptional regulation of PXR gene promoter. It is observed that NAD-dependent deacetylase SIRT1 positively regulates PXR gene promoter and is also involved in herbal drugs mediated PXR-promoter activation. Our study demonstrated that well known inhibitors of SIRT1, nicotinamide and sirtinol repressed the basal PXR-promoter activity in both, promoter cell lines Hepx-1096/+43 and the Hepx-497/+43. Furthermore, nicotinamide and sirtinol also inhibited the acacetin, resveratrol, piceatannol, kaempferol, genistein, quercetin and coumestrol induced PXR-promoter activity significantly in both the PXR-promoter cell lines. Collectively, these findings suggested that inhibition of SIRT1 activity by its inhibitors attenuated the PXR-promoter basal, as well as, herbal drug-induced activity. Our results also demonstrated that nicotinamide and sirtinol not only attenuate the PXR-mediated transcriptional activity but also PXR-promoter activity. In conclusion, these findings suggested that SIRT1 positively regulates the PXR gene transcription and is involved in herbal drugs mediated PXR-promoter activation.

- The role of cAMP signalling in regulation of PXR-promoter and its transcriptional functions is investigated. Our results indicated that inhibition of adenylyl cyclases by MDL-12,330A prevented activation of PXR-promoter. Since adenylyl cyclases are responsible for cAMP synthesis, therefore, the inhibitory effect of MDL-12,330A on PXR-promoter could be mediated by reduced cAMP levels. Thus, our results indirectly suggested that intracellular cAMP levels positively regulate basal and resveratrol, piceatannol, acacetin, kaempferol and genistein induced PXR gene promoter activity. These findings also suggest that resveratrol, piceatannol, acacetin, kaempferol and genistein may elevate the cAMP levels.

- We have also shown that cAMP signalling positively regulates PXR-mediated transcriptional activity in HepXREM cell line since MDL-12330A inhibited

the basal PXR transcriptional activity. Additionally, our results also exhibit that rifampicin, acacetin, resveratrol, piceatannol, kaempferol, genistein and forskolin augment PXR transcriptional activity dependent on cAMP levels. Taking together, our findings demonstrated that reduced cAMP levels not only attenuate the PXR-promoter activity but also inhibit PXR-mediated transcriptional activity. In conclusion, these results suggest the positive role of cAMP signalling in regulation of basal and drug-induced PXR-promoter activity and PXR-mediated transcriptional function.

- AMPK is emerging as a key regulator of whole-body metabolism and has been shown to increase NAD^+ levels and activate SIRT1 and PGC-1α. We speculated a role for AMPK in regulation of PXR-promoter expression. Our results indicated the differential regulation of PXR-promoter by AMPK in Hepx-1096/+43 and Hepx-497/+43 cell lines. It appears that AMPK positively regulates PXR-promoter and increased the PXR-promoter activity in Hepx-1096/+43 cell line while AMPK negatively regulates PXR-promoter and attenuated the PXR-promoter activity in Hepx-497/+43 cell line. It appears that the induction of PXR-promoter activity by resveratrol, piceatannol, acacetin, kaempferol and genistein may also be mediated through AMPK activation. The discrepancy between the results derived from the Hepx-1096/+43 and Hepx-497/+43 cell lines could be because of the cis-regulatory elements and dominancy of unknown trans-regulatory factors which may be regulated by AMPK activation or inhibition.
- Further, the role of AMPK was investigated in the regulation of PXR transcriptional function. Our results indicated that AMPK inhibition by dorsomorphin imparts positive effect on the basal PXR transcriptional activity in HepXREM cell line. Nonetheless, our results also indicate that inhibition of AMPK activity by dorsomorphin reduces the rifampicin, acacetin, resveratrol, kaempferol, genistein and forskolin increased PXR transcriptional activity. These findings suggest that AMPK activation could be involved in ligand-mediated PXR transcriptional activation. However, indirect activator of AMPK, AICAR did not affect the PXR transcriptional activity. Furthermore, AICAR impeded the acacetin, kaempferol and

genistein induced PXR transcriptional activity. Remarkably, forskolin induced PXR transcriptional activity was synergistically augmented by AICAR. Rifampicin-mediated PXR transcriptional activation was not altered by AICAR.

- Moreover, we have examined if the AMPK upstream kinase CaMKKβ is involved in regulation of PXR-promoter. Our results indicated that PXR-promoter activity is differentially regulated in CaMKKβ-dependent and -independent manner in Hepx-497/+43 and Hepx-1096/+43 cell lines respectively. Partial inhibition of kaempferol and acacetin augmented PXR-promoter activity in Hepx-1096/+43 and Hepx-497/+43 cells respectively by STO-609 (CaMKKβ inhibitor) indicated that involvement of CaMKKβ in the kaempferol and acacetin mediated PXR-promoter activation. However, the synergistic effect of STO-609 on piceatannol induced PXR-promoter activity in Hepx-497/+43 cells indicated that CaMKKβ inhibits the PXR-promoter activation by piceatannol. These findings also indicated that regulation of PXR-promoter by AMPK may involve the LKB1 pathway, another AMPK upstream kinase.

- Furthermore, we determined role of CaMKKβ in regulation of PXR-mediated transcriptional functions in HepXREM cell line. Our results indicated that CaMKKβ is not involved in the control of basal PXR transcriptional activity since STO-609, a CaMKKβ inhibitor did not influence this process. However, CaMKKβ partially regulated the acacetin and genistein mediated PXR transcriptional activity positively because STO-609 treatment attenuated this process. Nevertheless, piceatannol and forskolin mediated PXR transcriptional activation appeared to be regulated negatively by CaMKKβ as its inhibitor STO-609 treatment synergistically elevated the piceatannol and forskolin induced PXR transcriptional activity. Our findings also indicate the possibility of cross-talk between PKA and CaMKKβ signalling pathways in regulation of these drug-mediated PXR transcriptional activation. The results also indicated that the regulation of PXR transcriptional activity by AMPK may involve the LKB1 pathway.

- We investigated the possibility if cAMP-dependent PKA signalling is involved in the regulation of PXR gene transcription. Our findings indicated a differential regulation of PXR-promoter activity in both the cell lines by PKA. Nonetheless, results from Hepx-1096/+43 cell line which was integrated with larger fragment of PXR proximal promoter clearly indicated that PKA positively regulates PXR gene transcription by phosphorylating the trans-regulatory factors of PXR-promoter. Additionally, results indicated that PKA is also involved in resveratrol, piceatannol, acacetin, kaempferol and genistein mediated PXR-promoter activation. These findings suggested that these drugs may be involved in PKA activation.

- Furthermore, the role of PKA was investigated in the regulation of PXR-mediated transcriptional functions. Our findings indicated that PKA does not affect the PXR basal transcriptional activity. However, it positively regulates the rifampicin, acacetin, resveratrol, piceatannol, kaempferol, genistein and forskolin mediated PXR transcriptional functions since H89 pretreatment significantly diminished the induced PXR transcriptional activity in HepXREM cell line. The entire induction of PXR transcriptional activity by forskolin was inhibited by H89. Therefore, these results suggest that forskolin increased PXR transcriptional activity is with the involvement of PKA activation. Collectively, our results indicated that transactivation of PXR by rifampicin, acacetin, resveratrol, piceatannol, kaempferol and genistein involved the activation of PKA.

- Subsequently, we investigated if MAPK/ERK-1/2 pathway is involved in the regulation of PXR gene transcription. Our results indicated a differential regulation of PXR-promoter in both the cell lines by MAPK/ERK-1/2. Nonetheless, results from Hepx-1096/+43 cell line which was integrated with large fragment of PXR proximal promoter clearly indicated that MAPK/ERK-1/2 positively regulate PXR gene transcription possibly by phosphorylating the trans-regulatory factors of PXR-promoter. Besides, our results indicated that resveratrol, piceatannol, acacetin, kaempferol and genistein mediated PXR-promoter activation may partially involve MAPK/ERK-1/2.

- Further, the role of MAPK/ERK-1/2 was examined in the regulation of PXR-mediated transcriptional functions in HepXREM cell line. Our results indicated that MAPK/ERK-1/2 does not regulate the PXR basal transcriptional activity. Furthermore, our results also suggested that transactivation of PXR by acacetin involves the activation of MAPK/ERK-1/2 since pretreatment of ERK-1/2 selective inhibitor FR180204 significantly inhibited acacetin induced PXR transcriptional activity.
- Besides, we have investigated the role of PI3K/Akt pathway in the regulation of PXR gene transcription. Our findings strongly showed that PI3K/Akt pathway positively regulates the PXR gene transcription since inhibition of PI3K activity by its selective inhibitor LY294002 significantly repressed the PXR-promoter activity in both the PXR-promoter cell lines. Moreover, our results clearly indicated that PI3K/Akt pathway activation is also involved in resveratrol, piceatannol, acacetin, kaempferol and genistein mediated PXR-promoter activation since LY294002 pretreatment significantly diminished these drugs elevated PXR-promoter activity.
- Likewise, the role of PI3K/Akt pathway was explored to assessif it controls the PXR-mediated transcriptional functions. Our results indicated that PI3K/Akt pathway does not regulate the PXR basal transcriptional activity. However, we have shown that rifampicin, acacetin and genistein mediated PXR transcriptional activation also involves the PI3K/Akt pathway activation. Observations suggest the possibilities that PXR may be a direct target of PI3K/Akt pathway or regulated indirectly by downstream factors in response of these ligands.
- We investigated further to examine if the histone acetyltransferases (CPB/P300) are involved in the regulation of PXR gene transcription. Our results showed differential regulation of PXR-promoter activity in both the promoter cell lines by histone acetyltransferases (CPB/P300). Nevertheless, results from Hepx-497/+43 cell line indicated that histone acetyltransferases (CPB/P300) negatively regulates PXR gene transcription. Conversely, these results indicated that at least kaempferol and genistein

mediated PXR-promoter activation is also attributed to histone acetyltransferases (CPB/P300) but to marginal extent.

- Furthermore, we assessed the role of histone acetyltransferases (CPB/P300) in the regulation of PXR-mediated transcriptional functions. Our data suggest that inhibition of histone acetyltransferases (CPB/P300) increased the PXR basal transcriptional activity. Further more, our results also indicated that transactivation of PXR by genistein and acacetin involves the activation of histone acetyltransferases (CPB/P300). However, inhibition of histone acetyltransferases (CPB/P300) induces the synergistic effect on piceatannol induced PXR transactivation.

- Apart from the PXR-promoter activation by activators, we further investigated as to how forskolin inhibits the PXR-promoter activity. We have observed that the inhibition of PXR-promoter activity by forskolin is not mediated by activation of cAMP-dependent PKA pathway since pretreatment of PKA inhibitor (H89) did not showed any effect on this process. Moreover, inhibition of PXR-promoter activity by H89 and MDL-12,330A (adenylyl cyclase inhibitor) is significantly less than forskolin implying that PKA activation is not required for forskolin-mediated inhibition of PXR-promoter. Further, inhibition of adenylyl cyclase by MDL-12,330A exhibited additive suppressive effects of forskolin on PXR-promoter suggesting that elevated cAMP levels may be required for this action of forskolin. However, the downstream effect of forskolin on PXR-promoter appeared to be deviated from cAMP-dependent PKA pathway. Further, AMPK may be involved in this process since inhibition of AMPK by dorsomorphin reversed the forskolin effect on PXR-promoter. Though, the rescue effect by AMPK inhibition was small but was observed to be significant. Additionally, inhibition of PI3K/Akt pathway by LY294002 also contributed to the inhibitory effect of forskolin on PXR-promoter.

- Finally, we investigated the role of PXR in HepG2 cell proliferation, cell cycle progression and apoptosis. Our results indicated that PXR overexpression in HepG2 cell line, inhibited cell proliferation and arrested the cell cycle in G0/G1 phase (24 h) and S phase (48 h) in a time-dependent manner with

drugs treatments. Further, our results also suggest that the overexpression of PXR induces further apoptosis in HepXR cell line as compared to HepG2 cell line.

In brief, this part of our studies contributes to the molecular understanding of the regulation of PXR-promoter and its transcriptional regulation by novel agonists/antagonists, SIRT1, cAMP-dependent PKA, AMPK, MAPK/ERK 1/2 and PI3K/Akt signalling. In conclusion, the present study highlights some of the novel agonists and antagonists of PXR and modulators of PXR-promoter. The revelations from this study also provide an insight into the complex regulatory signalling mechanisms that determine regulation of PXR-promoter and its transcriptional function in response to anti-cancer herbal drugs. Overall, the work presented in the current study contributes to the understanding of the interfaces among the anti-cancer herbal drugs (ligands), various signal transduction pathways, PXR-promoter regulation and receptor transcriptional functions that are critical for the development of safe and effective therapeutic strategies.

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PUBLICATIONS

Patent:

1. Tyagi R. K., **Negi S.**, Kumari S., Saradhi M. and Mukhopadhyay G. Stable integration of a xenobiotic receptor promoter-reporter DNA construct into a human liver cell line for evaluating the drugs by high throughput screening. Indian Patent application number: **464/DEL/2010**.
International (PCT) Patent application number: **PCT/IN2011/000127**.
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1. **Negi S.**, Akbarsha M. A., Tyagi R. K. (2008). Clinical correlates in drug-herbal interactions mediated via nuclear receptor PXR activation and cytochrome P450 induction. J. Endocrinol. Reprod. 12(1): 1-12.

2. Chaturvedi N. K., Kumar S., **Negi S.**, Tyagi R. K. (2010). Endocrine disruptors provoke differential modulatory responses on androgen receptor and pregnane and xenobiotic receptor: potential implications in metabolic disorders. Mol. Cell. Biochem. 345(1-2): 291-308.

3. Kumar S., Jaiswal B., Kumar S., **Negi S.**, Tyagi R. K. (2010). Cross-talk between androgen receptor and pregnane and xenobiotic receptor reveals existence of a novel modulatory action of anti-androgenic drugs. Biochem. Pharmacol. 80(7): 964-976.

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Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

[Continued on next page]

(54) **Title**: STABLE INTEGRATION OF A XENOBIOTIC RECEPTOR PROMOTER-REPORTER DNA CONSTRUCT INTO A HUMAN LIVER CELL LINE FOR EVALUATING THE DRUGS BY HIGH THROUGHPUT SCREENING

Figure – 1

(57) **Abstract**: The present invention relates to a stably integrated pregnane and xenobiotic receptor (PXR) promoter-reporter construct-cell line comprising PXR promoter of -1096 to +43 region having sequence listing as illustrated in Figure 1, which is capable of assessing drug's capability to bind and induce PXR-promoter on its administration, and hence, is capable of indirectly influencing the protein level of the PXR-promoter on its administration so as to avoid failure of combination therapy. In one embodiment, it relates to a method of preparation of stably integrated pregnane and xenobiotic receptor (PXR) promoter-reporter construct-cell line. In another embodiments, it relates to a pregnane and xenobiotic receptor (PXR) promoter and to a pregnane and xenobiotic receptor (PXR)-promoter-reporter construct.

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J Endocrinol Reprod 12 (2008) 1: 1-12 **Review Article**

Clinical correlates in drug-herbal interactions mediated *via* nuclear receptor PXR activation and cytochrome P450 induction

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This article is dedicated to the memory of Late Prof. Ben M.J. Pereira of Indian Institute of Technology, Roorkee, who had been actively associated with SRBCE and the Journal of Endocrinology and Reproduction

Summary

Pregnane and Xenobiotic Receptor (PXR), a vital xenosensor, acts as master regulator of phase-I (cytochrome P450) and phase-II enzymes (glutathione *S*-transferases, sulfotransferases, and uridine 5'-diphosphate glucuronosyltransferases) as well as several drug transporters (multi-drug resistance protein, and multi-drug resistance-associated proteins). PXR can bind to a variety of chemically distinct endobiotics (steroids, bile acids and their derivatives, vitamins, etc.) and xenobiotics (prescription drugs, herbal medicines, endocrine disruptors, etc.). Activation of PXR by various compounds leads to trans-activation of PXR-target genes involved in detoxification machinery (phase-I and phase-II enzymes, and efflux proteins). Herbal medicines are readily used without prescription under the belief that anything natural is safe. These medicines contain active chemical constituents which execute distinctly different or similar pharmacological response(s). But, like prescription drugs, herbal drugs also have both therapeutic and, sometimes, adverse effects. Some of the herbal drugs induce drug metabolizing enzymes (especially CYP3A4) and drug efflux proteins *via* activation of PXR. Phase-I enzyme CYP3A4 is involved in the metabolism of 50-60% of clinical drugs as well as the chemical ingredients in herbal medicines. In addition to this, 25-30% of these compounds are metabolized by the CYP2B isoenzymes. The combined metabolic effects of phase-I and phase-II enzymes and drug transporters, following induction by therapeutic molecules, constitute the molecular basis for many drug-herbal interactions. For example, if one drug activates PXR, it can encourage the elimination of a co-administered drug that is also metabolized and eliminated by PXR-target gene products, thereby affecting the therapeutic efficacy of the drug in the context of combination therapy. The present review highlights some of the recent clinical correlates in drug-herbal interactions mediated primarily *via* PXR and cytochrome P450.

Keywords: Drug-herbal interactions, Pregnane and Xenobiotic Receptor (PXR), cytochrome P450 (CYP450), drug transporters.

1. Introduction

The history of herbal medicines is as old as human civilization. The documents, many of which are of great antiquity, reveal that plants were used as medicines in China, India, Amazon Basin, Egypt and Greece, long before the beginning of the Christian era. India is very rich in natural resources and traditional knowledge. The use of plants as a source of herbal medicine has been an innate and vital aspect of India's healthcare system. The three Indian traditional systems of medicine (Ayurveda, Siddha and Unani) have identified more than 1,500 medicinal plants, of which nearly 700 are commonly used (Agarwal and Raju, 2006). According to an estimate by the World Health Organization (WHO), 70-80% of the world population, especially in developing countries, relies on traditional medicines, mostly plant drugs, for their primary healthcare needs (WHO, 2002; Agarwal and Raju, 2006). Recent reports reveal that the worldwide market of herbal medicines is estimated to be around US $80 to 100 billion, and it is projected to reach up to US $2,500 billion by the year 2010 (Mathur, 2003; Agarwal and Raju, 2006).

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