University of Ioannina

School of Health Sciences

Faculty of Medicine

Section of Clinical and Basic Functional Science

Laboratory of Pharmacology

"Regulation of aldehyde dehydrogenases by nuclear receptors CAR and PXR in rats genetically predetermined in response to Phenobarbital"

Katerina Touloupi

Biologist, MSc

PhD Thesis

Ioannina 2015

University of Ioannina

School of Health Sciences

Faculty of Medicine

Section of Clinical and Basic Functional Science

Laboratory of Pharmacology

"Regulation of aldehyde dehydrogenases by nuclear receptors CAR and PXR in rats genetically predetermined in response to Phenobarbital"

Katerina Touloupi

Biologist, MSc

PhD Thesis

Ioannina 2015

«Η έγκριση της διδακτορικής διατριβής από το Τμήμα Ιατρικής του Πανεπιστημίου Ιωαννίνων δεν υποδηλώνει αποδοχή των γνωμών του συγγραφέα Ν. 5343/32, άρθρο 202, παράγραφος 2 (νομική κατοχύρωση του Ιατρικού Τμήματος)».

Ημερομηνία αίτησης της κ. Τουλούπη Αικατερίνης: 27-1-2011

Ημερομηνία ορισμού Τριμελούς Συμβουλευτικής Επιτροπής: 705[α]/8-2-2011

Μέλη Τριμελούς Συμβουλευτικής Επιτροπής:

Επιβλέπων

Παππάς Περικλής Επίκουρος Καθηγητής Φαρμακολογίας

Μέλη

Μαρσέλος Μάριος-Αθανάσιος Καθηγητής Φαρμακολογίας

Paavo Honkakoski Professor Faculty of Pharmacy University of Western Finland

Ημερομηνία ορισμού θέματος: 14-2-2011

«Ρύθμιση αλδεϋδικών αφυδρογονασών από πυρηνικούς υποδοχείς CAR και PXR σε επίμυες γενετικά προκαθορισμένους ως προς την απάντηση στη φαινοβαρβιτάλη».

ΟΡΙΣΜΟΣ ΕΠΤΑΜΕΛΟΥΣ ΕΞΕΤΑΣΤΙΚΗΣ ΕΠΙΤΡΟΠΗΣ : 781[α]/29-9-2015

1. Paavo Honkakoski Professor Faculty of Pharmacy University of Western Finland
2. Μαρσέλος Μάριος-Αθανάσιος τ. Καθηγητής Φαρμακολογίας του Τμήματος Ιατρικής του Παν/μίου Ιωαννίνων
3. Αγγελίδης Χαράλαμπος Καθηγητής Γενικής Βιολογίας του Τμήματος Ιατρικής του Παν/μίου Ιωαννίνων
4. Κωνσταντή Μαρία Καθηγήτρια Φαρμακολογίας του Τμήματος Ιατρικής του Παν/μίου Ιωαννίνων
5. Φριλίγγος Ευστάθιος Καθηγητής Βιολογικής Χημείας του Τμήματος Ιατρικής του Παν/μίου Ιωαννίνων
6. Παππάς Περικλής Αναπληρωτής Καθηγητής Φαρμακολογίας του Τμήματος Ιατρικής του Παν/μίου Ιωαννίνων
7. Λεονταρίτης Γεώργιος Λέκτορας Φαρμακολογίας του Τμήματος Ιατρικής του Παν/μίου Ιωαννίνων

Έγκριση Διδακτορικής Διατριβής με βαθμό **«ΑΡΙΣΤΑ»** στις **29-10-2015**

ΠΡΟΕΔΡΟΣ ΤΟΥ ΤΜΗΜΑΤΟΣ ΙΑΤΡΙΚΗΣ
Πασχόπουλος Μηνάς
Καθηγητής Μαιευτικής-Γυναικολογίας

Η Γραμματέας του Τμήματος
ΚΑΠΙΤΩΠΟΥΛΟΥ ΜΑΡΙΑ

This research was co-financed by the European Union (European Social Fund - ESF) and national funds through the Operational Programme "Education and Lifelong Learning" of the National Strategic Reference Framework (NSRF) - Research Funded Project: Heraclitus II Investing in society knowledge through the European Social Fund.

ACKNOWLEDGEMENTS

The present study was carried out in the Department of Pharmacology, Faculty of Medicine of the University of Ioannina, and part of it in the School of Pharmacy, Faculty of Health Sciences of the University of Eastern Finland, in Kuopio, Finland.The research was co-financed by the European Union (European Social Fund - ESF) and national funds through the Operational Programme "Education and Lifelong Learning" of the National Strategic Reference Framework (NSRF) - Research Funded Project: **Heraclitus II** Investing in society knowledge through the European Social Fund.

This PhD thesis was done in collaboration with the laboratory of Professor Paavo Honkakoski, member of the advisory committee. I wish to thank my supervisors for giving me the opportunity to fulfill my dream of becoming a real researcher, and work in laboratories such the aforementioned in Ioannina and Kuopio, where the high quality of training in advanced molecular techniques has met all my expectations.

First and foremost, I would like to thank Assistant Professor Periklis Pappas for believing in my capabilities from the outset of this project and choosing me as a recipient of this scholarship. Periklis has helped me through these 4 years to broaden my mind and my way of thinking, and always provided me with advice and assistance whenever needed, especially in experiments that required animal handling.

It has been a great pleasure for me to be supervised by Professor Paavo Honkakoski. I had the chance to work in his laboratory and be fully supported either in practical or theoretical parts of the research, even after leaving from Kuopio. Paavo was always willing to answer to all my questions, and our constructive talks helped me to fully elucidate substantial molecular techniques.

I would like to express my warmest thanks to Professor Marios Marselos for accepting me at the Department of Pharmacology, as well as for the critical comments on results arising during the research and his continuous encouragement for progress.

The examiners of this thesis, Professor Maria Konstandi, Professor Charalampos Aggelidis, Professor Eustathios Friliggos and Lecturer George Leontaritis are gratefully acknowledged for their careful and critical reading of the manuscript and their valuable comments for its improvement. Special thanks to Prof. Konstandi for her substantial guidance in primary hepatocytes isolation experiments.

For the demanding experiments of Chromatin Immunoprecipitation assays, the help and experience of Lecturer Angeliki Magklara was invaluable, not only during the setting of the protocol and experimental procedure, but also at the troubleshooting and interpretation of results, for which I am deeply grateful. I further thank Professor Katerina Antoniou for her masterful remarks on technical matters, and her willingness to share her time and knowledge with me.

It would be such ingratitude not to recognize the vital contribution of Assistant Professor Evangelos Kolettas for my reaching the goal of carrying out this PhD, since he was the one who proposed and promoted me for this position, which I truly appreciate.

I warmly wish to thank Yannis Simos, PhD, for his friendship and support, especially during the first years of this project, as well Marianthi Sotiropoulou, PhD, Leonidas Mavroeides, MD, Foteini Malliou, PhD candidate, and all undergraduate students that worked in the department, for the nice moments we shared and the companionship after long hours in the laboratory.

Special thanks to Mrs. Olga Tsoumani, Mrs. Akylina Mpalomenou and Mr. Thanasis Verros for technical and secretarial assistance, and for their warm advice and wishes during the years.

Not only had I the unique opportunity to visit Kuopio, in Finland, and gain a lot of experience, but I also met great people, each of whom helped me in their own way. I am very grateful for the guidance, positive criticism, support and friendship of Ferdinand Molnàr, PhD. Jenni Kublbeck, PhD, performed the luciferase- and co-transfection assays, for which I am very grateful. I would also like to thank Mrs. Lea Pirskanen for the technical support and her smiley face that cheered me up.

I was extremely lucky to be surrounded by lots of nice people, however I wish to specially thank my friends Pyry Välitalo, PhD, and Bjorn Peters, PhD Candidate, for helping me making my stay in Finland more ease and pleasant.

I would also like to thank my Greek family in Finland. Maria, Darin, Dennis and Stefanos, Leena, Dimitris, Orestis, thank you for hugging me and making me feel like home.

My acknowledgements end where my life begins… I am blessed for having you, and during these 4 years I realized once again that I would not have achieved anything in my life without them. Thank you for all these years of support, encouragement, and endless love…

To my beloved grandmothers who left...

To my lovely nieces and nephews that came

To our new life...

...thank you for being there for me. Always...

CONTENTS

ABBREVIATIONS

AhR	Aryl hydrocarbon receptor
AKRs	Aldo-Keto Reductases
ALDH	Aldehyde dehydrogenase
AP1	Activating Protein 1
CAR	Constitutive Androstane receptor
CCRP	CAR Cytoplasmic Retention Protein
CITCO	6-(4-chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde-(3,4-dichlorobenzyl)oxime
CLZ	Clotrimazole
CYP P450	Cytochrome P450
DBD	DNA Binding Domain
DHA	Docosahexaenoic acid
DRs	Direct Repeats
ERs	Everted Repeats
FXR	Farnesoid X Receptor
GFP	Green Fluorescent Protein
GRIP1	Glucocorticoid Receptor-Interacting Protein 1
GSTA1	Glutathione S-transferase A1
HSP90	Heat Shock Protein 90
IRs	Inverted Repeats
LBD	Ligand Binding Domain
LCA	Lithocholic acid
MCS	Multiple Cloning Site
MRP	Multidrug Resistant Protein
NCoR	Nuclear receptor Co-Repressor
NES	Nuclear Export Signal

NLS	Nuclear Localization Signal
NRs	Nuclear Receptors
PB	Phenobarbital
PCN	5-Pregnen-eβ-ol-20-one-16α-carbonitrile
PCR	Polymerase Chain Reaction
PP2A	Protein Phosphatase 2A
PPARα	Peroxisome-Proliferator-Activated Receptor α
PXR	Pregnane X Receptor
RE	Restriction Enzyme
REs	Response Elements
RXRα	9-*cis*-Retinoid X Receptor
SMRT	Silencing Mediator of Retinoid and Thyroid Receptor
SRC-1	Streoid co-activator 1
SXR	Steroid and Xenobiotic Receptor
TCPOBOP	1,4-bis[2-(3,5-dichloropyridyloxy)]benzene
TF	Transcription Factor
TPD	2,4,6-tryphenyldioxane-1,3
TR	Thyroid Receptor
TRP	tetratricopeptide repeat
TSS	Transcription Start Site
UGT1A1	UDP-glucoronosyltransferase 1A1
VDR	Vitamin D Receptor
WKY	Wistar Kyoto
XCPE	Activator-, mediator-binding factor core promoter element
XREM	Xenobiotic-Responsive Enhancer Module
XRS	Xenochemical Response Sequence

INTRODUCTION

The ALDH superfamily

The superfamily of aldehyde dehydrogenases (ALDHs) consists of enzymes that are enrolled with the irreversible oxidation of endogenous and exogenous aldehydes [Sophos et al., 2001]. The representation of ALDHs in all taxonomic groups (Archaea, Eubacteria and Eukarya) is indicative of the substantial role these enzymes play in organisms, however their functionality extends far beyond enzymatic detoxification [Jackson et al., 2011]. Not only they participate in cell growth, differentiation and survival, and the synthesis of biomolecules (e.g. retinoic acid) [Westerlund et al., 2005], but they further exhibit roles in protection of the harmful ultraviolet (UV) irradiation in the cornea, or even as biomarkers for a variety of diseases, including certain cancer types and Parkinson's Disease [Jackson et al., 2011; Westerlund et al., 2005]. Furthermore, ALDHs have been reported to possess structural, apart from the enzymatic, role; for example in eye lens and cornea [Alnouti and Klaassen, 2007].

ALDH1A1 and ALDH1A7

Members of ALDH superfamily with more than 40% homology belong to the same family, and those that share more than 60% identity are categorized in the same subfamily [Sophos et al., 2001]. Exception to this standard nomenclature rule is ALDH2, which sequence is 68% identical to ALDH1A1 sequence, and should be classified in the same family [Alnouti and Klaassen, 2007]. Among the 21 genes of ALDH superfamily that exist in the rat genome [Jackson et al., 2011], we focused on ALDH1A1 and ALDH1A7, two isozymes belonging to ALDH1A subfamily. ALDH1A1 is found in all species except of zebrafish, while ALDH1A7 is a noteworthy gene that has been conserved only in rats and mice, and is considered as one of the several gene duplications found in rodents (paralog) [Jackson et al., 2011; Black et al., 2009]. Both ALDH1A1 and ALDH1A7 genes are located in

Chromosome 1 and in close proximity to each other, though in opposite orientation. ALDH1A1 is a cytosolic enzyme that catalyzes oxidation of retinal to retinoic acid (RA). ALDH1A7 is a cytosolic isoform of ALDH1A1, also known as ALDH-PB, [Kathmann et al., 2000; Kathmann and Lipsky, 1997], based on the elevated expression levels detected in responsive rats after phenobarbital (PB) treatment [Deitrich, 1971]. Although the two isozymes are highly identical, concerning their sequence, they exhibit differential functionality in terms of catalytic effects, since ALDH1A7 does not participate in retinoic acid synthesis [Hsu et al., 1999; Kathmann et al., 2000; Marselos et al., 1987]. Aldehyde dehydrogenases demonstrate tissue-specific distribution, which is in relevance to their substrate preference and reflect their role in metabolism of xenobiotics in respective organs. ALDH1A1 and ALDH1A7 display similar expression patterns, with high mRNA levels reported in liver, lung and kidneys. Interestingly, highest ALDH1A1 expression in rats was measured in kidney tissue, whereas it was not detected in mice. Alike, no expression of ALDH1A7 was reporter in kidney of mice, while in rats corresponding mRNA reached high levels of expression [Alnouti and Klaassen, 2007]. ALDHs activity is not only in accordance to their distribution in organs, but is also prone to age-related changes [Yoon et al., 2005].

Xenobiotics and the role of Nuclear Receptors in drug elimination

Vast research has been done during the last decade on cytochrome P450 enzymes and their vital role in metabolism of endobiotics, such as hormones, vitamins, acid derivatives, or pharmaceutical drugs and other exogenous chemical compounds, also called xenobiotics [Honkakoski and Negishi, 2000;]. Many xenobiotics are usually implicated in the transcriptional regulation of CYP genes, (and especially members of the CYP1, CYP2, CYP3 and CYP4 families), that are required for the metabolism of themselves, by acting as ligands or activators of nuclear receptors. Nuclear receptors (NRs) are transcription factors that recognize and bind to specific DNA sequences upon ligand activation, and play a critical role in regulation of development and homeostasis [Mangelsdorf et al., 1995; Aranda et al., 2001]. The receptors therefore bind to upstream promoter sequences, thus inducing transcription regulation of various genes [Smirlis et al., 2000], and

especially P450 CYPs or other drug-metabolizing genes [Tompkins and Wallace, 2007]. Fine examples of CYP genes regulation by NRs, which are presented in many reviews and put together in a study by Muangmoonchai et al. (2001), are the following: Polycyclic aromatic hydrocarbons promote the expression of CYP1 via the aryl hydrocarbon receptor (AhR), while CYP2 is known to be regulated by numerous diverse compounds, including the barbiturate phenobarbital, or plant extracts, such as picrotoxin, pinenes camphor and limonene, with the implication of constitutive androstane receptor (CAR). CYP3 is activated by the pregnane X receptor (PXR, also known as steroid and xenobiotic receptor), in response to natural and synthetic steroids, and CYP4 genes are induced by drugs and carcinogens via the peroxisome-proliferator-activated receptor α (PPARα). Unraveling the impact of both upregulation or inhibition of P450 enzymes is of great magnitude, since undesirable effects might occur such as low drug efficacy, caused by either weak or rapid metabolism of a drug substrate [Konstandi et al., 2005; Daskalopoulos et al., 2012]. A spherical view of all CYP genes and their interaction with NRs is presented in table 1 by Honkakoski and Negishi (2000).

Table 1. Examples of interactions between CYP genes and nuclear receptors

Abbreviations: NSAIDS, non-steroidal anti-inflammatory drugs; MR, mineralocorticoid receptor.

CYP gene	CYP substrates/products that can serve as NR ligands	NR affected by the CYP substrate/product	NRs known to regulate *CYP* gene*	Response element†	NR effect‡	Cell specificity of NR action
CYP1A	Oestrogens, retinoids	ERs, RARs, RXRs	ER, GR RAR	Indirect DR4?	ER, GR ↑ ↓ RAR ↑ ↓	Ubiquitous Ubiquitous?
CYP1B	Oestrogens	ERs	ER, GR	Indirect	ER ↑ ↓, GR ↓	Ubiquitous
CYP2A	Androgens	AR	HNF-4	DR1	HNF-4 ↑	Hepatocytes
CYP2B	Xenobiotics§ Methoxychlor	Many	CAR (PXR)	DR4	CAR ↑	Hepatocytes
	Androgens§, oestrogens, retinoids	AR, ER, RARs, RXRs	GR	GRE half-sites?	GR ↑	Hepatocytes?
CYP2C	Xenobiotics§ NSAIDs Methoxychlor Androgens, retinoids, fatty acid derivatives	Many AR, ER, RARs, RXRs PPARs	HNF-4, orphan RAR	DR1 Unknown	HNF-4 ↑, orphan ↑ ↓ RAR ↑	Hepatocytes Hepatocytes
CYP2D	Xenobiotics§ Androgens, oestrogens, vitamin D_3	Many AR, ER	ER VDR HNF-4	Indirect Unknown DR1	ER governs GH secretion VDR ↓ HNF-4 ↑	Hypophysis Hepatocytes Hepatocytes
CYP3A	Xenobiotics§ Methoxychlor	Many	PXR (CAR) HNF-4, orphan	DR3, ER6 DR1	PXR ↑ HNF-4 ↑, orphan ↑ ↓	Hepatocytes, intestine Hepatocytes
	Androgens, corticoids, oestrogens, pregnanes§	AR, ER, GR	GR	GRE half sites?	GR ↑	Hepatocytes?
CYP4A	Fatty acid derivatives	PPARs	PPAR, (orphan)	Extended DR1	PPAR ↑, orphan ↓	Hepatocytes
CYP26	Retinoic acid	RARs, RXRs	RAR	Unknown	RAR ↑	Ubiquitous
CYP27B1	Vitamin D		VDR	Unknown	VDR ↓	Kidney
CYP24	Vitamin D	VDR	VDR (RXR, orphan)	DR3	VDR ↑, orphan ↓	Ubiquitous
Steroidogenic CYPs	Androgens, oestrogens, corticoids, pregnanes§	AR, ER, GR, MR	SF-1, orphan	Extended monomer	SF-1 ↑, orphan ↑ and ↓	Adrenals, gonads
CYP7A	Precursors for steroids		CPF LXR, FXR	Extended monomer DR4	CPF ↑ LXR ↑, FXR ↓	Hepatocytes Hepatocytes
Bile-acid-forming CYPs	Oxysterols	LXR, FXR	LXR?, FXR?	Unknown		
CYP51	Sterols		LXR	Unknown	Unknown	

* Nuclear receptors in parentheses have a tentative role.
† Binding elements related to AGGTCA motifs (? means role of the element is tentative or not clear; indirect or unknown effects described in the text).
‡ Activating (↑), repressing (↓) or conflicting (↑ ↓) effects on *CYP* gene expression.
§ Many compounds in this class are activators of PXR, CAR or PPAR.

(Honkakoski and Negishi, 2000)

NRs further act as therapeutic targets for more than 10% of widely used drugs, with many examples summarized in a review of Goodwin and Moore (2004), and shown in Table 2.

Table 2. The role of nuclear receptors as therapeutic targets

Nuclear Receptor	Therapeutic target
Estrogen Receptor (NR3A1)	Breast cancer
Retinoid Receptors (NR1Bs)	Leukemia
Peroxisome Proliferator-Activated Receptors PPARs (NR1Cs)	Dyslipidemia, Diabetes
Glucocorticoid Receptor (NR3C1)	Inflammatory disorders
Androgen Receptor (NR3C4)	Prostate cancer
Oxysterol receptors LXRα (NR1H3), LXRβ (NR1H2) and bile acid receptor FXR (NR1H4)	Atherosclerosis, Cholestasis

Structurally, NRs are composed of an N-terminal trans-activation domain (AF-1), a DNA binding domain (DBD), a hinge region and a C-terminal trans-activation ligand-binding domain (LBD) (see Fig.1A) [Honkakoski and Negishi, 2000]. NRs are usually retained in the cytoplasm of the cell, and translocate into nucleus after stimulation by the presence of endo- or xenobiotics, which bind, or not, to NRs. After that, the receptors form (either homo- or hetero-) dimers or tetramers, in order to bind to their cognate sequences that lie on the promoter of their target genes, and therefore turn on or up-regulate their transcription. Specific sequences, which are recognized by NRs, are characterized as response elements (REs) and are usually composed of two half-sites of the hexamer AGGTCA, or a closely related 6-nucleotide. There is a spacer sequence between the two half-sites that consists of a few base pairs (bps) (Fig. 1B). The half-sites are oriented as direct

repeats (DRs), everted repeats (ERs),or inverted repeats (DRs) [Honkakoski and Negishi, 2000]. The structural domains of a receptor, and the REs and possible orientation are shown in Fig.1B.

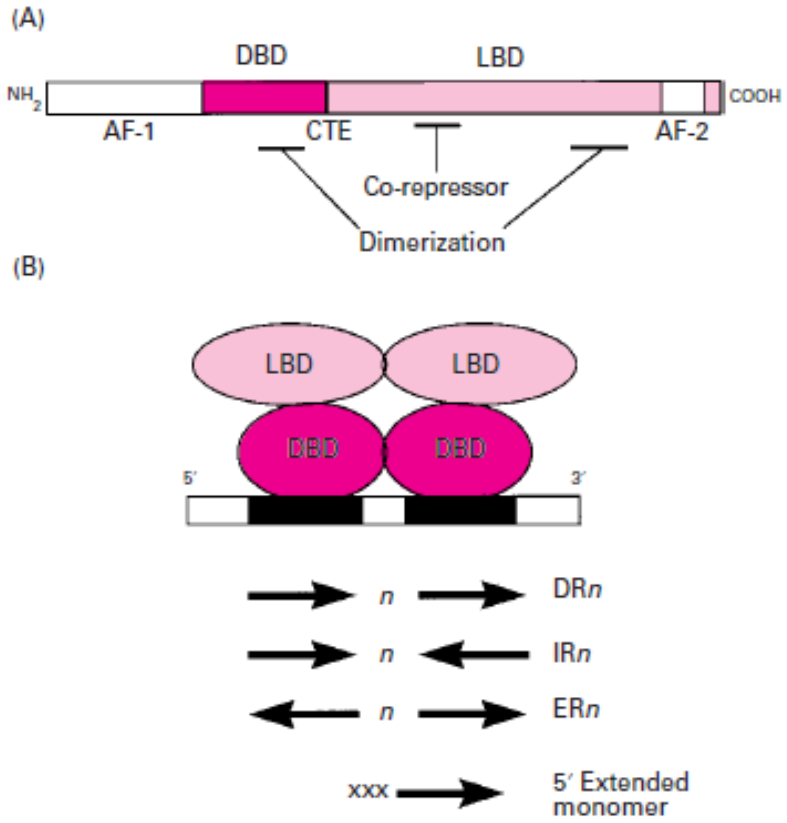


Figure 1. Structural domains in nuclear receptors and their binding sites. [Negishi and Honkakoski, 2000]

Phenobarbital (PB): a master player in hepatic drug metabolism

Various chemicals used in studies of CYPs expression regulation, were found to have similar impact on ALDH superfamily of genes as well. The best studied inducer of drug-metabolizing enzymes is the barbiturate phenobarbital (PB), which is a representative of a large group of structurally diverse compounds (characterized as PB-like inducers) that activate the expression of numerous genes, especially in the hepatic tissue. Phenobarbital is a sedative and anti-epileptic drug, with a great impact on liver homeostasis, including gross liver enlargement, tumor development and regulation of more than 30 genes' expression [Waxman and Azaroff, 1992; Meyer and Hoffmann, 1999; Bell and Michalopoulos, 2006]. Extensively studied target genes of the above inducers, which are highly up-regulated after administration of the drugs, are members of CYP2B and CYP3A subfamilies [Honkakoski et al., 1998 and 2003; Negishi and Honkakoski, 2000; Zelko and Negishi, 2000]. Genes from both subfamilies encode enzymes that are implicated in the metabolism of xenobiotics, especially barbiturates, pesticides, antibiotics and other drugs. Their ability to be induced by a variety of different substrates, mainly in liver, sets CYPs 2B and 3A as the most important enzymes in hepatic drug metabolism and elimination. They further demonstrate specificity, regarding sex, tissue, or even developmental stage [Honkakoski and Negishi, 2000].

Genes' responsiveness to PB administration is attributed to corresponding sequences on the promoters, known as response elements. The response elements consist of two half-sites AGGTCA as direct repeats (DR), inverted repeats (IR) or everted repeats (ER), with a few bases in between, and serve as binding sites for nuclear receptors [Zelko and Negishi, 2000].

The Phenobarbital-Responsive Enhancer Module (PBREM) is a sequence of 51-bp of DNA at around –2300 bp on the promoter of mouse Cyp2b10 and rat CYP2B2 gene, also present in the rat CYP2B1, and human CYP2B6 genes. PBREM element contains an NF1-binding site, flanked by two nuclear receptor (NR)-binding sites, yet only NR sites are critical for response to potent inducers like PB, PB-type inducers and 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) [Honkakoski et al.; 1998a; Honkakoski et al., 1998b; Sueyoshi et al., 1999; Ramsden et al., 1999], although NF1 site may also be essential for full activity of

the element [Zelko and Negishi, 2000]. Both NR1 and NR2 are DR4 motifs (direct palindromes with 4 bp spacing) [Honkakoski and Negishi, 2000; Sueyoshi and Negishi, 2001], on which nuclear receptors bind, after forming heterodimers with the 9-*cis*-retinoid X receptor alpha (RXR*α*) (see fig. 2) [Mangelsdorf and Evans, 1995; Negishi and Honkakoski, 2000].

The enhancer element for CYP3A genes is called Xenobiotic-Responsive Enhancer Module (XREM) and located around –7700 on the CYP3A4 gene. XREM consists of two NR-binding sites, NR1 which is a DR3 (direct repeat separated by 3 bases) and NR2 which is an ER6 (everted repeat separated by 6 bases), with 29 bases in between [Honkakoski and Negishi, 2000; Sueyoshi and Negishi, 2001]. NR1 sites in sequences of both gene subfamilies (DR4 and DR3 elements on promoter regions of CYP2B and CYP3A, respectively) are the specific binding sites recognized by two nuclear receptors, CAR and PXR, which are implicated in transcription regulation of the above-mentioned CYPs (see Fig.2) and the ALDHs, amongst other genes.

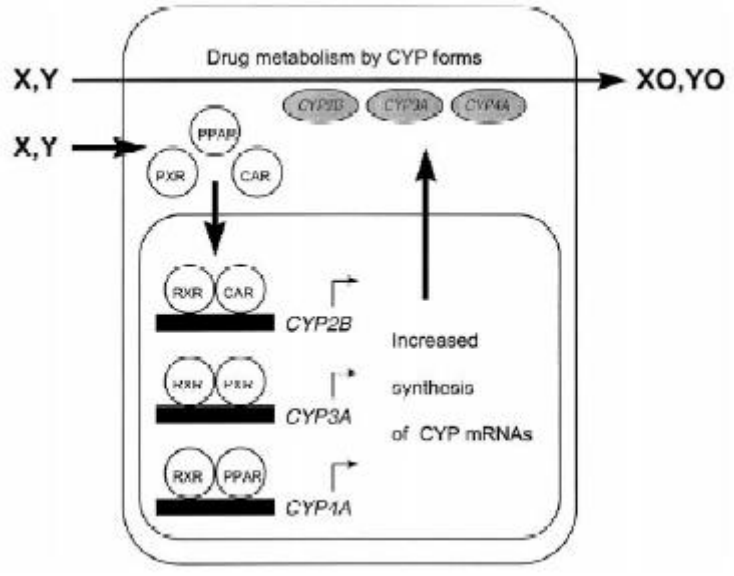


Figure 2. Schematic presentation of NRs-heterodimers with RXRα and their binding on promoter regions of target genes [Negishi and Honkakoski, 2000].

Nuclear receptors CAR and PXR: The 'adopted orphans'

For many years, CAR and PXR had been characterized as 'orphan' nuclear receptors, and classified together with other proteins that demonstrate the typical structure of NRs, but for which no endogenous ligand has been found thus far. Recently, physiological ligands of the two receptors have been identified; therefore they are now categorized in the group of 'adopted nuclear receptors'. Both of them are highly expressed in liver and intestine, and demonstrate cross-regulation of their target genes, as well as promiscuity of their binding capacity [Li and Wang, 2010]. A fine example of the cross-talk between the two NRs through their indiscriminate binding on responsive elements, although induced in a ligand-specific way, is the transcriptional activation of CYP2B1 and CYP3A1 by CAR and PXR, after administration of their selective agonists; PB or the synthetic pregnane Pregnenolone 16α-carbonitrile (PCN), respectively [Smirlis et al., 2001]. CAR, along with PXR act not only xenosensors, but are further responsible for physiological regulation of lipid homeostasis, glucose and energy metabolism, and bile acid elimination [Konno et al., 2008; Wada et al., 2009; Omiecinski et al., 2011]. Therefore, it has been proposed that CAR and PXR could potentially play substantial role in the therapy of serious metabolic disorders, such as diabetes and obesity [Tolson and Wang, 2010].

Common feature of the mechanism of action of these two receptors is their cytosolic sequestration under physiological conditions and their translocation into nucleus and binding on response elements on the promoter of their target genes under exposure of cell to multiple xenobiotics. Interestingly, despite PXR translocates into nucleus only after direct ligand-binding, CAR can further shuttle in the nuclear compartment either directly or indirectly, meaning that some xenobiotics serve as activators of the receptor, without binding to it [Tolson and Wang, 2010].

It should be borne in mind that, since nuclear receptors play an important role in gene regulation and at the same time serve as xenobiotic sensors in order to promote their elimination from the body, it is vital to fully understand the mechanisms that control NRs activity, in order to predict or target drug-drug interactions under pathological conditions. According to Lin (2006), co-

administration of drugs might have the opposite effect, for example inefficacy or even toxicity, in case of activation of CYP genes, since they are up-regulated by presence of xenochemicals so as to de-activate or eliminate them.

The Constitutive Androstane Receptor – CAR

Constitutive active/androstane receptor (CAR, NR1I3) is a liver-specific nuclear receptor that is activated by numerous compounds, including pharmaceuticals, natural products and xenochemicals [Chang and Waxman, 2006], although the best characterized activator is phenobarbital (PB) or other PB-type inducers. The finding that CAR is expressed only in the liver [Honkakoski et al., 1996; Choi et al., 1997] was further supported by studies of Bookout et al. (2006) for absence of CAR from mouse lungs, kidneys and brain, and barely detectable levels in rat lungs [Chirulli et al., 2003].

Upon activation, CAR binds to DR4 motifs on promoter sequences of genes which are regulated by a PBREM-enhancer element, leading to up-regulation of its targets. However, unlike other NRs, CAR demonstrates the unique property of binding to the regulatory module, subsequently activating the transcription of target genes, even in the absence of a selective ligand [Choi et al., 1997; Muangmoonchai et al., 2001]. NR1 motif of the PBREM enhancer is considered to be the most important for mediating trans-activation of CAR receptor's target genes [Sueyoshi et al., 1999; Wang and Negishi, 2003]. CAR is able to bind to NR1 motif of PBREM [Swales and Negishi, 2004] even without being induced by PB or a PB-type agonist, therefore it has been characterized as a constitutive activated receptor [Zelko and Negishi, 2000]. However, this property is exhibited in immortal cells *in vitro*, and not in vivo, which means that there is a mechanism of sequestering the receptor in the nuclear compartment in the absence of a selective agonist, in an inactive form [Honkakoski et al., 1998a]. Cultured cells transfected with CAR expression plasmids, due to low endogenous expression of the receptor, usually exhibit reduced response to CAR activators, due to spontaneous nuclear translocation and sequestration of the over-expressed receptor [Imai et al., 2013].

Indeed, in the absence of any ligands or activators, CAR resides in the cytoplasmic compartment of hepatocytes, and its retention is controlled by a protein complex, which is composed by CCRP (for CAR Cytoplasmic Retention Protein, also designated as DNAJC7), a tetratricopeptide repeat (TRP) and heat-shock protein 90 (HSP90), a chaperone for steroid receptors. CCRP forms a complex with HSP90 and then binds directly to the ligand-binding domain (LBD) of CAR

[Kobayashi et al., 2003, Yoshinari et al., 2003]. Nuclear translocation of CAR requires a step through which the receptor is released from the CAR:HSP90 complex, and is triggered by the recruitment of protein phosphatase 2A (PP2A) to the complex, after PB treatment [Yoshinari et al., 2003]. This phosphorylation/dephosphorylation step is crucial for liberation of CAR from the complex, nuclear translocation and gene induction [Kawamura et al., 1999; Mutoh et al., 2009], for example CYP2B and 3A PB-induced expression is modulated through this cascade in rat primary hepatocytes [Joannard et al., 2000]. Also, an AMP-activated protein kinase is implicated in translocation of CAR in the nucleus and induction of CYP2B genes [Shindo et al., 2007]. On the other hand, induction of CYP2B genes is attenuated during carcinogenesis as a consequence of defective CAR translocation in response to PB treatment, as shown in experiments with hepatic preneoplastic lesions [Numazawa et al., 2005]. Likewise, the nutritional factor DHA (docosahexaenoic acid) was found to attenuate the nuclear translocation of CAR and decrease the formation of CAR-NR1 complex with the target gene, thus resulting in the down-regulation of CYP2B1 in rat primary hepatocytes [Li et al., 2007].

Constitutive activation of CAR is hampered by endogenous androstane metabolites, such as androstanol (5α-androstan-3α-ol) and androstenol (5α-androst-16-en-3α-ol), which act as inverse agonists of CAR [Forman et al., 1998], through their interaction with a co-factor that binds to LBD of the receptor, called the steroid co-activator 1 (SRC-1). The role of SRC-1 is important, since both constitutive and xenobiotic-induced CAR activation are enhanced by this co-factor. Concurrent effect of the enrichment of transcription mediated by CAR is the interplay of Sp1 transcription factor with PBREM [Muangmoonchai et al., 2001]. Okadaic acid is a protein phosphatase inhibitor, that also serves as a repressor of CAR activation and induction of its target genes in mouse and rat primary hepatocytes, through repression of PP2A activity and blockage of receptor's nuclear translocation [Sidhu and Omiecinski, 1997; Honkakoski and Negishi, 1998; Kawamoto et al., 1999]. Noteworthy is also the finding that some compounds were found to play rather an agonistic or an antagonistic role, but actually they exhibit a dual action, since they interact with both co-activators (for example SRC1 for mCAR) and co-repressors (for example NCoR for mCAR). Not only oestrogens, but also the xenobiotics

clotrimazole and methoxychlor are recognized to display this double activity [Mäkinen et al., 2003]. Interestingly, synthetic ligands of the Peroxisome Proliferator-Activating Receptor (PPAR) subfamily of nuclear receptors act as CAR antagonists (inverse agonists), while functioning as agonists of PPARα genes, exhibiting a dual action and suggesting a cross-talk of the xenobiotic sensors [Guo et al., 2007]. Moreover, the nuclear receptors PPARα and FXR demonstrate suppressive role against CAR-mediated induction of genes, through the competition of NRs for co-activators or binding sites [Mäkinen et al., 2002]. To summarize, there are many molecules interacting with CAR, either up- or down-regulating the activity of the receptor, and are finely presented in a recent review by Molnàr and his colleagues (2013) (see Table 3).

Table 3. List of CAR interacting proteins

CAR-interacting protein		Group/function
Full name	**Gene symbol[a]**	
Steroid receptor co-activator 1 (SRC1)	*NCOA1*	p160 Co-activator
Transcriptional intermediary factor 2 (TIF2)	*NCOA2*	p160 Co-activator
Receptor-associated co-activator 3 (RAC3)	*NCOA3*	p160 Co-activator
Activating signal co-integrator 2 (ASC2)	*NCOA6*	General NR co-activator
PPAR-binding protein (PBP)	*MED1*	Mediator TRIP/TRAP co-activator
PPARα-interacting cofactor 320 (PRIC320)	*CHD9*	General transcription machinery-interacting protein
PPARγ co-activator 1α (PGC-1α)	*PPARGC1A*	General NR co-activator
Forkhead box O1 (FoxO1)	*FOXO1*	Metabolic transcriptional factor
Growth arrest and DNA damage-inducible 45β (Gadd45β)	*GADD45B*	Cell cycle-regulating factor
Protein phosphatase 1 regulatory subunit 16A (PPP1R16A)	*PPP1R16A*	Regulator of signal transduction
Splicing factor 3a, subunit 3, 60 kDa (SF3a)	*SF3A3*	Splicing/inhibitor of CAR signaling
Nuclear receptor corepressor (NCoR)	*NCOR1*	General NR corepressor
Silencing mediator for retinoid or thyroid hormone receptors (SMRT)	*NCOR2*	General NR corepressor

For references, see the section Interactions of CAR with other nuclear proteins. [a]Approved by the HUGO Gene Nomenclature Committee (http://www.genenames.org/).

(Molnàr et al., 2013)

CAR activation is conducted either directly via binding of a selective ligand (for example TCPOBOP [Tzameli et al., 2000; Kretschmer and Baldwin, 2005]), or indirectly via a phosphorylation-dephosphorylation cascade, triggered by its prototypic activator PB or other, PB-type inducers [Moore et al., 2000; Goodwin and Moore, 2004]. Apart from PB, CAR is also activated indirectly by endogenous

activators. Bilirubin was the first physiological ligand identified [Yamamoto et al., 2003], whereby CAR is implicated in regulation of stress response in cases of hyperbilirubinemia [Goodwin and Moore, 2004; Kanno and Inouye, 2008]. Also, various herbal medicines have recently been identified to serve as CAR modulators, and the best characterized activator is *yin zhi huang*, a Chinese herbal beverage consisting of various plant extracts, used for treatment of neonatal jaundice [Chang, 2009].

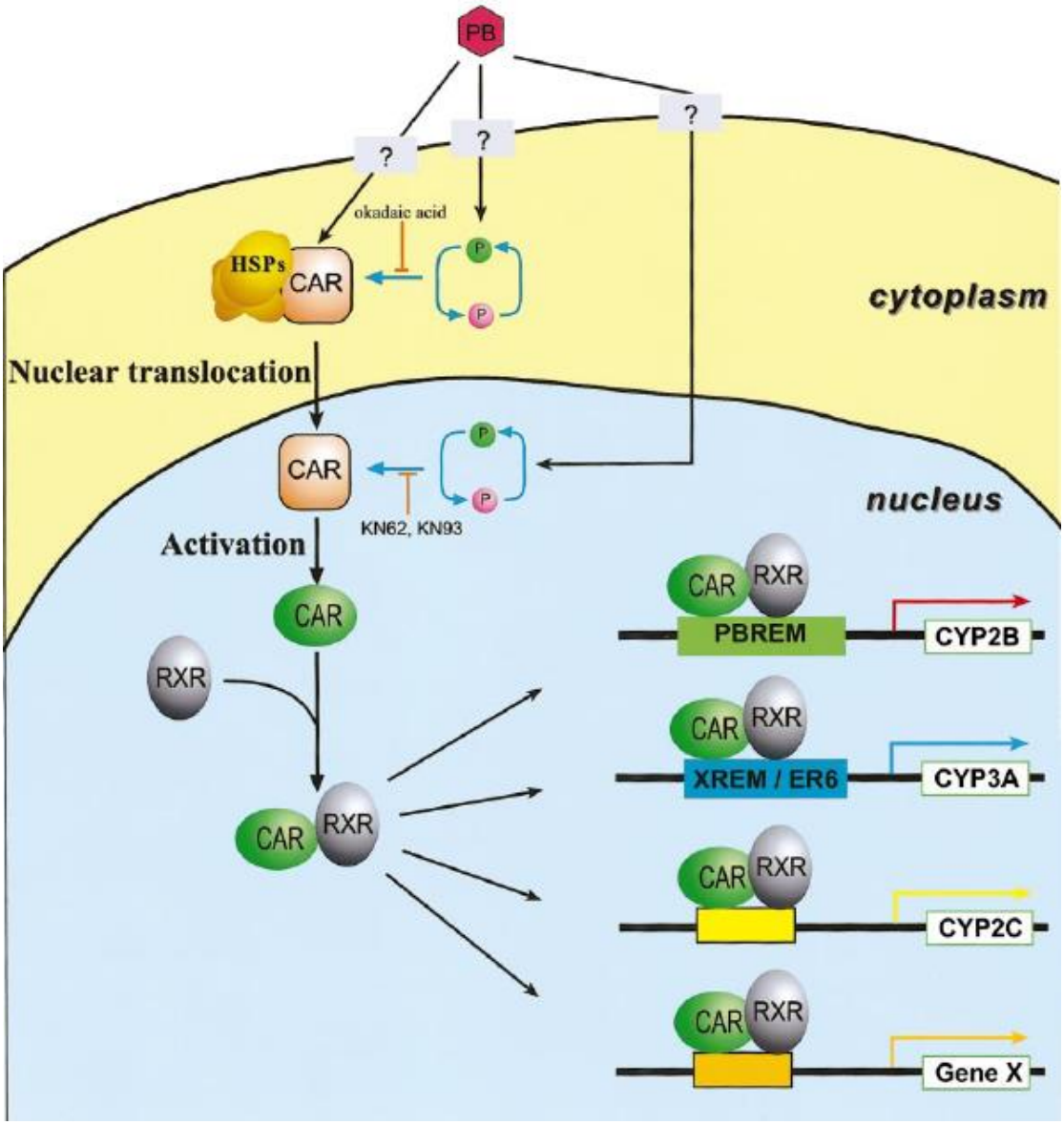


Figure 4. Proposed mechanism of CAR activation via PB induction [Zelko and Negishi, 2000].

As fore-mentioned, CAR forms heterodimers with the RXRα receptor, and the dimer recognizes and binds to DR-4 motifs on PBREM element, through which it regulates transcription of genes that are controlled by this enhancer module. On top of that, other functional response elements have been identified as recognition and binding sites for CAR/RXRα heterodimer, including ER-6 motif (an everted repeat with 6 base pairs in between, modulating the transcription of CYP3A4 gene) [Sueyoshi et al., 1999], ER-8 (everted repeat with 8-bp spacer), and DR-2, DR-3, DR-5 (direct repeats with 2-, 3-, 5-bp spacer, respectively) [Handschin and Meyer, 2003]. Studies in rat liver and rat primary hepatocytes have shown that CAR/RXRα can form a complex with NR1 site of PBREM after PB treatment or exposure to other PB-type xenochemicals [Pustylnyak et al., 2005; Li et al., 2007]. Actually, PB administration leads to nuclear accumulation of CAR even within 1 hour of treatment in mouse livers [Kawamoto et al., 1999]. Except for the formation of CAR/RXRα heterodimer and binding on PBREM sequence, other receptors such as thyroid receptors (TRs) have been found to bind to this complex, forming a polymer and enhance up-regulation of CYP2B1 [Bing et al., 2014].

The presence of CAR in the nucleus does not entail induction of target genes [Zelko and Negishi, 2000], since nuclear translocation could also be triggered by an inverse agonist (for example, androstenol), without consequent gene activation [Forman et al., 1998]. In transfected HepG2 cells, CAR spontaneously translocates into nucleus even in the absence of an activator, such as PB, resulting in the constant induction of CYP2B expression [Sueyoshi et al., 1999, Kanno and Inouye, 2008]. Also, basal levels of nuclear CAR have been detected in untreated hepatocytes [Hosseinpour et al., 2006; Li et al., 2007]. These findings imply that CAR shuttling presumably is ligand independent; although it has been reviewed that endogenous compounds emanating from portal vein could explain nuclear CAR in some periportally located cells, but not in all hepatocytes [Kawamoto et al., 1999]. Xia and Kemper (2005) have proposed a PB-independent nuclear accumulation and retention of mCAR by GRIP1 (Glucocorticoid Receptor-Interacting Protein 1). CAR, like other proteins that shuttle between cytosol and nucleus, has both NLSs (Nuclear Localization Signals) and NESs (Nuclear Export Signals) [Xia and

Kemper, 2007; Kanno and Inouye, 2008]. In case of immortal cell lines that transiently express CAR, the receptor is localized in the nucleus without any ligand stimulation, and is probably caused by an ineffective block of NLS activity, or impaired NES functionality [Kanno et al., 2005].

CAR is a major player in transcription regulation of hepatic metabolizing genes, especially those encoding Phase I (CYPs) and Phase II (GSTA1 – Glutathione S-transferase A1 and UGT1A1 – UDP-glucoronosyltransferase 1A1) drug metabolizing enzymes [Pascussi et al., 2003; Reschly and Krasowski, 2006, Dekeyser and Omiecinski, 2010], as well as Phase III uptake and efflux transporters (multidrug resistance proteins). A nice review of CAR's target genes belonging to Phase I, II and III are presented in Table 4 [Molnàr et al., 2013]. Apart from being a xenobiotic sensor, CAR is further implicated in the regulation of hepatic fatty acid oxidation, gluconeogenesis [Miao et al., 2006], hepatocarcinogenesis [Yamamoto et al., 2004], response to inflammation [Moreau et al., 2008], hepatic energy metabolism [Konno et al., 2008] and the metabolism of steroid hormones and bilirubin [Yamamoto et al., 2003].

Table 4. CAR target genes related to drug metabolism and disposition

Target gene	Gene symbol[a]	Species
Phase I		
Aldehyde dehydrogenases	*Aldh1a1, 1a7*	*M. musculus*
Cytochrome P450s	*CYP2A6, 2B6, 2B10, 2C9, 2C19, 3A4, 3A11*	*H. sapiens*
	Cyp1a1, 2a4, 2b10, 3a11	*M. musculus*
	Cyp2b1, 2b2, 2c6, 2c7, 3a1	*R. norvegicus*
P450 (cytochrome) oxidoreductase	*Por*	*M. musculus*
Phase II		
Glutathione *S*-transferases	*Gsta1, a2, a3, m1, m2*	*M. musculus*
	Gsta1, a2, a3, m1	*R. norvegicus*
Sulfotransferases	*Sult1a1, 2a1, 2a9*	*M. musculus*
UDP-glucuronosyltransferases	*UGT1A1*	*H. sapiens*
	Ugt1a1	*M. musculus*
	Ugt1b2	*R. norvegicus*
Phase III		
ATP-binding cassette family	*ABCB1, C2, C3*	*H. sapiens*
	Abcb1a, c1, c2, c3, c4	*M. musculus*
	Abcc2	*R. norvegicus*
Solute carrier transporters	*Slco2a1*	*M. musculus*
		R. norvegicus

Data were compiled from di Masi et al. [4] and Tirona and Kim [9]. *M. musculus, Mus musculus; H. sapiens, Homo sapiens; R. norvegicus, Rattus norvegicus*. [a]Approved by the HUGO Gene Nomenclature Committee (http://www.genenames.org/).

(Molnàr et al., 2013)

Based on CAR's unique capability of binding to PBREM enhancer element [Honkakoski et al., 1998c], and the lack of its expression in most stable cell lines [Sueyoshi et al., 1999], Honkakoski et al., (2001) developed a cell line in which luciferase gene expression is controlled by the nuclear receptor CAR and its enhancer module PBREM. This cell system can be used as a tool for studies of gene transcription regulation after administration of drugs that act as ligands. In another study based on tag-fused CAR proteins, Imai et al. (2013) have sought to establish a novel reporter assay system, by use of which chemical compounds would be identified as species-specific CAR activators.

There are species differences regarding the compounds that activate CAR [Moore et al., 2003], which could be attributed to sequence differences of the ligand binding domains of the receptor [Reschly and Krasowski, 2006; Pustylnyak et al., 2007]. For example, 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) serves as a selective ligand only for mouse CAR [Moore et al., 2000; Tzameli et al., 2000], 2,4,6-tryphenyldioxane-1,3 (TPD) is a PB-type CAR inducer only for rats [Pustylnyak et al., 2009], while 6-(4-chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde-(3,4-dichlorobenzyl)oxime (CITCO) activates only human CAR, but not the rodent equivalents [Maglich et al., 2003]. Correspondingly, androstanol and androstenol serve as inverse agonists for mouse CAR but not human CAR, whereas clotrimazole (CLZ) is a repressor for human CAR but not for mouse CAR [Li and Wang, 2010]. Also, meclizine is a drug that displays activating role for mCAR, which was considered to act as an antagonist to hCAR activation [Chang et al., 2009], although recent studies debunk this finding. Apart from the selectivity of CAR agonists in relation to various species, CAR exhibits differential activation even among strains of same species, or gender-dependent expression within the same animal strain. For instance, Xiong et al. (2002) have revealed CAR activation deficiency in obese Zucker rats, and Yoshinari et al., have proposed that differential expression of CAR between male and female WKY rats could give an explanation for the discrepancy in CYP2B genes induction by PB. Constitutive activation of CAR has been studied in experiments with CAR/GFP phusion protein, and revealed the presence of CAR in the nucleus of untreated HepG2 cells [Kawamoto et al., 1999]. Honkakoski et al., (1998b) and Sueyoshi et al., (1999) have proven with

both *in vivo* and *in vitro* experiments that PB or other similar inducers trigger the transcription of target genes only via CAR or close-related nuclear receptors.

Many studies (presented at Negishi and Honkakoski, 2000) support the idea that CAR cross-reacts with PXR (as both can potentially be activated by the same chemicals and induce the same target genes), and probably one receptor affects the efficiency of the other. This cross-talk is further supported by the fact that CAR has the potentiality to bind to XREM, the response DNA element for PXR [Sueyoshi et al., 1999; Moore et al., 2000], thus activating the CYP3A genes as well. Another fine example of the cross-talk between the two receptors has been shown by Goodwin B. et al., (2001) in tumor-bearing animals, where CYP2B gene was induced after PB administration via PXR receptor, since the CAR nuclear translocation was impaired. Noteworthy, although both PBREM and XREM enhancers can be induced by PB agonists, the expression of CAR is essential so that this induction is exhibited, at least in rodents [Zelko and Negishi, 2000].

The Pregnane X Receptor – PXR

The Pregnane X receptor (PXR, NR1I2), also known as SXR (for steroid and xenobiotic receptor), or PAR (for pregnane-activated receptor) belongs to the same subfamily of nuclear receptors with CAR, however the primarily characterized as 'orphan' PXR has now been 'adopted', as an endogenous agonist of the receptor has been identified. PXR is a promiscuous receptor, since it is activated by many, structurally diverse, compounds; glucocorticoids and antiglucocorticoids, such as the Pregnenolone 16α-carbonitrile (PCN), and several pesticides [Honkakoski and Negishi, 2000; Kretschmer and Baldwin, 2005]. Further, vitamins, steroids, antimycotics and antibiotics, anticancer compounds, endocrine disruptors, PPAR and nuclear receptor antagonists are potent PXR ligands [reviewed in Chen et al., 2012]. The known endogenous ligands that activate PXR are the lithocholic acid (LCA), a bile acid which activates also the receptors VDR (Vitamin D Receptor) and FXR (Farnesoid X Receptor) [Bailey et al., 2011], naturally occurring steroids (e.g. progesterone, corticosterone) [Timsit and Negishi, 2007], and the herbal St. John's wort, which contains hyperforin and up-regulates targets of PXR (e.g. CYP3A4) via direct binding to the receptor [Chang et al., 2009].

Upon ligand binding, PXR triggers the transcription of CYP3A genes, through binding to XREM cognate elements [Honkakoski and Negishi, 2000]. Apart from CYP3A, PXR regulates the expression of other CYPs (CYP2Bs, CYP2Cs, CYP2A6 and CYP4F12, all reviewed in Tolson and Wang, 2010) and other Phase I enzymes, such as aldo-keto reductases (AKRs) and aldehyde dehydrogenases [Alnouti and Klaassen, 2008, Chen et al., 2012], especially ALDH1A1 and ALDH1A7 [Tolson and Wang, 2010]. PXR further mediates the induction of Phase II (UGTs, GSTs and SULTs) and Phase III (MRPs) enzymes [Kretschmer and Baldwin, 2005], as well as genes responsible for energy homeostasis [Wada et al., 2009] and bile acid metabolism, or even some types of cancer [Carnahan and Redinbo, 2005]. On the other hand, various compounds act as antagonists for PXR, including the dietary sulforaphane [Zhou et al., 2007] and coumestrol [Wang et al., 2008].

Worth mentioning is the implication of PXR in the transcription regulation of its own gene, via a negative feedback loop. Upon treatment with an agonist, PXR gene expression is down-regulated and, consequently the amount of activated

PXR is reduced. By attenuating the PXR-mediated activation of target genes, the organism prevents excess response to a stimulus that may lead to disruption of homeostasis [Bailey et al., 2011]. In response to PCN, PXR gene expression is attenuated, therefore over-expression of CYP3A is restrained. In this view, PXR serves both as a 'xenosensor' for external stimuli, but also as an 'endosensor', in order to respond to internal changes in chemical balance.

The structure of PXR is alike to other nuclear receptors (presented above in Fig. 1). The N-terminal transactivation domain is followed by the highly conserved DNA binding domain (DBD), through which the receptor binds to response elements, in order to regulate expression of target genes. A ligand-binding domain (LBD), which includes the C-terminal activation domain, is responsible for the nuclear translocation of the receptor, dimerization with RXRα and recruitment of co-activators and co-repressors [Li and Wang, 2010]. PXR and CAR share same co-regulators, for example the well-studied co-activator SRC-1 and the nuclear co-repressor NCoR2/SMRT (for Nuclear receptor Co-repressor 2 / Silencing Mediator of retinoid and Thyroid Receptor) [Hariparsad et al., 2009]. Notably, the promiscuity for ligand binding of PXR is attributed to the shape and size flexibility of the LBD [reviewed in Li and Wang, 2010].

PXR, like CAR is predominantly expressed in liver and intestine [Li and Wang, 2010]. What differentiates PXR from CAR is that PXR is activated only via direct binding to a selective ligand [Sueyoshi and Negishi, 2001]. Although early studies supported the notion that PXR resides in the nucleus, either in absence or in presence of an inducer [reviewed in Sueyoshi and Negishi, 2001], mouse PXR has later been identified to be localized in the cytoplasm, until activated by a ligand and translocated into nucleus [Kawana et al., 2003; Matic et al., 2009].Indeed, mPXR is sequestered in the cytoplasm in the absence of a ligand, bound to a complex consisting of the receptor, the co-chaperone CCRP (for CAR Cytoplasmic Retention Protein, also designated as DNAJC7) and the heat shock 90 (Hsp90) protein. Nuclear translocation of PXR is, likewise for CAR, the essential step for binding on regulatory elements and promotion of gene transcription. PXR's shift into nucleus in response to PCN treatment is not controlled by the mPXR:CCRP:Hsp90 complex, but rather by the XRS (for Xenochemical Response Sequence), an NLS (for Nuclear Localization Signal) and AF-2 domain of the receptor [Squires et al.,

2004]. Nevertheless, there is still limited knowledge about hPXR localization, and so far it seems that the receptor is predominantly located in nuclear compartments of all human cells lines studied [reviewed in Li and Wang, 2010].

Interestingly, nuclear sequestration is not synonymous to gene transactivation, but direct binding of a selective ligand is a prerequisite [Lehmann et al., 1998; Kliewer et al., 1998]. PXR, upon activation by a selective ligand, forms heterodimer with RXRα and bind to response elements that lie on promoter regions of target genes. XREM is the enhancer module through which PXR regulates the induction of CYP3A genes. PXR/RXRα complex binds to DR3 and ER6 motifs [Tolson and Wang, 2010], but also recognize and bind to DR4, DR5, IR0, IR6 and ER8 [reviewed in: Li and Wang, 2010; Chen et al., 2012].

Species-specific induction of human and mouse/rat PXR is attributed to the relatively low-conserved LBDs. Thus, rifampicin and hyperforin selectively activate the human PXR but not the rodent counterparts [LeCluyse, 2001; Tirona et al., 2004], 5β-pregnane-3,20-dione is an activator of PXR in mouse and human, while PCN is a selective ligand for PXR in rodents [Honkakoski and Negishi, 2000; Sueyoshi and Negishi, 2001]. CYP3A is expressed in a similar to PXR's species-specific fashion, lending strong support to the notion that CYP3A induction is mediated by PXR in a species-specific manner [reviewed in Chen et al., 2012]. PB activates hPXR, but has little or no effect on rPXR [Jones et al.,2000; Moore et al., 2000; Sueyoshi and Negishi, 2001]. Moreover, clotrimazole (CLZ) and PK11195 are potent activators of hPXR, although they serve as deactivators for hCAR [Moore et al., 2000; Li et al., 2008]. Besides species-specificity regarding selective ligands for PXR, tissue-specific activation of the receptor has also been observed [Chang et al., 2009].

As mentioned beforehand, there is a cross-talk of PXR with CAR receptor, mainly due to promiscuity of their binding capacity on DNA elements. Subsequently, they share many target genes, although their inductive potential may vary, dependent on the receptors' abundance and the response element. Albeit their binding on the same elements, the transactivation is governed by selective activators for each of the receptors [Smirlis et al., 2001]. For example, both CAR and PXR can mediate the transcription of CYP2B and CYP3A via binding to respective regulatory sites; the DR4 motif within the PBREM or DR3 motif within the XREM.

However activation of the receptors is triggered in a xenobiotic-specific manner, since CAR is activated by PB and PXR by PCN [Smirlis et al., 2001]. These findings suggest that there is a competition between the receptors regarding their binding to the same sites on genes' promoters, but also for their dimerization partner, RXRα [Smirlis et al., 2001].

Apart from being transcription mediators of their target genes, CAR and PXR may act as suppressors of gene expression, by binding either directly or indirectly to response elements of other receptors and recruiting co-repressors, in order to attenuate the transcription. Konno et al., (2009) have described the attenuation of CYP24A1 gene upon vitamin D_3 activation, via recruitment of SMRT co-repressor by PXR and CAR. This is indicative of the impact these receptors may exert upon treatment with drugs or other substrates, and should raise concerns regarding possible drug-drug interactions and adverse side effects. For instance, many chemotherapeutics have been reported to activate hPXR and hCAR, thus contributing to acceleration of drug metabolism and disposition mediated by the receptors or even development of multi-drug resistance of cancer cells, which means a less effective cancer therapy [Chen et al., 2012]. The mechanisms of CAR and PXR activation via ligand direct or indirect binding of selective activators, and the subsequent promotion of their target genes' transcription is presented in Figure 4.

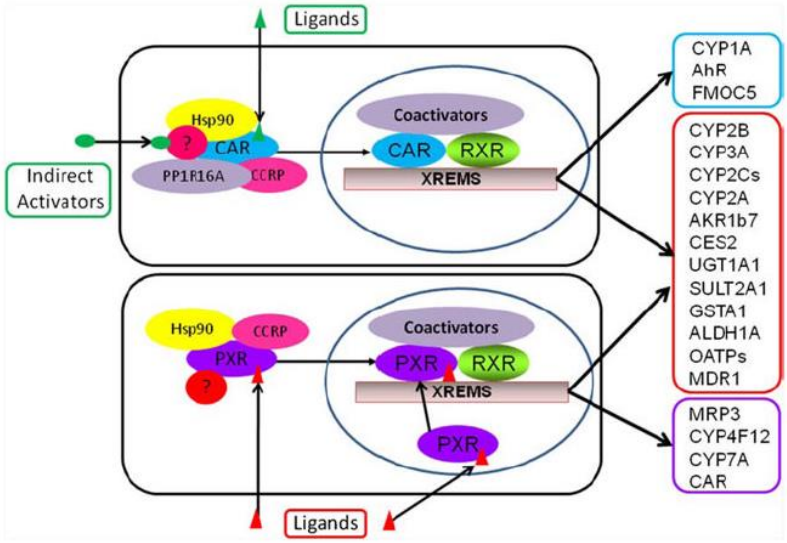
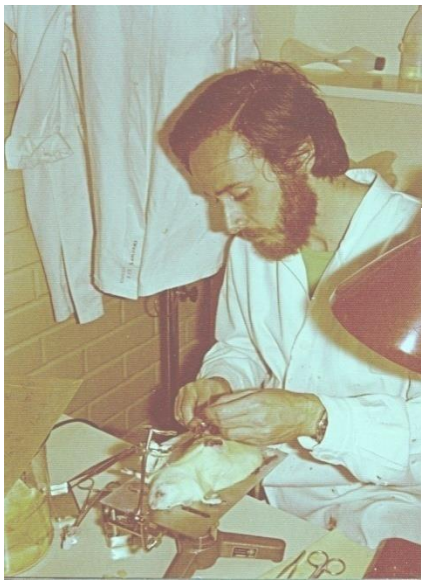


Figure 4. Schematic illustration of the activation mechanisms and target genes of CAR and PXR. [Tolson and Wang, 2010].

A unique experimental model for studying the regulation of ALDH1A genes

The present study was based on a unique experimental animal model that has been preserved in our laboratory, which consists of two rat strains that exhibit differential induction of ALDHs, not only in basal levels, but also under treatment with the barbiturate Phebobarbital (PB). The two populations of Wistar/Af/Han/Mol/Kuo/Io rats were identified and separated 40 years ago by Professor Marios Marselos in the University of Kuopio in Finland, (currently named the University of Eastern Finland, in Kuopio, Finland), who performed open liver biopsies in male rats treated with PB (Fig. 4). Further to biopsies, aldehyde dehydrogenases' activity was determined on liver samples and the population of rats responding to PB treatment (designated **RR** for responders, presented on the right side of the following panel, Fig.5) was distinguished from the non-responders (designated **rr,** presented on the left side of the panel, Fig.5). The distinct populations of **RR** and **rr** rats were reproduced by inbreeding, thus creating the respective rat strains.



Acta pharmacol. et toxicol. 1976, **39**, 186–197.

From the Department of Physiology, University of Kuopio, SF-70101 Kuopio 10, Finland

Genetic Variation of Drug-Metabolizing Enzymes in the Wistar Rat

By

Marios Marselos

(Received December 15, 1975; Accepted January 26, 1976)

Figure 4. Snapshot of Prof. Marios Marselos perfoming open liver biopsies on male Wistar rats treated with phenobarbital (PB), and the prototypic publication of this work (kindly provided by Prof. M. Marselos).

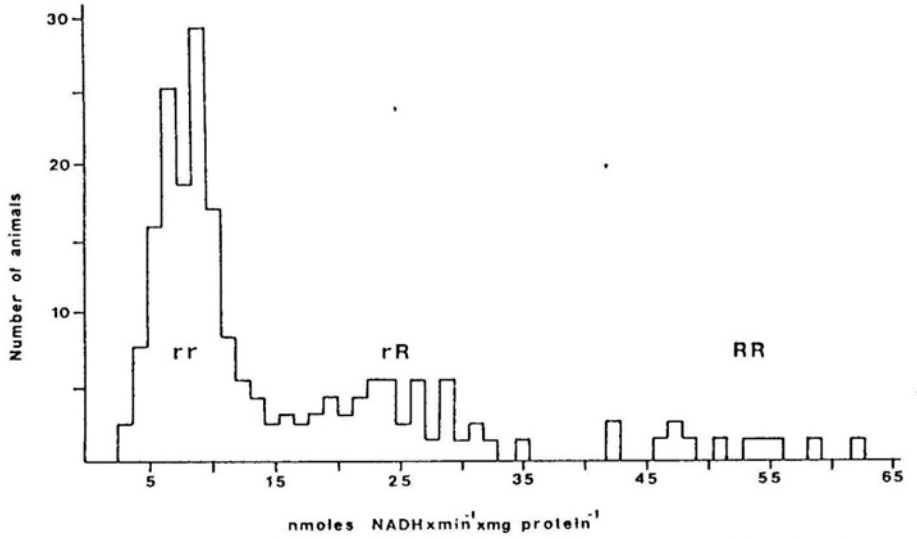


Figure 5. Measurement of cytosolic aldehyde dehydrogenases in liver samples from biopsies of randomly chosen male Wistar rats treated with phenobarbital (PB), as presented in the prototypic publication (kindly provided by Prof. M. Marselos).

Wistar/Af/Han/Mol/Kuo/Io rats, exhibit differential expression patterns of ALDH1A1 and ALDH1A7 either at basal or PB-induced levels. The induction of the cytosolic ALDH1 after PB treatment is being studied, mainly due to the genetic predisposition exhibited by specific strains of rats [see Kathmann et al, 2000]. Different levels of ALDH induction in these strains after PB administration seem to be controlled by two alleles, the **R** and **r**, of a single autosomal locus. The dominant allele **R** is responsive to the PB induction of ALDH, whereas the **r** is the recessive allele and is non-responsive. Homozygous responders (**RR**) exhibit maximum ALDH induction after PB, while in heterozygous (**Rr**) rats a moderate induction occurs, and non-responders homozygous (**rr**) show only a small increase of ALDH enzymatic activity. **RR** and **rr** rat strains express different mRNA levels of the ALDH1 gene, which is induced by PB, whereas no difference is exhibited in expression levels of other proteins induced by PB, for example CYP2B1/2B2, thus implying a selective effect [Dunn et al, 1989; Pappas et al, 1998 and 2001].

Aim of the study

Our study was aimed at understanding how PB and PB-like chemicals regulate ALDH1A genes (ALDH1A1 and ALDH1A7 in particular) and unraveling the role of nuclear receptors CAR and PXR in the activation of these genes, utilizing the characteristic genetic differentiation between the two strains of Wistar/Af/Han/Mol/Kuo/Io rats.

Even though ALDHs are important drug metabolizing enzymes, as well as CAR and PXR are well-defined and thoroughly studied factors, the mechanism of ALDH induction by PB and PCN and the possible implication of the above receptors remains unknown.

Consequently, we focused on resolving whether the differences between ALDH1A1 and ALDH1A7 gene expression is a result of structural alterations in the promoters of these genes or the differential induction is attained via selective binding of CAR, PXR or even other gene transcription regulators.

MATERIALS AND METHODS

Chemicals

Drugs administered to animals were: Phenobarbital (P-1636) and Clotrimazole (C6019-5G) by SIGMA, 5-Pregnen-eβ-ol-20-one-16α-carbonitrile (sc-227010) by SantaCruz. All other chemicals were of highest grade available.

Experimental model

Two substrains of Wistar/Af/Han/Mol/Kuo/Io rats have been isolated and reproduced by inbreeding, creating inbred lines of RR (responsive) and rr (non – responsive) rats, regarding the induction of aldehyde dehydrogenase family 1A, after administration of phenobarbital (PB).Animals were maintained on a 12-h light/dark cycle at ambient temperature and provided with typical chow and tap water ad libitum. All in vivo and in vitro experiments performed in the present study were reviewed and approved by the Institutional Animal Care and Use Committee of the Medical School of the University of Ioannina.

Animal treatment

- ***Multiple – dose treatment with specific agonists of CAR and PXR nuclear receptors***

For the multiple-dose experiment we used **RR** and **rr** male rats, aged 3 months old. Rats were treated with three (3) doses of phenobarbital – PB (80mg/kg bw; ip, in saline, every 24 hours), four (4) doses of 16α-pregnenolone carbonitrile – PCN (50mg/kg, ip, in olive oil, every 24 hours), as well as three doses of saline or four doses of olive oil, respectively, in rats that were used as controls. Each group consisted of four or five rats. Animals were sacrificed by decapitation 6 hours after last treatment. Liver sections were removed and total RNA, cytosolic, nuclear and total proteins were isolated, and further used for gene expression studies based on

the qPCR method and the Western blotting technique, respectively, as described below.

- ***Time – response effects of single – dose treatment with PB or PCN***

For the time-response study, male **RR** and **rr** rats of 3 months old, were divided into groups of four and treated with a single dose of phenobarbital – PB (80mg/kg bw; ip, in saline) or 16α-pregnenolone carbonitrile – PCN (50mg/kg, ip, in olive oil). Untreated rats of both strains were also injected with saline or olive oil,and used as controls. Animals were sacrificed by decapitation after 1, 3, 6, 12 or 24 hours of PB treatment, or 6, 12 or 24 hours of PCN treatment. Liver sections were removed and total RNA, cytosolic, nuclear and total proteins were isolated, and further used for gene expression studies based on the qPCR method and the Western blotting technique, respectively, as described below.

- ***Developmental expression of aldehyde dehydrogenases 1A1/1A7 and nuclear receptors CAR and PXR***

Male rats of the two strains, aged 1, 10, 20, 30 and 40 days old were sacrificed by decapitation. In the case of 1 and 10 days old rats, samples were pooled, due to the small size of liver (for protein extraction). Liver sections were used for isolation of total RNA and further used for gene expression studies based on the qPCR method, as described below.

Isolation of rat primary hepatocytes and primary culture development

The experimental procedure of isolating primary hepatocytes was based on the Klaunig et al. method [Klaunig et al., 1981], with some modifications, as described by Daskalopoulos et al. (2012).

Step 1: Rat was anaesthetized with ether in a specific glass chamber and immobilized on a special metallic base.

Step 2: The abdomen part of the rat was cleaned with ethanol solution (75%) and opened with sterile forceps and scissors. The stomach and intestines were moved out of the abdominal cavity and the portal vein was cannulated. A piece of thread was used, in order to secure the cannulation on the vein.

Step 3: Through the portal vein, 250 ml of a perfusion buffer (with calcium and magnesium-free Hanks' salt solution) was diffused into liver, via a pump with a manually tuned flow-rate (15 ml/min). Buffer was kept in a water bath at 37oC during the diffusion. This step was essential, in order to remove the blood from the liver. As a result, the liver colour turned from dark brown to light brown (yellowish).

Step 4: When liver started changing colour, post-caval veins were cut, so that the blood supply of liver stopped, and the animal died.

Step 5: After perfusion buffer, the liver was diffused with 250 ml of a medium containing collagenase, which was also kept in a water bath at 37^{o}C during the whole process, and pumped with a flow-rate gradually tuned from 15 to 30 ml/min. In the meanwhile, the tissue was gently massaged in order to help the fluid circulate within all liver lobes. Collagenase buffer changed the colour of the liver to white yellow and promoted the detachment of hepatocytes.

Step 6: By the end of the diffusion of buffers, the whole liver tissue was subtracted from the animal and placed on a sterile petri dish, then cut into small pieces by use of sterile forceps and scissors and divided in two falcons.

Step 7: 35 ml of DPBS were added in each falcon and the material was gently mixed. Afterwards, the fluid was filtered through 70 μm filters into new falcons.

Step 8: Samples were centrifuged at 1000 rpm for 3 minutes at 4^{o}C.

Step 9: Supernatant of each tube was discarded and pellet was gently re-suspended in 15 ml of DPBS, by use of a pipette. The following steps were done in the hood, in order to avoid any contamination.

Step 10: Samples were centrifuged at 1000 rpm for 3 minutes at 4^{o}C.

Step 11: Supernatant of each tube was discarded and pellet was gently re-suspended in 10 ml of DPBS, as described on step 9.

Step 12: Samples were centrifuged at 1000 rpm for 3 minutes at 4^{o}C.

Step 13: Supernatant of each tube was discarded and pellet was gently re-suspended in 10 ml of DPBS. Then, 1 ml of HBSS and 9 ml of Percoll were added in each falcon and gently mixed. The addition of Percoll was critical for the viability of cells, as most of them died during this step.

Step 14: Samples were centrifuged at 1000 rpm for 3 minutes at 4^{o}C.

Step 15: Supernatant of each tube was discarded and pellet was gently re-suspended in 10 ml of DPBS, as described on step 9.

Step 16: Samples were centrifuged at 1000 rpm for 3 minutes at 4^{o}C.

Step 17: Supernatant of each tube was discarded and pellet was gently re-suspended in 1 ml of William's E medium (with 1% L-Glutamine and 1% Penicillin/Streptomycin added, kept at 37^{o}C in water bath).

Step 18: Cells were counted under microscope, by use of a special chamber for measuring of cells (Neubauer chamber). The final cell title was estimated by multiplying the number of cells counted to 10^4. Depending on the number of cells needed for the experiment (approximately $5x10^5$cells/dish), the material was diluted in medium and cells were plated on collagene-coated petri dishes.

Step 19: Assessment of viability of hepatocytes above 85% is considered indicative of a successful cell culture, and was based on the use of trypan blue dye. The dye gave a blue colouring only to the cytoplasm of cells with ruptured membranes, which were dead, discriminating them from the living ones with intact cellular membrane.

Cells were cultured for 24 hours in William's E medium, then 1mM PB was administered for 6 hours, while cells used as controls were supplemented with fresh medium. Cells were incubated in 37^{o}C for 6 hours. Total RNA was isolated by treated and untreated cells, and further used for cDNA synthesis and gene expression study, based on the qPCR method.

Buffers and chemicals used for isolation and culture of primary hepatocytes are listed below:

- ✓ **Perfusion buffer** (250 ml): 250 ml HBSS, 1mM Na_2EDTA
- ✓ **Collagenase buffer** (250 ml): 250 ml HBSS, 0.14 gr $CaCl_2$, 0.125 gr Collagenase I

- HBSS – No Calcium, No Magnesium, No Phenol Red (500 ml) – GIBCO (14175-053)
- DPBS – No Calcium, No Magnesium (500 ml) – GIBCO (14190-094)
- William's E medium – With Sodium Bicarbonate, Without L-glutamine – SIGMA (W4128)
- Percoll (100 ml) – SIGMA – P1644
- Collagenase Type I (1g) – GIBCO – 17100

Figure 1. Isolation of primary hepatocytes from **rr** rat.

Protein Extraction

Protein extraction from tissue

For protein extraction from tissue, rat liver sections were taken immediately after decapitation, and placed into ice cold PBS. Then, we followed the protocol written below, with variations dependent on the desired protein fraction.

For total protein extraction:

1) First step was to chop the tissue in small pieces with sterile scissors or blade, then pieces were placed into sterile eppendorf, with addition of 1 ml of RIPA Buffer and protein inhibitors (1 μl/ml).
2) Samples were homogenized using (KINEMATICA) for 1 minute at speed 8, then placed on ice for 25-30 minutes. During this time, the samples were mixed every 10 minutes by vortexing.
3) Samples were centrifuged for 20 minutes at 14000 rpm, at 4^{o}C.
4) Supernatants were collected in sterile eppedorfs, their optical density (O.D.) was measured at 595 mn, and stored at -80^{o}C.

For cytosolic protein extraction:

1) Tissue was chopped in small pieces with sterile scissors or blade, then pieces were placed into sterile eppendorf, with addition of 1 ml of ice cold Hypotonic Buffer.
2) Samples were homogenized using homogenator for 1 minute at speed 8, then centrifuged for 3 minutes at 4000 rpm, at 4^{o}C .
3) Pellets were re-suspended in 300 μl of ice cold Hypotonic Buffer+0,1% NP-40. Eppendorfs were tapped until pellet was completely suspended into buffer.
4) Samples were kept on ice for 10 minutes, and vortexed 2-3 times for 20 seconds during this time.
5) Centrifugation of samples for 10 minutes at 13000 rpm, at 4^{o}C.
6) Supernatants, constituting the cytoplasmic fraction, were collected in sterile eppendorfs, their optical density (O.D.) was measured at 595 mn, and stored at -80^{o}C.

NOTE: Pellet was kept, in case of nuclear protein extraction, described above.

For nuclear protein extraction:

7) Pellet from the previous step (6) of cytosolic protein extraction was re-suspended in 1 ml of ice cold Hypotonic Buffer. Tapping of eppendorfs until the pellet was completely suspended into buffer.
8) Centrifugation of samples for 10 minutes at 13000 rpm, at 4^{o}C.
9) Pellets were re-suspended in 90 μl of ice cold High Salt Extraction Buffer, added drop-by-drop while the sample was vortexed.
10) Samples were kept on ice for 30 minutes, and vortexed every 5 minutes for 30 seconds during this time.
11) Centrifugation of samples for 30 minutes at 13000 rpm, at 4^{o}C.
12) Supernatants, constituting the nuclear fraction, were collected in sterile eppendorfs, their optical density (O.D.) was measured at 595 mn, and stored at -80^{o}C.

All measurements mentioned above were based on the Braddford method and the protein content was determined by use of standard curves.

All buffers mentioned above (underlined), and the recipes for their preparation are shown below:

Table 1. Protein extraction buffers

PBS (1x) pH=7.4	**RIPA buffer (for 60 ml)**	**Hypotonic buffer pH=7.9**	**High salt extraction buffer**
In 800 ml ddH_2O dissolve:	• 3 ml Tris-Cl 1 M pH 7.5	In 90 ml ddH_2O dissolve:	• 20 mM HEPES pH 7.9
• 8gr NaCl	• 1.8 ml NaCl 5 M	• 238.3 mg Hepes	• 25% Glycerol
• 0.2 gr KCl	• 0.6 ml Triton X-100	• 30.49 mg $MgCl_2$	• 450 mM KCl
• 1.44 gr Na_2HPO_4	• 1.5 ml Na-Deoxycholate	• 37.28 mg KCl	• 1mM EDTA
• 0.24 gr KH_2PO_4	• 0.06 ml SDS	Adjust final volume to 100 ml	
Adjust pH=7.4, then add ddH_2O up to 1 lt	Add 53.04 ml sterile ddH_2O		

All buffers were sterilized in autoclave at 121ºC for 20 minutes prior to use.

Essential step before the use of buffers in the above-mentioned protocols for protein extraction was the addition of protein inhibitors, as shown below:

Table 2. Ready-to-use protein extraction buffers

For ready-to –use Hypotonic buffer (for 30 ml)	**For ready-to –use Hypotonic buffer+NP-40 (for 5 ml)**	**For ready-to –use High salt extraction buffer (for 5 ml)**
• 30 μl aprotinin	• 5 μl aprotinin	• 5 μl aprotinin
• 30 μl leupeptin	• 5 μl leupeptin	• 5 μl leupeptin
• 150 μl PMSF	• 25 μl PMSF	• 25 μl PMSF
• 150 μl Na_3VO_4	• 25 μl Na_3VO_4	• 25 μl Na_3VO_4
• 300 μl DTT	• 50 μl DTT	• 50 μl DTT
	• 500 μl 1% NP-40	• 500 μl 1% NP-40
In 29.340 ml hypotonic buffer	In 4.390 ml hypotonic buffer	In 4.390 ml high salt extraction buffer

Western blotting

Quantitative and qualitative study of protein expression was based on the Western blotting technique, which is shared in various steps; preparation of the appropriate gel, loading of samples, electrophoresis of proteins, blotting of the gel onto a specific membrane, incubation with specific antibodies and visualization of products.

Gel preparation

The density of gel used for the electrophoresis of proteins is crucial, since protein mobility is proportionately dependent on their molecular weight. The proteins we wanted to study ranged from 40 kDa to 67 kDa; therefore we worked with 10% poly-acrylamide gel, also known as SDS-PAGE gel (for Sodium DodecylSulfate PolyAcrylamide Gel Electrophoresis). After the polymerization of separating gel, we added a second gel and shaped wells on it, for the loading of samples. The recipes for separating and stacking gels are shown on the following table:

Table 3. Separating and Stacking gel for protein electrophoresis

10% acrylamide gel (15 ml – for 2 gels)	**Stacking gel (5 ml – for 2 gels)**
• 5.9 ml ddH_2O	• 3.4 ml ddH_2O
• 5 ml acrylamide mix (30%)	• 0.83 ml acrylamide mix (30%)
• 3.8 ml Tris 1.5 M (pH 8.8)	• 0.63 ml Tris 1.0 M (pH 6.8)
• 0.15 ml SDS 10%	• 0.05 ml SDS 10%
• 0.15 ml ammonium persulfate 10%	• 0.05 ml ammonium persulfate 10%
• 0.006 ml TEMED	• 0.005 ml TEMED

Preparation and loading of samples

For the preparation of samples we used a loading buffer (Laemmli buffer) at a standard ratio of 1:4 and RIPA buffer (recipe given above), added to 50 – 100 μg of protein extract. Samples were boiled and loaded onto gel. Before loading of proteins, the gels were placed into a tank filled with Electrophoresis Buffer. Apart from the samples, a protein marker was loaded as well (Broad Range Marker, sc-2361), so that the correct protein band could be monitored.

Protein electrophoresis and transferring on membranes

Electrophoresis was carried out at 100 Volt, until the bands were properly separated. When the "migration front" of the dye molecule reached the bottom of the gel, we proceeded immediately at blotting of gels onto nitrocellulose membranes. The gel and membrane were sandwiched between sponge and paper as following:

sponge – paper – paper – gel – membrane – paper – paper – sponge

Sandwiches were placed into tank filled with ice cold Transfer Buffer, and transferring of the proteins was conducted at 100 Volt for 1 hour and 20 minutes at room temperature, or overnight at 35 Volt at 4^{o}C. After the completion of transferring, membranes were stained by use of Ponseau Red dye (SIGMA-7170) (after incubation for 5 minutes at room temperature on an agitator), in order to ensure that the proteins were successfully transferred onto nitrocellulose membranes. The recipes for the Electrophoresis Buffer (mentioned in the above paragraph) and the Transfer Buffer are shown on the following table:

Table 4. Electrophoresis and Tranfer buffers

Electrophoresis buffer (10x)	Transfer buffer
• 30.3 gr Tris	• 14.4 gr Glycin
• 144.1 gr Glycin	• 3.03 gr Tris
• 10 gr SDS	• 800 ml ddH_2O
Dissolve in 1 litre of ddH_2O for 10x stock. Use in 1:10 dilution in ddH_2O	• 200 ml Methanol

Immuno-staining of proteins with specific antibodies

Before using the specific antibodies in order to detect the desired protein bands, membranes were washed with TPBS (buffer containing PBS + 0.05% Tween-20 (SIGMA-P9416)), and incubated for 2 hours at room temperature in TPBS containing 5% non-fat milk powder, in order to block non-specific binding of antibodies. After blocking, a specific primary antibody was dissolved in fresh milk-TPBS buffer and incubated with agitation for 2 hours at room temperature, or overnight at 4^{o}C, depending on the antibody. In the present study we used anti-rabbit ALDH1/2 antibody (sc-50385, Santa Cruz) in dilution 1:500 and anti-mouse CAR (PP-N4111-00, Perseus Proteomics), anti-mouse PXR (PP-H4417-00, Perseus Proteomics) antibodies in dilutions 1:1000. Also, β-actin (SIGMA-A5316) and Lamin-B (C-20, sc-6216) were used as control proteins for normalizing loading of samples.

After incubation with primary antibodies, membranes were washed three times with TPBS buffer and the proper secondary antibody was added. Goat anti-rabbit (sc-2004) and goat anti-mouse (sc-2005) antibodies were used as secondary antibodies, diluted 1:5000. Membranes were incubated for 2 hours at room temperature with agitation, and then washed three times with TPBS.

Visualization of proteins

Secondary antibodies used above were HRP-conjugated, therefore immune detection of bands was based on the method of enhanced chemiluminescence, ECL. Development of the proteins was attained with a camera that captured digital images, or following the traditional method of X-ray film, using a developing agent (KODAK 190 0943) and a fixation agent (KODAK 190 1875).

Total RNA isolation

Total RNA isolation was performed by use of Nucleospin® RNA II - Total RNA isolation kit by Macherey-Nagel, following the appropriate protocol, according to the starting material.

RNA isolation from tissue:

Liver sections were removed after decapitation of animals and preserved in RNAlater® (Ambion - 7020) in -80ºC, until use. For the isolation, we proceeded on with the protocol below:

1) Frozen samples were left on ice into a sterile petri dish, and after thawed, they were chopped into small pieces with sterile blade or scissors and forceps treated with DEPC and sterilized. The following steps were performed by use of buffers and columns of the RNA isolation kit.
2) 350 μl of RA1 buffer was added in each sample, which was homogenized using homogenator for 1 minute at speed 8.
3) Samples were transferred into DEPC (for DiEthyl PyroCarbonate, SIGMA-D5758) treated tubes and 3.5 μl of β-mercapto-ethanol (SIGMA-M3148) was added in each sample, then vortexed vigorously.
4) Samples were loaded on columns with violet ring and centrifuged for 1 minute at 11000 x g.
5) The flow-through was kept and 350 μl of 70% ethanol was added.
6) Samples were vortexed and loaded on columns with blue ring, then centrifuged for 30 seconds at 11000 x g.
7) 350 μl of MBD was added and samples were centrifuged for 1 minute at 11000 x g.
8) 95 μl of DNase reaction mixture containing rDNase enzyme and was added in each sample and incubated at room temperature for 15 minutes.
9) 200 μl of RAW2 were added and samples were centrifuged for 30 seconds at 11000 x g.
10) 600 μl of RA3 were added and samples were centrifuged for 30 seconds at 11000 x g.
11) 250 μl of RA3 were added and samples were centrifuged for 2 minutes at 11000 x g.

12) 30-60 μl of H_2O were added in order to elute RNA, then samples were incubated at room temperature for 1 minute and then centrifuged for 1 minute at 11000 x g.
13) Samples were collected in eppendorf tubes, their concentration and purity were measured by use of Nanodrop 2000 (ThermoScientific), then stored at -80^{o}C.

In case of RNA isolation from primary hepatocytes, cells were collected from the culture and we proceeded immediately with the following protocol:

RNA isolation from cells:

1) Medium was removed and cells were washed twice with 5 ml of PBS.
2) 1 ml trypsin per plate was added, then the plates were placed for about 5 minutes in the incubator.
3) 5 ml of medium was added and the content of each dish was collected in falcon tube.
4) Samples were centrifuged for 5 minutes at 1500 rpm, at 4^{o}C, and then medium was removed.
5) 5 ml of PBS were added.
6) Samples were centrifuged for 5 minutes at 1500 rpm, at 4^{o}C, and then medium was removed. The following steps were performed by use of buffers and columns of the RNA isolation kit.
7) Cell pellets were re-suspended into 350 μl of RA1 buffer and transferred into eppendorf tubes.
8) 3.5 μl of β-mercapto-ethanol was added in each sample, and the samples were vortexed vigorously.
9) Steps **4-11** of the above-mentioned protocol were followed.
10) Samples were collected in eppendorf tubes, their concentration and purity were measured by use of Nanodrop, then stored at -80^{o}C.

cDNA synthesis

RNA samples were used as templates for cDNA synthesis, using Quanti-Tect® Reverse Transcription kit by Qiagen, based on the protocol described below in detail.

1. RNA templates were kept on ice, until thawed.
2. Genomic DNA elimination reaction was prepared on ice, according to Table 5.

Table 5. Genomic DNA elimination reaction components.

Component	Volume/reaction
gDNA Wipeout buffer, 7x	2 µl
Template RNA	Variable (up to 1 µg)
RNase-free water	Variable
Total volume	**14 µl**

3. Samples were incubated for 2 minutes at 42°C, then placed immediately on ice.
4. Reverse-transcription master mix was prepared on ice, according to Table 6.

Table 6. Reverse-transcription reaction components.

Component	Volume/reaction
Reverse-transcription master mix	
Quantiscript Reverse Transcriptase	1 µl
Quantiscript RT Buffer, 5x	4 µl
RT Primer Mix	1 µl
Entire genomic DNA elimination reaction (step 3)	14 µl (add at step 5)
Total volume	**14 µl**

5. Template RNA from step 3 (14 µl) was added to each tube containing reverse-transcription master mix.
6. Samples were incubated at 42ºC for 15 minutes.
7. Samples were then incubated at 95ºC for 3 minutes and Quantiscript Reverse Transcriptase was inactivated.
8. 105 µl of RNase-free water was added in each tube, and samples were aliquoted and stored at -80ºC.

Polymerase Chain Reaction (PCR)

With the Polymerase Chain Reaction a certain sequence of a template DNA is amplified, which is selected by use of specific primers that have complementary sequences with the target. Particular PCR protocols were conducted for various experiments, according to the template used and the desired product, which are described below. All reactions were performed by use of the CFX96™ Real-Time System and the C1000™ Thermal Cycler, by Biorad.

Quantitative real-time PCR

For studies of real-time gene transcription regulation, analysis of mRNA levels was performed with real-time RT-PCR, based on the TaqMan protocol, by use of KAPA PROBE FAST qPCR Master Mix (KAPA BIOSYSTEMS, KK4702) and TaqMan® Gene Expression Assay primers for *r*ALDH1A1, *r*ALDH1A7, *r*CAR, *r*PXR, *r*CYP2B1, *r*CYP3A23 and *r*b-actin genes. The reaction components and the cycling protocol are shown on the following tables:

Table 7. Reaction components for qRT-PCR

Reaction Components	Per 15 μl reaction
PCR grade water	1.75 μl
KAPA PROBE FAST qPCR Master Mix (2x)	7.5 μl
TaqMan® Gene Expression Assay primer	0.75 μl
Template cDNA	5 μl

Table 8. Cycling conditions for qRT-PCR

Step	Temperature	Duration	Cycles
Enzyme activation	95ºC	3 min	Hold
Denature	95ºC	3 sec	40
Anneal/Extend/Acquire	60ºC	30 sec	

In order to study the binding potential of nuclear receptors on template DNA generated from Chromatin Immuno-Precipitation experiments, we followed the above protocol with some differentiations. In this case, the KAPA SYBR® FAST qPCR Master Mix (KAPABIOSYSTEMS, KK4601) was used, together with specific primers that amplified certain regions of a given sequence, specifically the ALDH1A7 promoter from **RR** and **rr** rats. Primers were designed with the **NetPrimer** (www.premierbiosoft.com/netprimer) and the **BLAST Primer designing tool** (www.ncbi.nlm.nih.gov/tools/primer-blast/). The list of primers is presented in the section of ChIP assays experiments, whereas the reaction components and the cycling protocol are shown on the following tables:

Table 9. Reaction components for qRT-PCR for ChIP

Reaction Components	Per 10 μl reaction
PCR grade water	3.3 μl
KAPA SYBR® FAST qPCR Master Mix (2x)	5 μl
Primer mix (forward+reverse) 5 μM	0.7 μl
Template DNA	1 μl

Table 10. Cycling conditions for qRT-PCR for ChIP

Step	Temperature	Duration	Cycles
Enzyme activation	95ºC	3 min	Hold
Denature	95ºC	3 sec	35
Anneal/Extend/Acquire	60ºC	30 sec	

- Quantification has been done by use of standard curves.

Common PCR for amplification of selected fragments of ALDH1A7 promoter

Various fragments of ALDH1A7 promoter region, 450 bp to 2700 bp long, were selected for amplification, using Phusion High-Fidelity DNA Polymerase (Finnzymes, F-350S) or KAPA HiFi (Hot start) DNA Polymerase (KAPABIOSYSTEMS, KK 2102). OLIGO-6 software was available at the University of Eastern Finland, by use of which we designed specific primers amplifying selected regions on the ALDH1A7 promoter, producing 5' and 3' ends with cutting sites for enzymes that were afterwards used in order to clone deletion fragments into plasmid vectors. Below are presented the reaction components and the cycling protocol, as well as the list of primers using for the amplification of fragments:

Table 11. Reaction components for common PCR reaction

Reaction Components	Per 25 µl reaction
PCR grade water	14.75 µl
5x KAPA HiFi Fidelity	5 µl
KAPA dNTP Mix (10 mM each dNTP)	0.75 µl
Primer mix (forward+reverse) 10 µM	1.5 µl
Template DNA (10-50 ng)	2.5 µl
KAPA HiFi DNA Polymerase (1U/µl)	0.5 µl

Table 12. Cycling conditions for common PCR reaction

Step	Temperature	Duration	Cycles
Initial denaturation	95ºC	3 min	1
Denaturation	98ºC	20 sec	35
Primer annealing	Optimal Ta* (60ºC)	15 sec	
Extension	72ºC	30 sec/kb**	
Final extension	72ºC	5 min	1
Cooling	4ºC	Hold	1

* In some cases, a gradient PCR was a necessary step, in order to decide the appropriate annealing temperature. For a gradient PCR, samples were loaded in a vertical position in the PCR machine, and the annealing temperature of each well from A to H was set at a different value. The temperature range is shown below:

Well	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**
Ta	72.0ºC	70.9ºC	68.6ºC	64.6ºC	59.8ºC	55.9ºC	53.3ºC	52.0ºC

**Extension times for the fragments:

Fragment **4** (~2700 bp)	90 sec
Fragments **5** (~1550 bp) &**6** (~1950 bp)	60 sec
Fragment **7** (~450 bp)	15 sec

Table 13. Set of Primers used for the amplification of fragments

Deletion Fragment	**Forward Primer**
Fragment **4 (RR sequence)**	5'-ACC ACG CGT TGT ATT TTA TG-3'
Fragment **4 (rr sequence)**	5'-AAG ACG CGT AGA ACT AAC AGA-3'
Fragment **5 (RR and rr)**	5'-GTC ACG CGT CTC TCT ATC AA-3'
Fragment **6 (RR and rr)**	5'-CTC TCA CGC GTC ACT TAT AA-3'
Fragment **7 (RR and rr)**	5'-AAA ACG CGT ACT CTG ATC TG-3'

Reverse Primer (used for all fragments above)
5'-TGT GTC TCG AGT TCT GAA AG-3'

Colony PCR from liquid starter culture

The deletion fragments described above were cloned into plasmid vectors, and bacteria cells were transformed with the vectors. Then, the transformed bacteria were used for reporter gene assays. One way to spot transformed bacteria colonies was to look for a deletion fragment sequence into bacterial DNA, by using the DreamTaq DNA Polymerase (ThermoScientific, EP0701) and following the Colony PCR protocol. The reaction components, the primers used and the cycling protocol are shown on the following tables:

Table 14. Reaction components for Colony PCR

Reaction Components	Per 15 µl reaction
Starter culture (DNA template)	1 µl (or 10 ng of template)
10X Taq Buffer	1.5 µl
10 mM dNTPs	0.3 µl
10 µM primer mix	0.3 µl
DreamTaq DNA Polymerase (0.3U)	0.06 µl
Milli-Q Water	11.84 µl

Table 15. Cycling conditions for Colony PCR

Step	Temperature	Duration	Cycles
Enzyme activation	94ºC	2 min	1
Denaturation	94ºC	30 sec	35
Annealing	55ºC	30 sec	
Extension	72ºC	1 min/kb*	
Final extension	72ºC	5 min	1

Table 16. * Extension times for the fragments

Fragment **4** (~2700 bp)	3 min
Fragments **5** (~1550 bp) &**6** (~1950 bp)	2 min
Fragment **7** (~450 bp)	30 sec

Table 17.Set of Primers used for the amplification of fragments

Forward	**Reverse**
pGL3-Basic forward **(1F)**	RR7/rr7 reverse **(1R)**
RR7/rr7 forward **(2F)**	pGL3-Basic reverse **(2R)**

The abbreviations in bold are also depicted in the following drawing. Both pairs of primers (red and pink pairs) were used, for the determination of positive colonies, after colony PCR and electrophoresis of the PCR products on agarose gel.

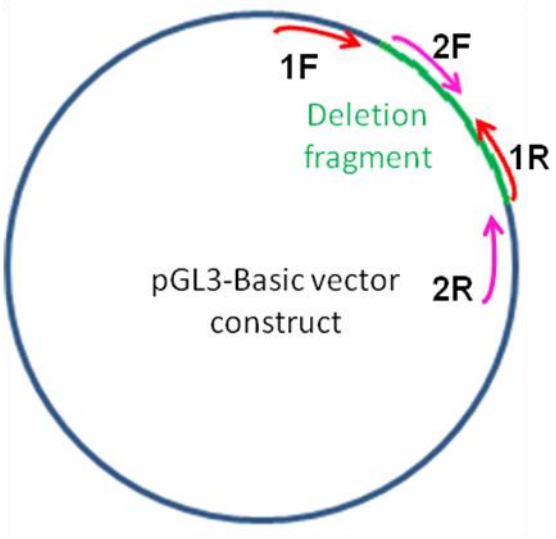


Figure 2. Schematic view of a deletion fragment inserted into plasmid vector, and the pairs of primers used for identification of insertion (positive colony).

Genomic DNA (gDNA) isolation of RR and rr rat strains

A small sample of tissue (about 25 mg of liver from male rats, 3 months old) was removed under sterile conditions, for isolation of genomic DNA. The sample processing was performed according to the protocol of NucleoSpin ® Tissue kit by Macherey Nagel. Particularly, the steps followed are described below:

Step 1: Rat was decapitated and the abdominal area was cleaned with ethanol solution (75%) and opened with sterile forceps and scissors.

Step 2: 25 mg of liver tissue was removed, placed on a sterile petri dish, and chopped by use of sterile scissors and forceps

Step 3: Pieces were placed in an eppendorf tube, then 180 μl of T1 buffer and 25 μl of Proteinase K were added, and the tubes were vortexed until samples were mixed and fully covered with lysis solution.

Step 4: Samples were incubated in a water bath, at 56^{o}C for 1 to 3 hours, until complete lysis was obtained. In the meanwhile, tubes were tapped or mildly vortexed every 10 – 15 minutes.

Step 5: Afterwards, 200 μl of B3 were added in each tube, and samples were further incubated in a water bath, at 70^{o}C for 10 minutes.

Step 6: DNA binding conditions were adjusted, by addition of 210 μl of 96 – 100% ethanol per tube.

Step 7: The whole material of each tube was loaded into a column containing a silica membrane.

Step 8: Samples were centrifuged at 11000 x g for 1 minute.

Step 9: Silica membrane was washed with 500 μl of BW added in each column.

Step 10: Samples were centrifuged at 11000 x g for 1 minute.

Step 11: Silica membrane was washed with 600 μl of B5 added in each column.

Step 12: Samples were centrifuged at 11000 x g for 1 minute.

Step 13: Samples were further centrifuged at 11000 x g for 1 minute, in order to dry the silica membrane from the residual ethanol.

Step 14: 100 μl of BE (pre-heated at 70ºC) were added in each column and the samples were incubated for 1 minute at room temperature.

Step 15: Samples were centrifuged at 11000 x g for 1 minute, and highly pure DNA was eluted.

Step 16: Qualitative and quantitative measurement of samples was performed using a NanoDrop® 2000.

Step 17: DNA quality was further checked by electrophoresis of the samples in 1% agarose gel, in order to check whether it was intact or broken.

Electrophoresis of DNA on agarose gel

Agarose gels (0.8 – 1 %) were used for electrophoresis of DNA, either to check the quality of sample (after gDNA isolation) and/or the size of chromatin (after sonication of cells during Chromatin ImmunoPrecipitation) or the correct size of fragments (after digestion with restriction enzymes, in order to verify successful cloning of promoter fragments into plasmid vectors).

For the preparation of gels, we dissolved agarose (0.8 – 1% of final volume) into TAE buffer (1x) into a microwave oven, then let the mixture cool down at 40 – 45ºC and added 5% Ethidium Bromide, stirred well and placed the mixture in a specific plate, until it was polymerized and turned into a gel.

The plate holding the gel was placed into a tank that contained TAE buffer (1x). On the following, samples were prepared, by addition of 1 part of loading dye: 4 parts of DNA, and the samples were loaded into gel wells, together with a DNA marker (Fermentas, SM-0241). Electrophoresis was attained at 100 Volt and gels were observed under Ultra Violet light, using a MiniBIS Pro Camera.

Reporter gene assays

Chemicals

The human CAR agonist 6-(4-chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime (CITCO) was from BioMol Int (Plymouth Meeting, PA). The synthesis of the mouse CAR agonist 1,4-bis[(3,5-dichloropyridyloxy)]benzene (TCPOBOP) has been described [Honkakoski et al., 1996]. The human and mouse PXR agonists rifampicin (RIF) and mifepristone (RU486) were from Sigma-Aldrich (St. Louis, MO). All other chemicals were at least of analytical grade from major vendors.

ALDH1A7 promoter constructs

The ALDH1A7 promoters (10 kbp; 240,591,833 – 240,601,833) were amplified from genomic DNA isolated from both **RR** and **rr** rat strains (as described in section above), sequenced by standard DNA sequencing analysis(Epoch Life Science Inc., Missouri, USA), and inserted into pGL3-Basic plasmid vectors (Promega, Madison, WI) (Fig. 3A-3C).

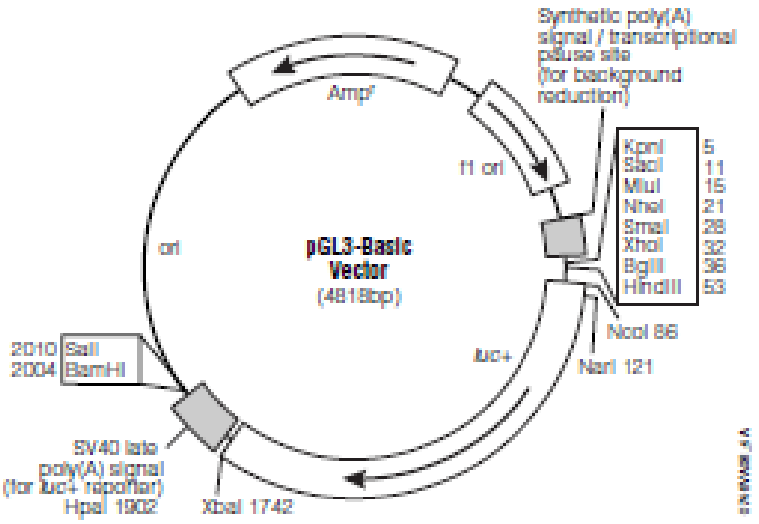


Figure 3A. pGL3-Basic plasmid vector map.

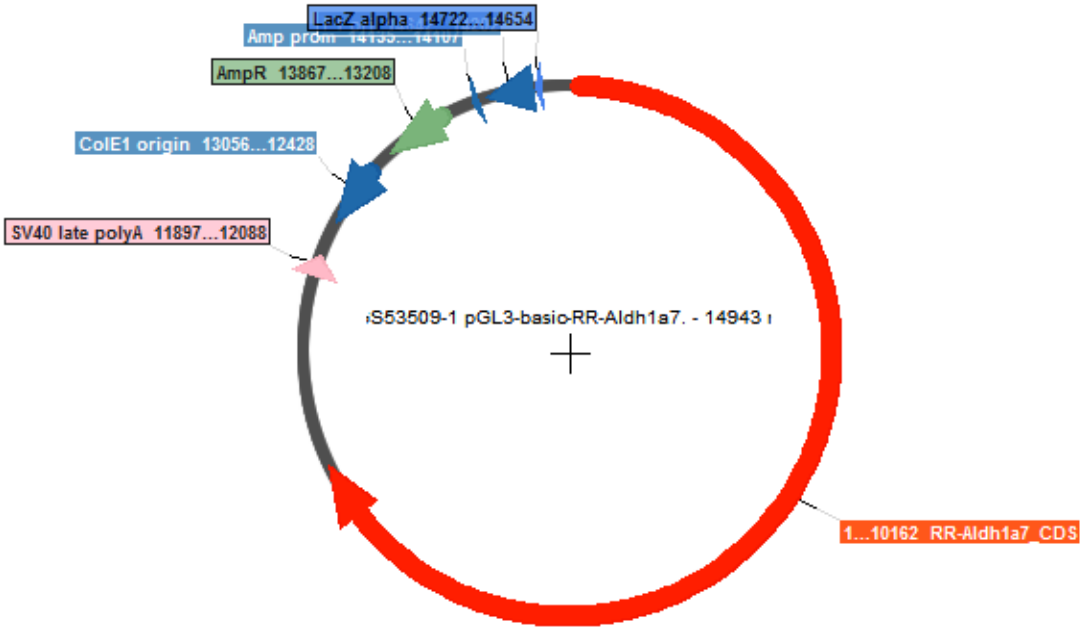


Figure 3B. Visualization of 10 kbp **RR**-ALDH1A7 promoter sequence cloned into pGL3-Basic plasmid vector.

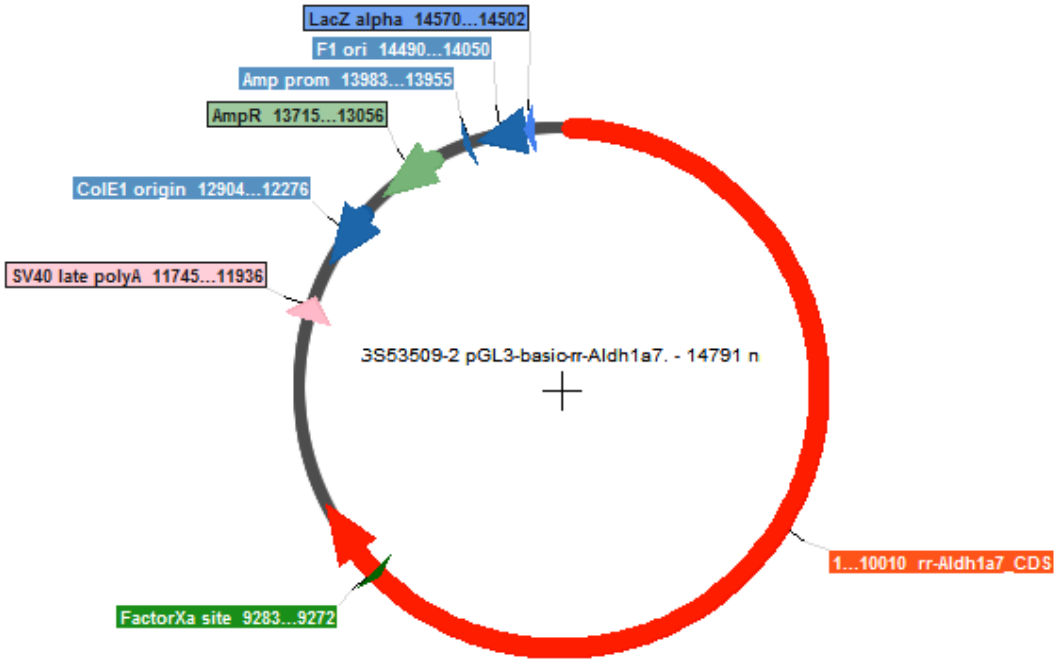


Figure 3C. Visualization of 10 kbp **rr**-ALDH1A7 promoter sequence cloned into pGL3-Basic plasmid vector.

Sequence alignment and comparison of ALDH1A7 promoter regions from **RR** and **rr** strain were done with NCBI BLAST (http://blast.ncbi.nlm.nih.gov), intimating the differences between the sequences. We confined our study to the region from -2700 bps to +1 (TSS). 4 sets of primers were designed and used for cloning of the deletion fragments into pGL3-Basic vectors. The amplicons were cloned into the MSC site of the vector after digestion with restriction enzymes (RE). The appropriate RE should be chosen among the long catalog of enzymes that recognize specific sites, either within the vector, or the selected fragment, without destroying the sequence of any of them. For that reason, SerialCloner® and OLIGO-6 softwares were used, in order to select preferential REs and design the desired primers, respectively. The primers were oligos of 20-25 nucleotides, consisted of a part which was complementary either to the sense or anti-sense promoter sequence (about 10 nucleotides), an RE recognition cut site (6 nucleotides), and 4-6 nucleotides on the 5' end, which were essential for efficient cut by restriction enzymes. In all of the fragments, the 5' ends were comprised of a recognition cut site for MluI (forward primers used), while on their 3' ends the corresponding cut site was recognized by XhoI (reverse primers used) (Fig. 4).

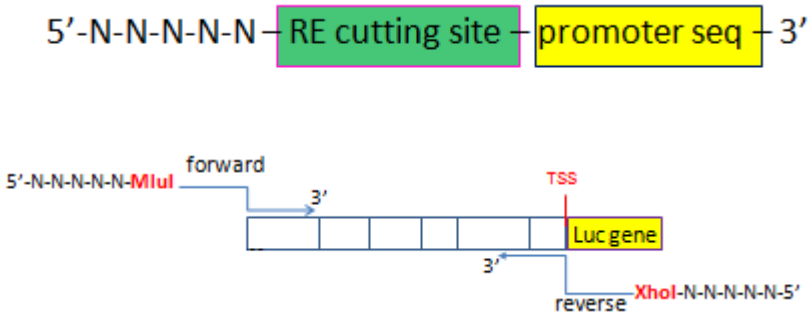


Figure 4. Visualization of the primers (upper sketch) used for cloning of the deletion fragments upstream of the luciferase gene, into pGL3-Basic plasmid vector (lower sketch).

Figure 5 is a schematic presentation of the deletion fragments designed for **RR** promoter at positions -2667, -1954, -1566, -452 upstream from TSS. The respective fragments were designed for **rr** as well (at -1550, -437), and all of the fragments were subcloned into XhoI site of MCS (Multiple Cloning Site) of the luciferase reporter vector pGL3-Basic.

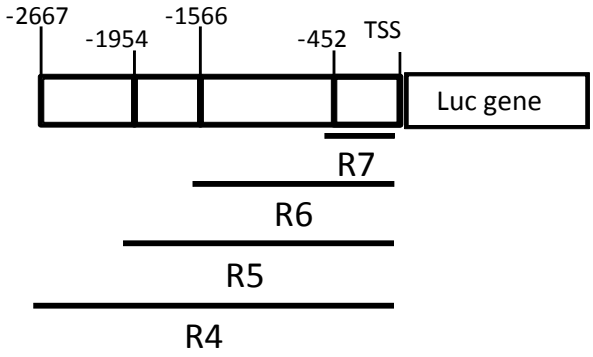


Figure 5. Fragments of **RR**-ALDH1A7 cloned into pGL3-Basic plasmid vector.

After amplification, the PCR products were purified using the E.Z.N.A. Cycle-Pure Spin kit (OMEGA bio-tek, D-6492). Subcloning was accomplished by digestion of the amplicons with restriction enzymes MluI (ThermoScientific, ER0561) and XhoI (ThermoScientific, ER0691) producing 5' and 3' overhangs.pGL3-Basic vectors were also digested with the above enzymes and ligation reactions were carried out by use of T4 DNA Ligase (ThermoScientific, EL0014). Thus, deletion fragments were inserted into the vector (Fig. 6).

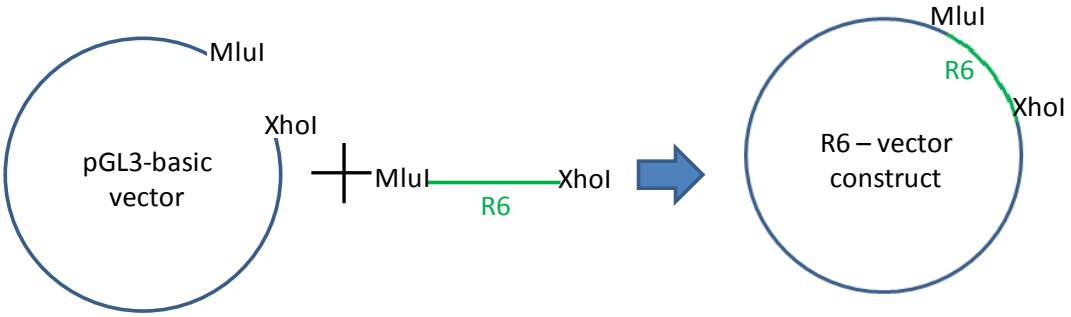


Figure 6. Schematic presentation of the cloning of deletion fragment RR6 into pGL3-Basic plasmid vector.

Competent *E. coli* bacteria (XL-10 or DH5α) were transformed with the constructs or empty vehicles and plated colonies were selected for isolation and purification of plasmid DNA, by use of Qiagen buffers P1, P2, P3(19051, 19052,

19053). The samples were further double-digested with MluI and SalI (ThermoScientific, ER0641), and pDNA from colonies that were possibly hosts of a deletion construct were further purified with QIAprep® Spin Miniprep kit (QIAGEN, 27104) and verified by dideoxy sequencing (DNA Analysis Facility, Yale University, USA).

Bacteria colonies that were transformed with the deletion constructs were also detected by performing colony PCR, as described in the corresponding section (see fig. 2). The products were electrophorized in agarose gel and positive colonies were selected for ultra-purification and sequencing, as above.

NR expression vectors and their reporters

The full-length human and mouse CAR and PXR constructs have been described [Mäkinen et al., 2002]. The reporters for the full-length CAR (PBREM-tk-luc) and full-length PXR (XREM-3A4-luc) have been described in references [Honkakoski et al., 2001; Goodwin et al., 1999], respectively.

Co-transfection and reporter assays

C3A hepatoma cells were seeded on 48-well plates (about 180,000 cells/cm^2) and co-transfected with either empty NR expression vector, full-length CAR or PXR construct (100 ng/well), plus either individual ALDH1A7 promoter construct, control pGL3-Basic plasmid, human PBREM-tk-luc or human XREM-3A4-luc reporter, respectively (450 ng/well) and the transfection control plasmid pCMVβ (900 ng/well) using the calcium phosphate method essentially as described [Küblbeck et al., 2008]. After transfection for 4 hours, the medium was removed and fresh DMEM supplemented with 5% delipidated serum (HyClone, Logan, UT) instead of fetal bovine serum and including either vehicle control (DMSO, 0.1%) or NR ligands (1 μM CITCO for human CAR; 1 μM TCPOBOP for mouse CAR; 10 μM RIF for human PXR; 10 μM RU486 for mouse PXR) were added to wells. After exposure of cells to chemicals for 24 hours, they were lysed and assayed for luciferase and β-galactosidase activities as before [Honkakoski et al., 2001].

Chromatin Immunoprecipitation (ChIP) assay

Our studies on the binding capacity of nuclear receptors were based on the Chromatin Immuno-Precipitation protocol, which is divided in several parts:

Part 1: Cross-linking reaction

- Male rats were treated with a single injection of saline (control animals) or PB (80 mg/kg, i.p. in saline) and sacrificed with decapitation 3 hours later.
- Liver tissue was subtracted from the animal under sterile conditions and sliced into ice cold sterile PBS (on a petri dish placed on ice).
- Liver sections were fixed in 10 ml Cross-linking Solution Buffer for 15 minutes at room temperature with agitation.
- Cross-linking reaction was quenched by addition of 0.125M glycine (1/10 of reaction volume) and incubation with agitation for 5 minutes at room temperature.
- Samples were centrifuged for 5 minutes at 1500 rpm, at 4^{o}C.
- Supernatant of each sample was aspirated and pellet was rinsed with ice cold PBS+Protease Inhibitors (P.I.). Centrifugation of samples for 5 minutes at 1500 rpm, at 4^{o}C. Repetition of this step one time.
- The supernatants were aspirated and the pellets were then homogenized in sucrose buffer (Buffer I)+P.I. and filtered by use of 0.7 μm filters (BD Falcon 352350).
- Centrifugation of samples for 5 minutes at 1500 rpm, at 4^{o}C.

Part 2: Cell lysis and sonication

- The pelleted homogenates were resuspended in 1% SDS Cell Lysis Buffer+P.I. and incubated on ice for 10 min. Samples were stirred every 3 minutes for a few seconds during this time.
- Centrifugation of samples for 5 minutes at 1500 rpm, at 4^{o}C.
- Lysates were resuspended in 2 ml of Sonication Buffer+P.I.
- Sonication of samples until DNA was sheared to an average length of 300–800 base pairs. Samples were checked with electrophoresis on agarose gel.
- Centrifugation of samples for 15 minutes at 13000 rpm, at 4^{o}C.

Part 3: Immuno-precipitation of chromatin

- Supernatants were diluted with ChIP Dilution Buffer +P.I. (buffer volume was proportional to the number of chip reactions).
- Samples were pre-cleared with addition of 60 μl per reaction of Protein A/G PLUS-Agarose beads (Santa Cruz, sc-2003) and incubated with rotation at 4°C for 1 hour.
- Beads were removed with centrifugation of samples for 5 minutes at 13000 rpm, at 4°C.
- 1/10 of the genomic DNA was retained as input control for qPCR analysis (for normalization of primer efficiency), and kept at -20°C until use.
- Pre-cleared chromatin samples were incubated at 4°C overnight, with the addition 2 μg of polII antibody (sc-899, X-TransCruz N-20) and 2 μg of RXRα antibody (sc-553, D-20) or without antibody, in order to be used as negative control.

Part 4: Elution of chromatin and reverse cross-linking

- 60 μl of agarose beads were added in each reaction tube, and the chromatin-DNA complexes were precipitated after 3h of incubation with rotation, at 4°C.
- Centrifugation of samples for 20 seconds at 12000 rpm, at 4°C.
- Pellets were washed with 1ml of Low Salt Buffer+P.I. and incubated on ice for 10 minutes.
- Centrifugation of samples for 20 seconds at 12000 rpm, at 4°C.
- Pellets were washed with 1ml of High Salt Buffer +P.I. and incubated on ice for 10 minutes.
- Centrifugation of samples for 20 seconds at 12000 rpm, at 4°C.
- Pellets were washed with 1ml of LiCl Wash Buffer +P.I. and incubated on ice for 10 minutes.
- Centrifugation of samples for 20 seconds at 12000 rpm, at 4°C.
- Pellets were washed with 1ml of TE Buffer +P.I. and incubated on ice for 10 minutes.
- The last two steps were repeated once.
- Centrifugation of samples for 20 seconds at 12000 rpm, at 4°C.

- Re-suspension of pellets with 100 μl of Elution Buffer. Samples were mixed well and incubated with agitation for 15 minutes at 37ºC or for 30 minutes at room temperature.
- Centrifugation of samples for 5 minutes at 1000 rpm, at room temperature.
- Supernatants were collected in new eppendorf tubes, and the remaining pellets were re-suspended with 100 μl of Elution Buffer. Samples were mixed well and incubated with agitation for 15 minutes at 37ºC or for 30 minutes at room temperature.
- Centrifugation of samples for 5 minutes at 1000 rpm, at room temperature.
- Collection of second eluted supernatant in the same tube for each sample (200 μl of eluted sample per chip reaction).
- Inputs were left to melt at room temperature and 200 μl of Elution Buffer were also added in each sample.
- 8 μl of 5M NaCl were added in each reaction and samples were incubated at 65ºC overnight.

Part 5: DNA extraction

- Preparation of a Master Mix containing EDTA/Tris/Proteinase K and addition of 13 μl in each reaction tube.
- Incubation of samples for 2 hours at 55ºC.
- 200 μl of phenol–chloroform–isoamyl alcohol extraction were added per reaction tube and samples were vortexed vigorously for 15 seconds.
- Centrifugation of samples for 5 minutes at 13000 rpm, at room temperature.
- The liquid phase of each sample was subtracted and transferred into a clean eppendorf tube. This material was further purified with addition of 200 μl of phenol–chloroform–isoamyl alcohol extraction. Samples were vortexed vigorously for 15 seconds.
- Centrifugation of samples for 5 minutes at 13000 rpm, at room temperature.
- Again, the liquid phase of each sample was subtracted and transferred into a clean eppendorf tube. 20 μl (or 1/10 of sample volume) of 3M Sodium Acetate were added and samples were mildly vortexed.
- 600 μl (or 3 times the volume of sample) of Ethanol Absolute were added and samples were mildly vortexed.

- DNA precipitation was achieved with incubation of samples at -20^{o}C overnight, or at -80^{o}C for 30 minutes.
- Centrifugation of samples for 30 minutes at 13000 rpm, at 4^{o}C.
- Supernatants were removed and 200 μl of 75% Ethanol were added in each tube.
- Centrifugation of samples for 5 minutes at 13000 rpm, at 4^{o}C.
- Supernatants were completely removed and pellets were left to dry on air for maximum 10 minutes.
- The extracts were diluted in 60-100 μl of sterile water and incubated for 10-15 minutes at 55^{o}C.

Part 5: DNA extraction

- Amplification of samples with the qPCR method, as described in the corresponding section. Samples were tested in triplicates, and the results were statistically processed with Excel or SPSS software.

Bioinformatics analysis of putative nuclear receptor (NR) and transcription factor (TF) binding sites were done with NUBISCAN (www.nubiscan.unibas.ch/) and MatInspector (www.genomatix.de) programs, in order to spot the regions with putative NR binding sites. The algorithms used for designing the specific PCR primers has been described in previous section, and primers sequences are shown on the table below (synthesized by VBC-Biotech, Vienna, Austria or Eurofins Genomics):

Table 18. Sets of Primers used for amplification of immuno-precipitated DNA

Name of site	*Primers' sequence*
ALDH1A7 proximal promoter	Forward primer: 5'-CATTTAAAGGCAAAGGCTCCC-3' Reverse primer: 5'-GCACTTGCTCCTTTTTATCTGCT -3'
CAR1	Forward primer: 5'-TGTGTTGACCTTCATAAAAAGTTCT-3' Reverse primer (RR): 5'-TTCAAGACCTACCCTTACAGCC-3' Reverse primer (rr): 5'-TCTGTGATTTACCCATGACAATAAT-3'
CAR2	Forward primer (RR): 5'-AAAGAGTTCACACACACACAAATG-3' Reverse primer (RR): 5'-CCTACCTCTTGTGAACATTTTTTT-3' Forward primer (rr): 5'-ACTTCACACACACACACACAAATG-3' Reverse primer (rr): 5'-CCTACCTCTTGTTGAACATTTCTTT-3'
CAR3	Forward primer: 5'-TTCTCACCTCATTCTCTACAAGT-3' Reverse primer: 5'-GCAGGCATTGTGGTCAGTCA-3'
Actin (proximal promoter)	Forward primer: 5'-GGTGACCCCCAGAATACAGG-3' Reverse primer: 5'-GAAGAGTTTGGCGATGGGTG -3'
PBREM positive	Forward primer: 5'-CGTGGACACAACCTTCAAG-3'* Reverse primer: 5'-GAGCAAGGTCCTGGTGTC-3'*

* Primers for PBREM are selective for the -2320/-2197 bp region of the rat CYP2B2 gene, as reported by Pustylnyak et al. (2011).

The recipes for all of the buffers used in the above protocol (underlined) are given below:

Table 19. Buffers for the Cross-linking reaction (Part 1)

Cross-linking Solution Buffer (for 10 ml)	**Buffer I** (for 50 ml stock)
270 µl formaldehyde 37%	5.125 gr sucrose
500 µl Hepes-KOH 1M pH 7.5	1 ml KCl 3M
200 µl NaCl 5M	150 µl NaCl 1M
20 µl EDTA 0.5M	250 µl $MgCl_2$ 1M
15 µl EGTA 0.5M	10 µl EGTA 0.5M
8.995 ml milli-Q H_2O	750 µl Tris 1M
	30 µl DTT 0.83M
	50 µl PMSF 0.1M
	47.76 ml milli-Q H_2O

Table 20. Buffers for Cell Lysis, Sonication and Immunoprecipitation (Parts 2&3)

Cell Lysis Buffer (for 5 ml)	**Sonication Buffer#** (for 2 ml)	**Chip Dilution Buffer#** (for 20 ml stock)
500 µl SDS 10%	100 µl Hepes 1M	2.2 ml Triton-X 10%
100 µl EDTA 0.5M	56 µl NaCl 5M	48 µl EDTA 0.5M
250 µl Tris 1M pH 8	4 µl EDTA 0.5M	334 µl Tris 1M pH 8
4.15 ml milli-Q H_2O	200 µl Triton-X 10%	668 µl NaCl 5M
	56 µl NaCl 5M	20 µl SDS 10%
	0.002 gr Na-Deoxycholate	16.75 ml milli-Q H_2O
	20 µl SDS 10% (Final con. 0.1%)*	
	1.62 ml milli-Q H_2O	

*Final SDS concentration ranged from 0.1-1%, and was determined based on the sensitivity of antibody.

Table 21. Buffers for Elution of chromatin

Low Salt Buffer# (for 10 ml stock)	**High Salt Buffer#** (for 10 ml stock)	**LiCl Wash Buffer#** (for 10 ml stock)
100 µl SDS 10%	100 µl SDS 10%	62.5 µl LiCl 4M
1 ml Triton-X 10%	1 ml Triton-X 10%	1 ml NP-40 10%
40 µl EDTA 0.5M	40 µl EDTA 0.5M	0.1 gr Na-Deoxycholate
200 µl Tris 1M pH 8	200 µl Tris 1M pH 8	20 µl EDTA 0.5M
300 µl NaCl 5M	1 ml NaCl 5M	100 µl Tris 1M pH 8
8.36 ml milli-Q H_2O	7.66 ml milli-Q H_2O	8.8175 ml milli-Q H_2O

Table 22. Buffers for Elution of chromatin and DNA extraction

TE Buffer# (for 25 ml stock)	**Elution Buffer** (for 1 ml)	**Master Mix** (for 13 µl)
250 µl Tris 1M pH 8	800 µl TE Buffer (w/o P.I.)	4 µl EDTA 0.5M
50 µl EDTA 0.5M	100 µl SDS 10%	8 µl Tris 1M pH 6.5
24.7 ml milli-Q H_2O	100 µl Na-Bicarbonate 1M	1µl Proteinase K

#In all buffers noted with the symbol, 1 µl of Protease Inhibitors (P.I.) per ml of buffer were added. The Protease Inhibitors used were: a mix of Aprotinin and Leupeptin (Protease Inhibitor Coctail, SIGMA, P2714) and PMSF (SIGMA – P7626).

Measurement of ALDH1As activity after administration of PB or PCN

Three-months-old **RR** and **rr** male rats were treated with three (3) doses of phenobarbital – PB (80mg/kg bw; ip, in saline) or four (4) doses of 16α-pregnenolone carbonitrile – PCN (50mg/kg, ip, in olive oil) and sacrificed with decapitation 6 hours after last injection. Liver tissue sections were homogenized in cold sucrose and centrifuged at 20,000xg for 30 minutes. Supernatants were collected and used for the measurement of enzymatic activity, according to the protocol based on the oxidation of NAD (β-Nicotinamide Adenine Dinucleotide). Particularly, 850 μl of ice cold cocktail mix containing0.1 M Na-pyrophosphate buffer (pH 8), 20 mM Pyrasole and 20 mM NAD was added in 50 μl of sample and tubes were incubated at room temperature for 5 minutes. After addition of 100 μl of 100 mM Propionaldehyde, the velocity of enzyme activation was measured in a Shimadzu UV1601 spectrophotometer at 340 nm and 37°C, under specific kinetics parameters of the reaction.

Protein concentration measurement was carried out according to the Lowry method. The following buffers were prepared before measurement and mixed together immediately before they were used:

Buffer A: 500 ml 2% Na_2CO_3 and 0.1 M NaOH
Buffer B: 20 ml 1% $CuSO_{4\cdot5}H_2O$
Buffer C: 20 ml 2% Sodium Tartrate ($Na_2C_4H_4O_6$)
Buffer D: Mix Buffer B and Buffer C prior to use

Step 1: 1 ml of Buffer E was added in 200 μl of sample. Buffer E was prepared immediately before use and consisted of 50 ml of Buffer A and 1 ml of Buffer D.
Step 2: Samples were incubated at room temperature for 10 minutes.
Step 3: Addition of 100 μl of Follin pigment (diluted 1:1 into H_2O).
Step 4: Samples were incubated at room temperature for 30 minutes.
Step 5: Samples were measured at 550 nm, within 2 hours after incubation.

Estimation of the enzymatic potency of ALDH1As was based on the above measurements.

Statistical analysis

Data where expressed as means ± SD and significant differences were studied by use of Student's *t*-test. The significance level was considered as a probability of less than or equal to 0.05.

RESULTS

Multiple – dose treatment with specific agonists of CAR and PXR nuclear receptors.

The results demonstrate low levels of detection of ALDH1/2 in the cytoplasm of both rat strains. Phenobarbital treatment exhibited a great induction of protein levels in **RR** rats, whereas lower induction was observed in **rr** rats. Similar expression pattern was observed after treatment with PCN (Figure 1). No significant change in expression levels of CAR and PXR was observed, but the receptors were accumulated into nucleus after administration of PB (Figure 1).Administration of Clotrimazole (selective agonist of CAR receptor) led to similar inductive effects, alike to PB treatment, though to a lesser extent (data not shown).

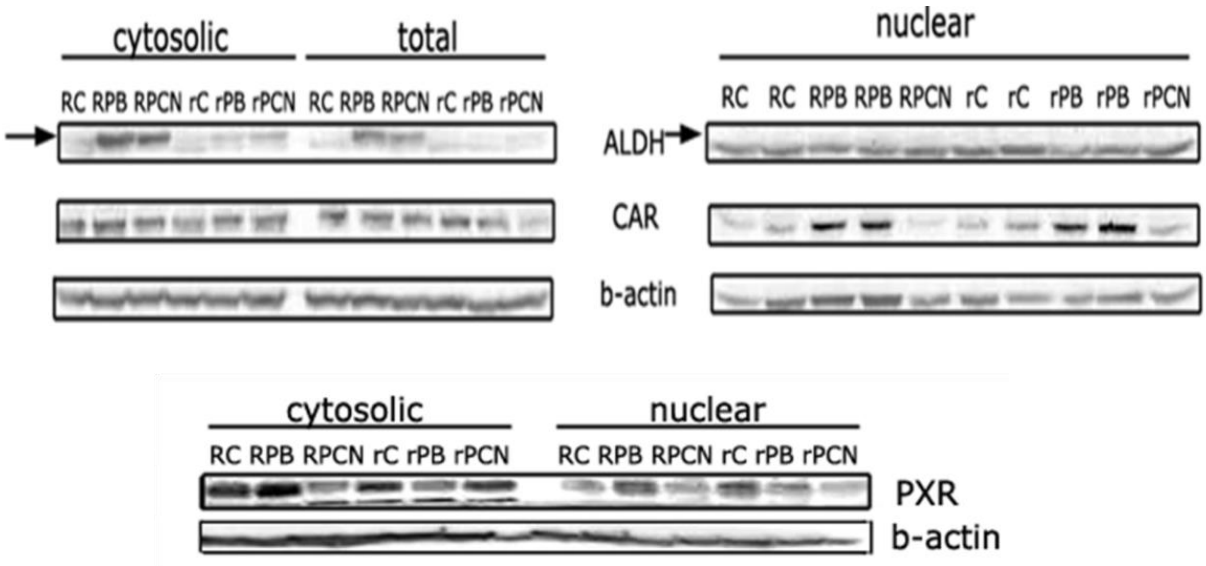


Figure 1. Western blotting of ALDH1/2, CAR and PXR proteins in cytosolic, nuclear and total liver extracts from **RR** and **rr** rats. β-actin was used for the normalization of samples.

Expression patterns of the above samples were further studied, based on the real-time qPCR method. The results were in accordance to the findings from immunodetection experiments. By use of primers that amplify rALDH1A1 and rALDH1A7 distinctively (and not as a subfamily ALDH1/2 like the antibody used in WB) we were able to study the mRNA expression of the two genes in control state and after administration of the specific inducers (Figures 2A-2D).

Particularly interesting results were revealed from the study of ALDH1A7. Gene expression was completely absent from **rr** rats, while it was expressed in **RR** rats, and upregulated after PB or PCN treatment. In particular, 4 doses of PCN led to 20-fold mRNA levels of the enzyme, and 3 doses of PB raised expression levels to 100 times higher, compared to control (Figure 2A). On the other hand, ALDH1A1 was present in both rat strains, and mRNA levels were significantly elevated after administration of PB, as well as with PCN(Figure 2B).

However, nuclear receptors CAR and PXR showed a similar expression pattern in **RR** and **rr** rats, with no discernible effect after administration of the agonists, apart from a minor decrease of CAR mRNA levels with PCN treatment. (Figure 2C and 2D).

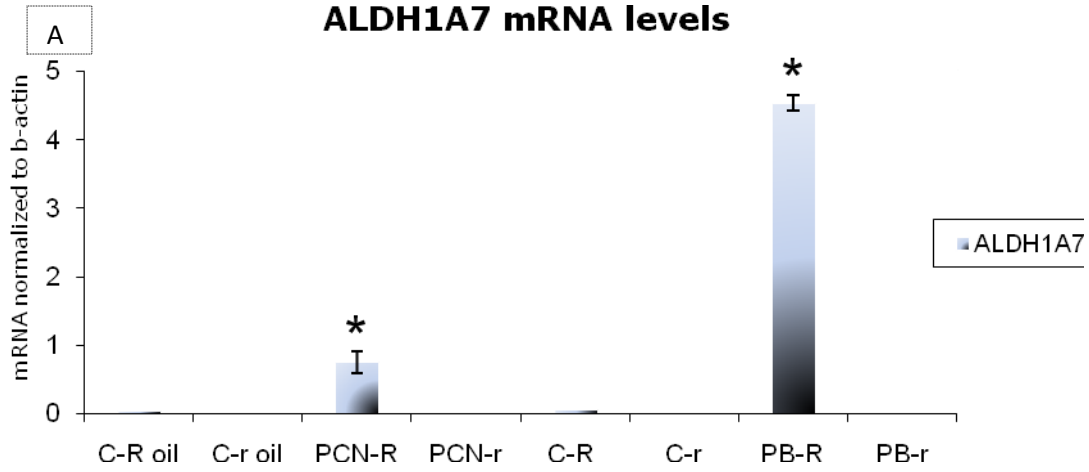


Figure 2A. ALDH1A7 mRNA expression in **RR** and **rr** rats, in control state and after administration of PB or PCN.* control vs treated samples cells, $p<0.05$.

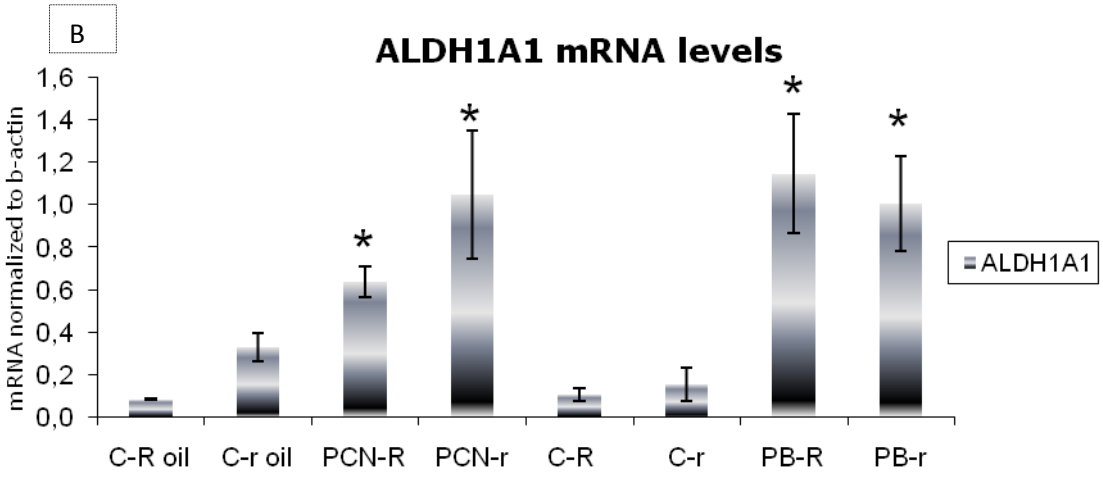


Figure 2B. ALDH1A1 mRNA expression in **RR** and **rr** rats, in control state and after administration of PB or PCN.* control vs treated samples cells, $p<0.05$.

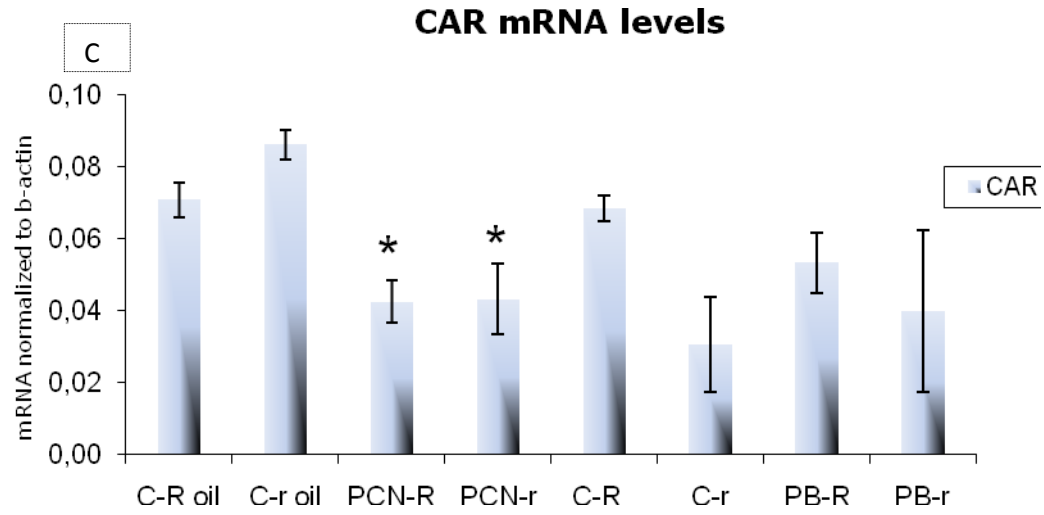


Figure 2C. CAR mRNA expression in **RR** and **rr** rats, in control state and after administration of PB or PCN.* control vs treated samples cells, $p<0.05$.

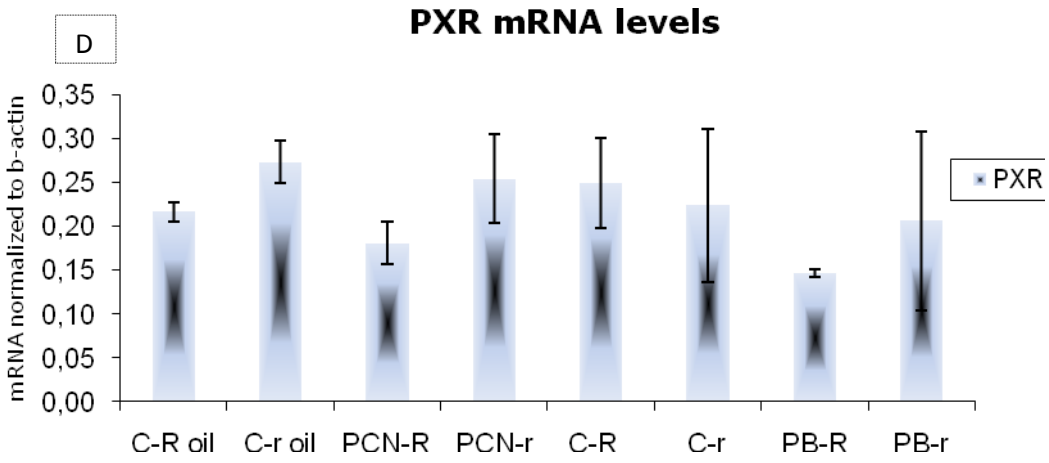


Figure 2D. PXR mRNA expression in **RR** and **rr** rats, in control state and after administration of PB or PCN.* control vs treated samples cells, $p<0.05$.

Time – response effects of single – dose treatment with PB or PCN

Together with the dose – dependent impact of agonists on ALDH1A7, ALDH1A1, CAR and PXR, we further studied the effects of a single dose treatment with PB or PCN, in order to study the time – dependent induction of the enzymes and transcription factors in **RR** and **rr** rats. The effect of drug was studied at 1, 3, 6, 12 and 24 hours after administration of PB, or 6, 12 and 24 hours after injection with PCN.

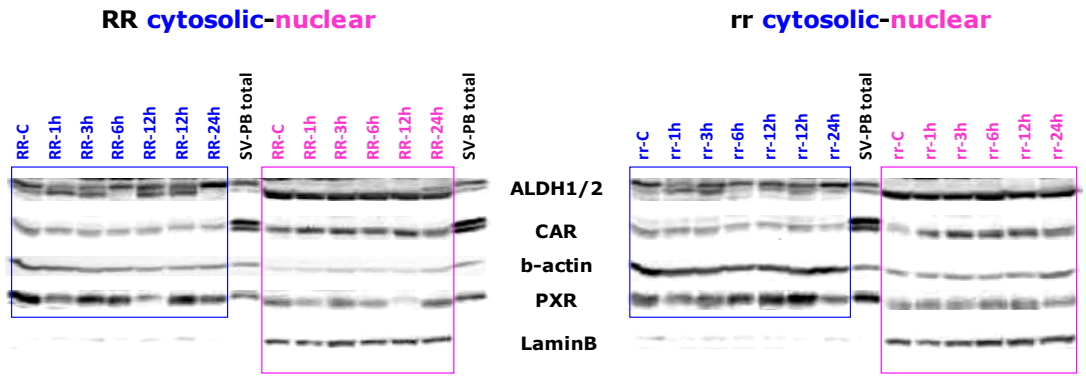


Figure 3. ALDH1/2, CAR and PXR protein expression in cytosolic and nuclear liver extracts from **RR** and **rr** rats, 1,3,6,12 and 24 hours after PB administration. β-actin and lamin-B were used for the normalization of samples. 'SV-PB total' represents a sample from SV mice treated with PB, that were used as controls.

Based on the results presented in figure 3, ALDH1/2 expression was depended on the time period that PB acts in the animal. As shown in the dose – dependent study, ALDH1/2 was detected in the cytoplasm of both strains in control rats, and PB administration triggered the expression of the genes in a time – dependent way. ALDH1/2 expression in total protein extracts was higher than the controls already at 12 hours in **RR** rats, whereas in **rr** rats notable difference was observed after 24 hours. Comparison of ALDH1/2 expression in total extracts between the two strains showed higher levels in **RR** rats, as expected (Figure 4).

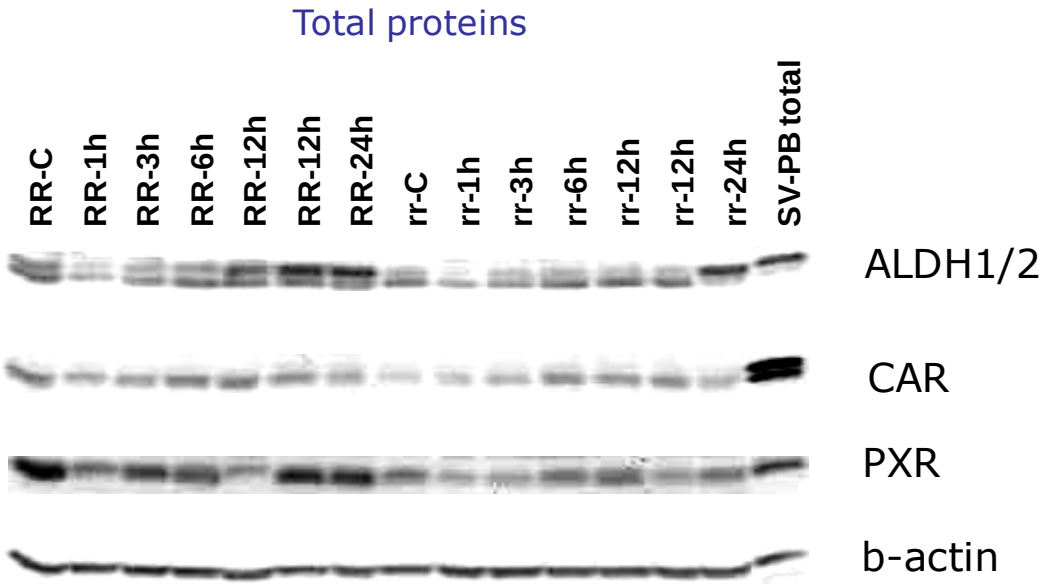


Figure 4. ALDH1/2, CAR and PXR protein expression in total liver extracts from **RR** and **rr** rats, 1, 3, 6, 12 and 24 hours after PB administration. β-actin and lamin-B were used for the normalization of samples. 'SV-PB total' represents a sample from SV mice treated with PB, that were used as controls.

Regarding the expression of nuclear receptors after administration of PB, protein levels of CAR were decreased in the cytoplasm, while raised in the nucleus in quite short time (already after 1 hour of administration). This indicated the translocation of the receptor in presence of the selective agonist (Fig. 3). However, cytosolic CAR levels were only slightly decreased (less than expected), due to up-regulation of CAR expression 6 hours after treatment, which was further supported by the study of total proteins (Fig. 4). PXR expression was up-regulated in both sub-cellular compartments, especially after 6 hours of PB administration, although PCN constitutes a specific agonist for rPXR. Indeed, PXR accumulated into nucleus 6 hours after injection of rats with PCN (50 mg/kg). On the contrary, a single – dose treatment proved to be insufficient to led to translocation of CAR, thus enhancing the selectivity of PCN as an inducer (Fig. 5). Noteworthy, low levels of ALDH1/2 were detected only after 24 hours of administration, and only in **RR** rats, whereas at 6 and 12 hours after treatment PCN demonstrated a rather negative effect

on ALDH expression. Coordinately, higher PXR levels were noticed at that time point (Fig.5).

Results from time- and dose-dependent studies suggest that Phenobarbital is a strong mediator of ALDH induction, via mobilization of nuclear receptors, while Pregnenolone-16α Carbonitrile shows similar, though milder, involvement in regulation of these genes.

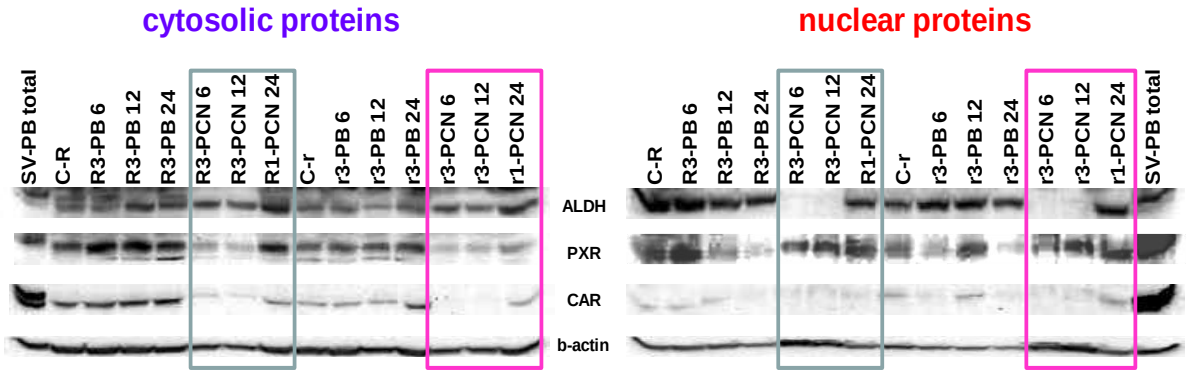


Figure 5. ALDH1/2, PXR and CAR protein expression in cytosolic and nuclear liver extracts from **RR** and **rr** rats, 6, 12 and 24 hours after PCN administration. β-actin was used for the normalization of samples. 'SV-PB total' represents a sample from SV mice treated with PB, that were used as controls.

Time – response effects of a single – dose treatment on mRNA expression levels of ALDH1A1, ALDH1A7, CAR and PXR genes were analyzed with qRT-PCR (administration of PB or PCN for 6, 12 and 24 hours).

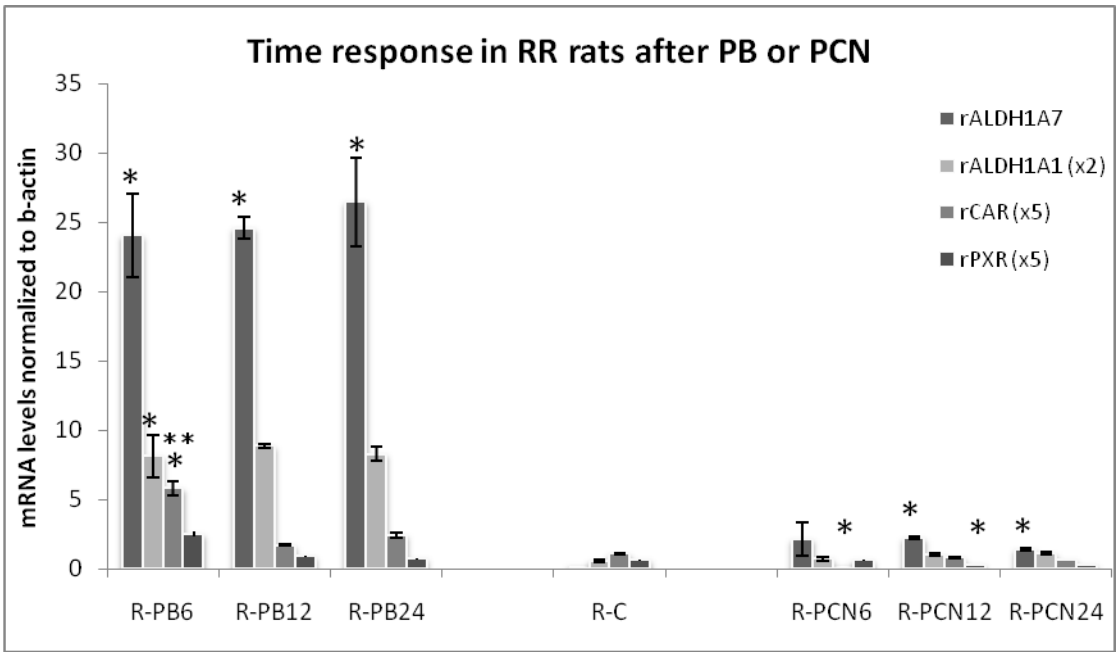


Figure 6. ALDH1A7, ALDH1A1, CAR and PXR mRNA expression in **RR** rats after PB or PCN administration for 6, 12 and 24 hours.* control vs treated samples cells, $p<0.05$.

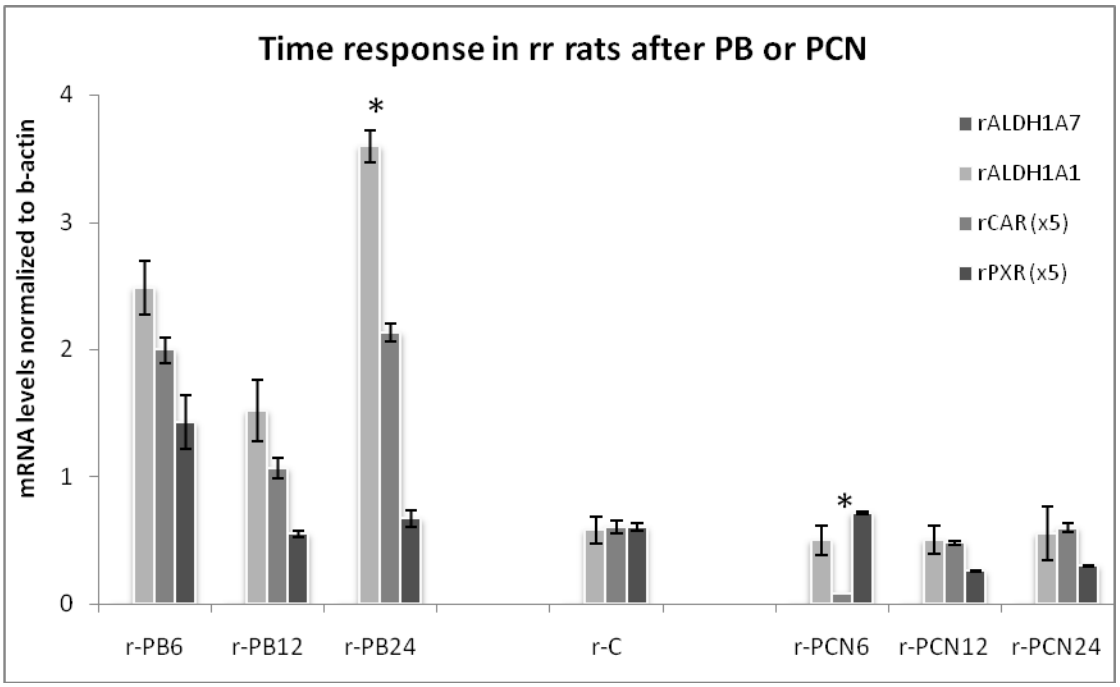


Figure 7. ALDH1A7, ALDH1A1, CAR and PXR mRNA expression in **rr** rats after PB or PCN administration for 6, 12 and 24 hours.* control vs treated samples cells, $p<0.05$.

The results showed expression of ALDH1A7 exclusively in **RR**, which was induced shortly after PB administration, since only 6 hours later mRNA levels were 100 times higher than in control rats and remained at high levels even after 24 hours of treatment. PCN treatment demonstrated lower levels of induction of ALDH1A7 after 12-24 hours of administration, while ALDH1A1 levels significantly increased only after administration of PB for 6 hours in **RR** and 24 hours in **rr** strain. No significant change was observed in mRNA levels of the nuclear receptors (Fig. 6 & 7).

Study of time- and dose- effects of PB treatment.

We further compared the protein expression of ALDH1/2, CAR and PXR in rats treated with one (1), two (2) or three (3) doses of PB for 6 hours and one (1) or two (2) doses for 24 hours.

In **RR** rats, cytosolic levels of ALDH1/2 in untreated rats were compared to alike samples from rats treated with 1, 2 or 3 doses of PB for 6 hours. It was shown that a single PB dose was not sufficient to raise expression levels of ALDH1/2, whereas significant up-regulation of the genes was observed after two doses. Furthermore, 3 PB doses led to even higher levels, which suggest that PB up-regulates ALDH1/2 in a dose-dependent way. This finding was further supported by the comparison between 1 or 2 doses of PB for 24 hours, were dose – response effect was also observed (Fig. 8). Same results were shown with total protein extracts (Fig. 9).

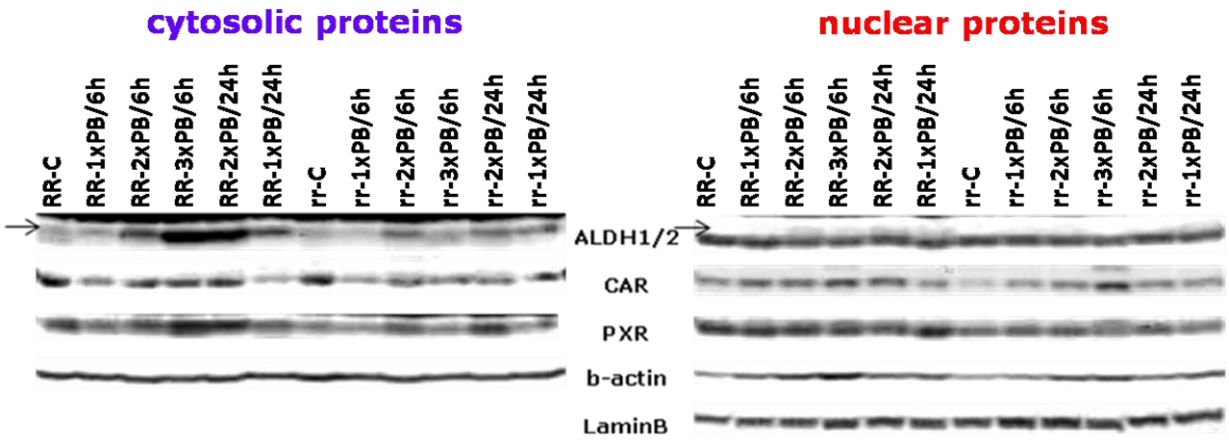


Figure 8. Time- and dose – dependent effect of PB on ALDH1/2, CAR and PXR protein expression in cytosolic and nuclear extracts from **RR** and **rr** rats. PB was administered in 1, 2 or 3 doses for 6 or 24 hours.

Figure 9. Time- and dose – dependent effect of PB on ALDH1/2, CAR and PXR protein expression in total extracts from **RR** and **rr** rats. PB was administered in 1, 2 or 3 doses for 6 or 24 hours.

Results presented in Fig. 8 and 9 also include time – dependent effects of PB on ALDH1/2 expression. With a single PB injection for 6 or 24 hours, protein levels were remarkably elevated during time. Same expression pattern was observed with 2 PB doses for 6 or 24 hours (Fig. 8 and 9).

CAR translocation was also dose- and time-dependent, indicating a relationship between the receptor's presence into nucleus and ALDHs' induction in cytosolic samples in all time and dose-cases studies mentioned above (Fig. 8).

Same expression patterns of ALDH and CAR were detected in samples from **rr** rats, although cytosolic and nuclear protein levels were lower, compared to those from **RR** rats (Fig. 8). These differences are depicted in Fig. 9 as well, in total protein extracts.

Confirmation of translocation of CAR and PXR into nucleus and involvement in gene regulation.

By use of specific antibodies for CAR and PXR, the quantitative and qualitative study of the proteins showed that the receptors translocate into nucleus, especially after administration of agonists. These results prompted us to verify the nuclear localization of the receptors and their participation in metabolic pathways, such as expression regulation of drug metabolizing genes in liver. The study was carried out by measuring mRNA levels of two target genes; CYP2B1 for CAR receptor and CYP3A1 (CYP3A23 in rats) for PXR receptor. For this purpose, samples from the above-mentioned experiments for time- and dose-response effects of PB or PCN were used.

A rise of CAR mRNA levels was detected already at 6 hours after PB administration. At the same time, CYP2B1 levels also reach remarkably high levels, which remain constant even 24 hours after injection with the drug. Interestingly, CAR levels decreased beyond the 6-hour treatment, which probably suggests that up-regulation of CAR gene occurred at an early stage, after which the receptor accumulated into nucleus and formed heterodimer with RXR. Binding of the complex on regulatory elements of CYP2B1 promoter led to up-regulation of the target gene. The results demonstrate same expression pattern between the two rat strains, though in **rr** samples mRNA levels were lower. PCN is not a selective agonist for CAR receptor, thus no alterations were observed in CAR or CYP2B1 levels (Fig. 10A and 10B).

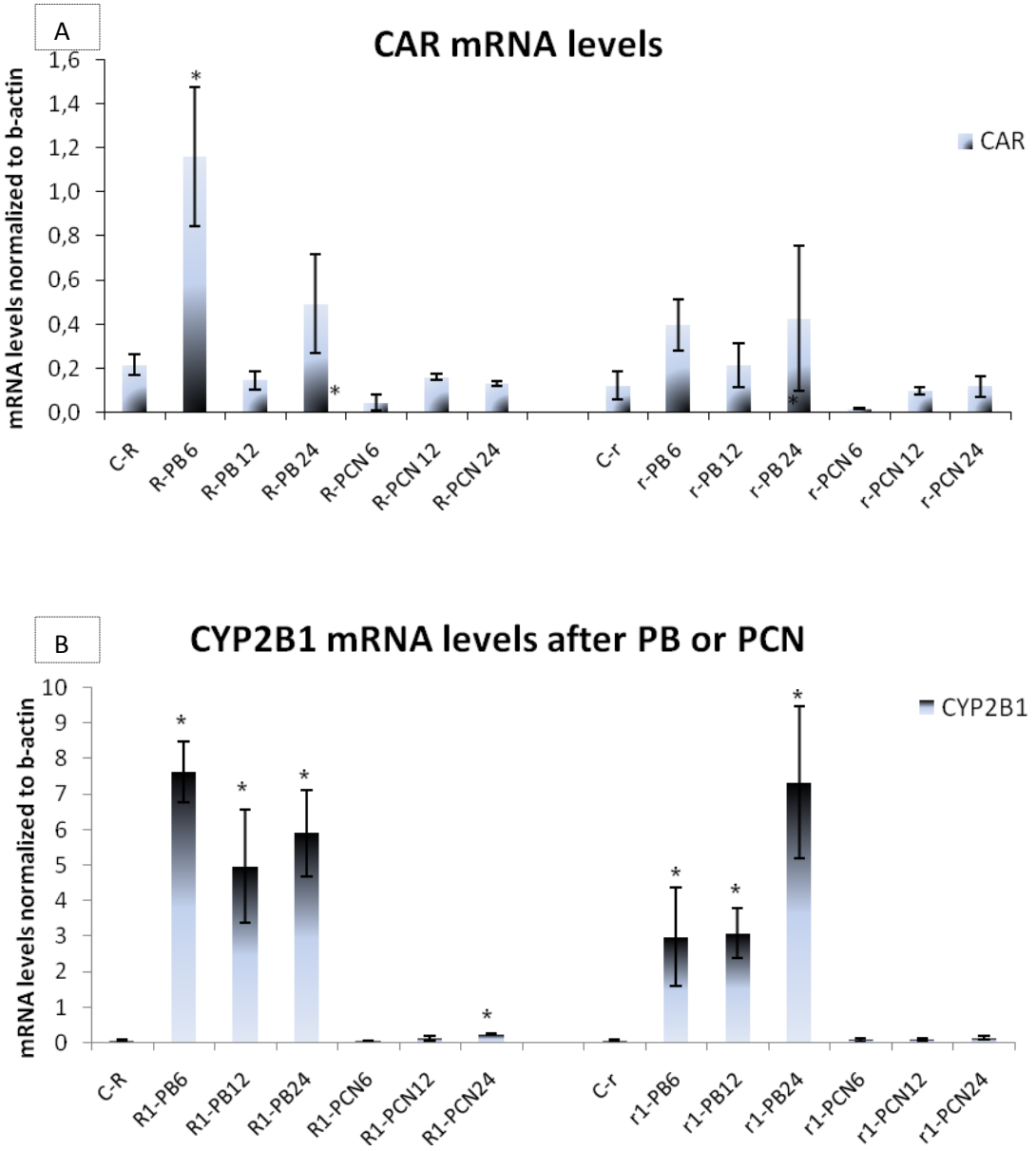


Figure 10A and 10B. Modifications in mRNA levels of CAR and its target-gene CYP2B1, after a single-dose treatment with PB or PCN for 6, 12 or 24 hours. * control vs treated samples cells, $p<0.05$.

Fig. 10A and 10C demonstrate the mRNA levels of CAR or PXR next to the respective levels of their gene-targets (Fig. 10B and 10D) on the time-response course, as measured in the same samples.

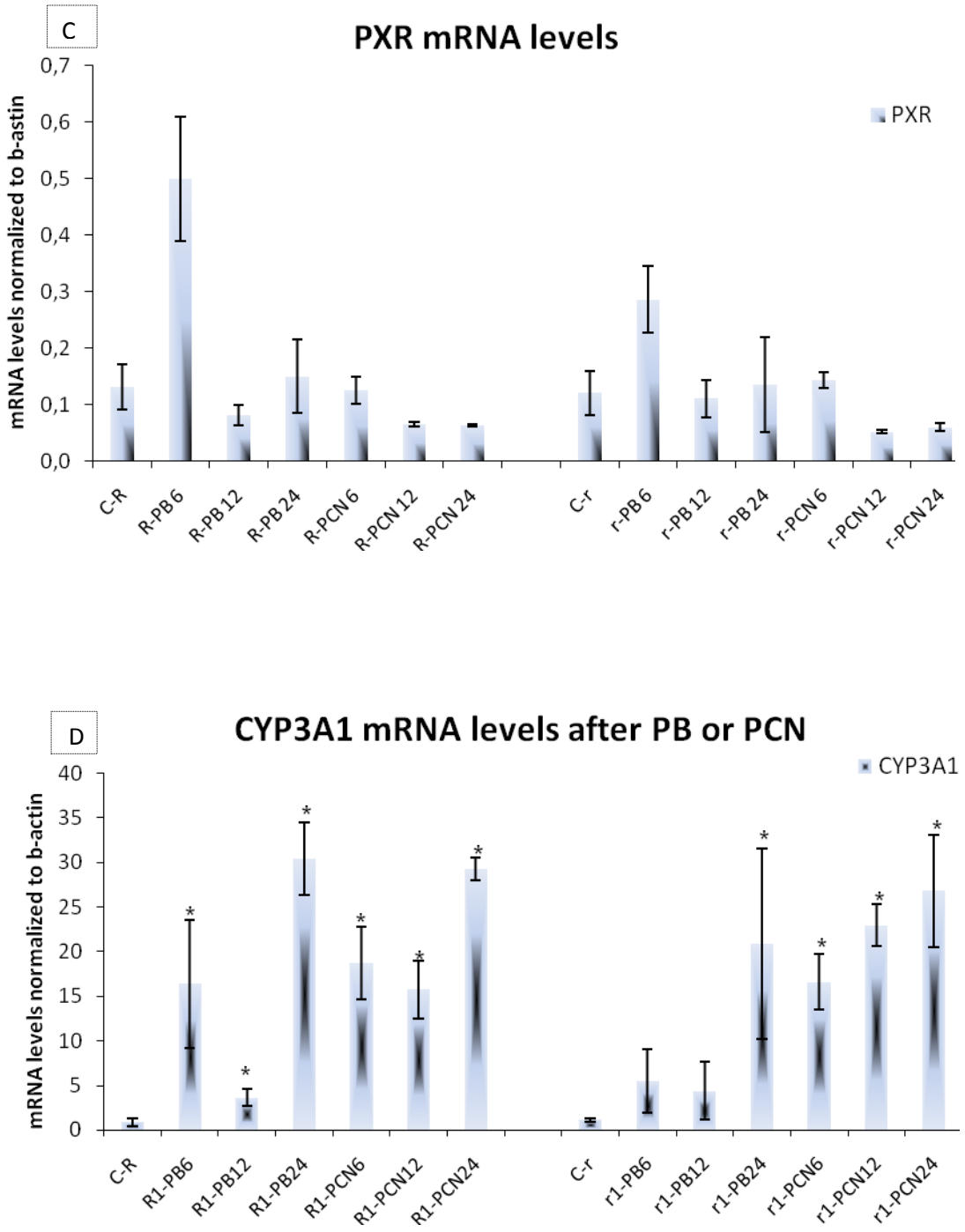


Figure 10C and 10D. Modifications in mRNA levels of PXR and its target-gene CYP3A1, after a single-dose treatment with PB or PCN for 6, 12 or 24 hours. * control vs treated samples cells, $p<0.05$.

Despite the fact that PCN acts as selective agonist for PXR, when administered in a single dose could not activate the receptor's expression (in samples from either **RR** or

rr rats). Nevertheless, our aim was to confirm the induction of CYP3A1 gene, which levels were greatly raised due to translocation of PXR into nucleus, already after 6 hours of treatment. Constantly high levels of CYP3A1, even 24 hours after injection, intimate presence of the receptor in the nuclear compartment and formation of PXR-RXR heterodimers that bound to transcription elements on the promoter of CYP3A1, and consequently up-regulated the target gene (Fig. 10C and 10D). Noteworthy is the up-regulation of CYP3A1 via PXR after administration of PB (especially after 24 hours), since PB is not considered a selective agonist for PXR, however there is a broad range of factors that can be activated, directly or indirectly, by PB.

Study of CYP2B1 and CYP3A1 expression in samples derived from rats treated with the dose-dependent experimental protocol (3 doses of PB and 4 doses of PCN), showed that no alteration in mRNA levels of the receptors' was exhibited after administration of their selective agonists; yet translocation of CAR and PXR led to induction of their target genes. Moreover, no discernible difference in transcriptional levels of both CYP2B1 and CYP3A1 genes was observed between **RR** and **rr** rats (Fig. 11 A-D).

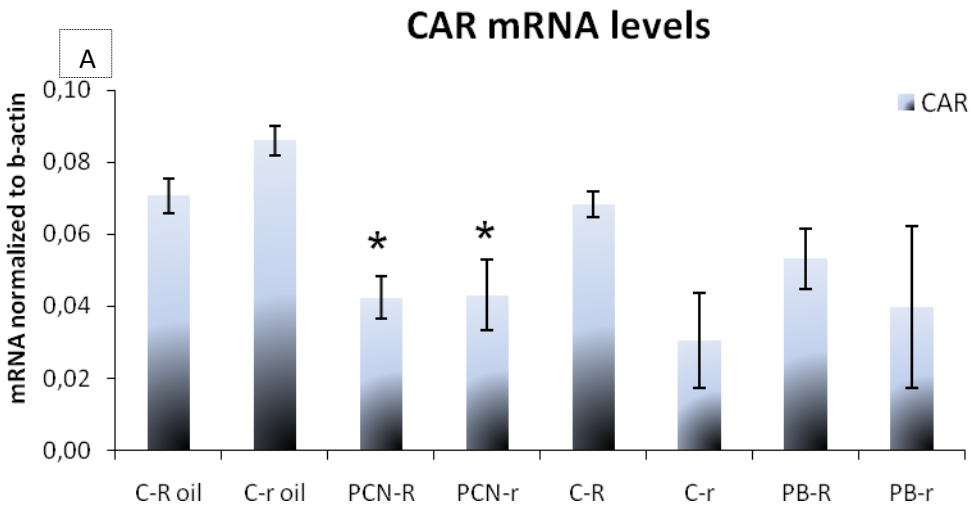


Figure 11A. Modifications in mRNA levels of CAR after multiple dose treatment with selective agonists (3 PB doses or 4 PCN doses). * control vs treated samples cells, $p<0.05$.

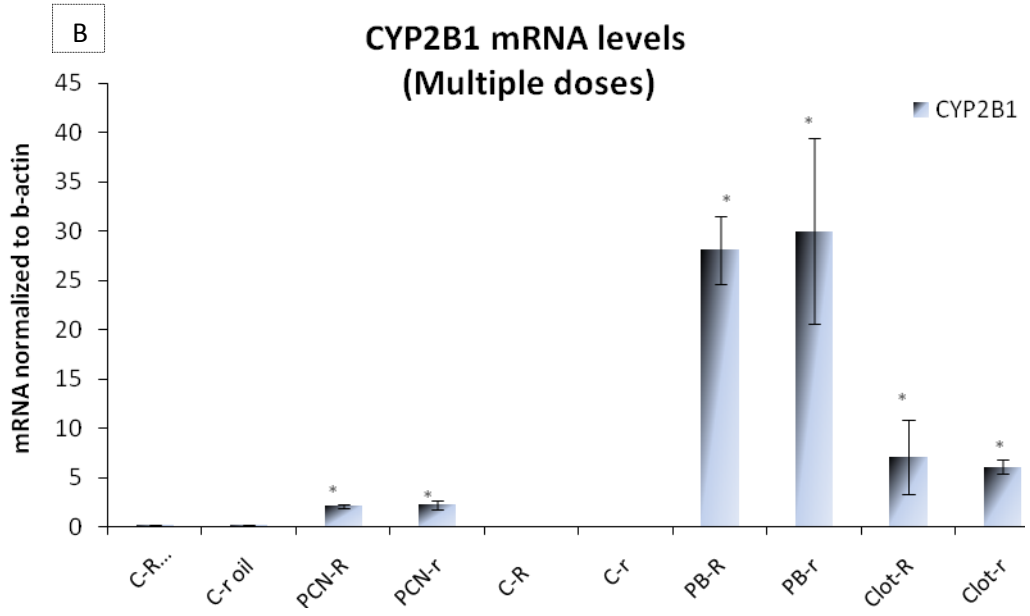


Figure 11B. Modifications in mRNA levels of CAR's target-gene CYP2B1, after multiple dose treatment with selective agonists (3 PB doses or 4 PCN doses). * control vs treated samples cells, $p<0.05$.

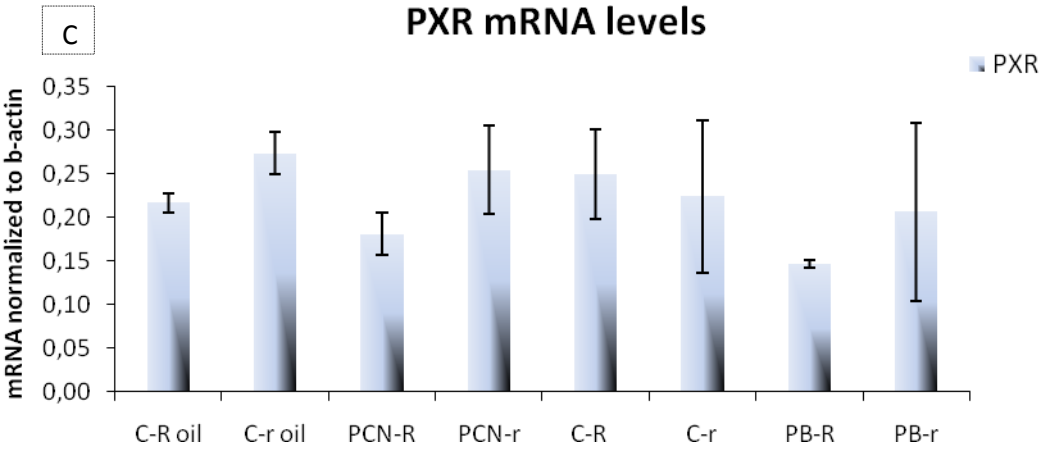


Figure 11C. Modifications in mRNA levels of PXR after multiple dose treatment with selective agonists (3 PB doses or 4 PCN doses). * control vs treated samples cells, $p<0.05$.

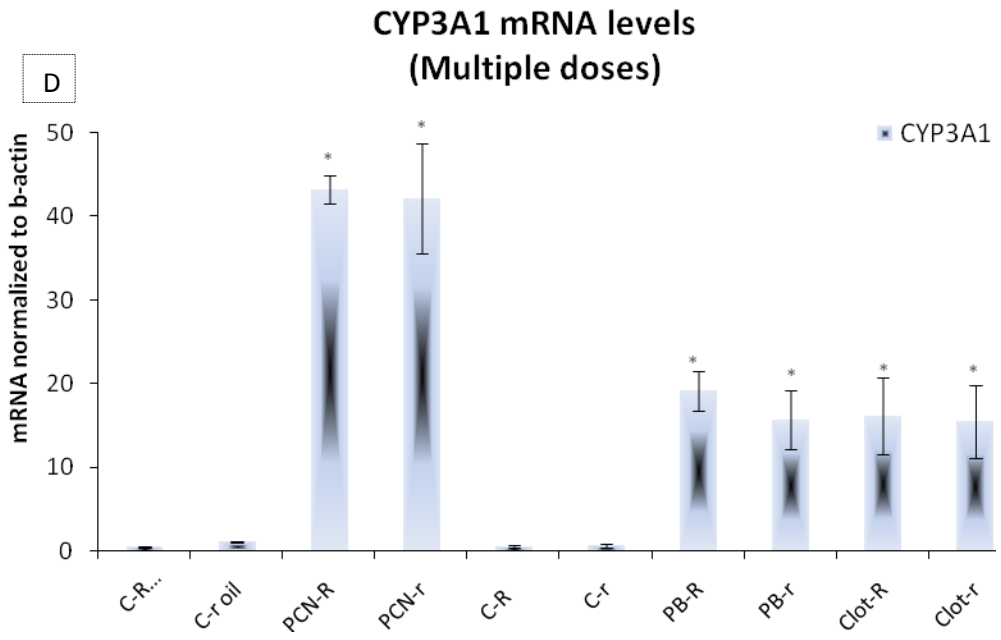


Figure 11D. Modifications in mRNA levels of PXR's target-gene CYP3A1, after multiple dose treatment with selective agonists (3 PB doses or 4 PCN doses). * control vs treated samples cells, $p<0.05$.

Developmental expression of aldehyde dehydrogenases 1A1/1A7 and nuclear receptors CAR and PXR.

In order to form a spherical view of the differential expression of ALDH1A1 and ALDH1A7 genes in both strains and possible participation/contribution of CAR and PXR receptors in this process, we further studied the ontogeny of aldehyde dehydrogenases 1A1/1A7 genes, as well as CAR and PXR nuclear receptors' genes, during the development of **RR** and **rr** rat strains.

The results obtained from qRT-PCR of samples from male rats of both strains aged 1, 10, 20, 30 and 40 days old, revealed a tremendous increase of ALDH1A7 in **RR** rats, in contrast to the absence of expression in **rr** rats. mRNA levels in **RR** are 100 times higher at day 40^{th}, compared to the 1^{st} day after birth. Regarding the ALDH1A1 gene, there is a critical point between 20^{th} and 30^{th} day, in which mRNA levels significantly increased in both rat strains. More significant in the development of nuclear receptors seemed to be the second ten days, during which a smaller-scale increase in mRNA levels occurred (Fig. 12A and 12B).The low CAR levels observed in newborn rats has also been reported to human neonates, wherein low CAR levels might account for neonatal jaundice [Goodwin and Moore, 2004].

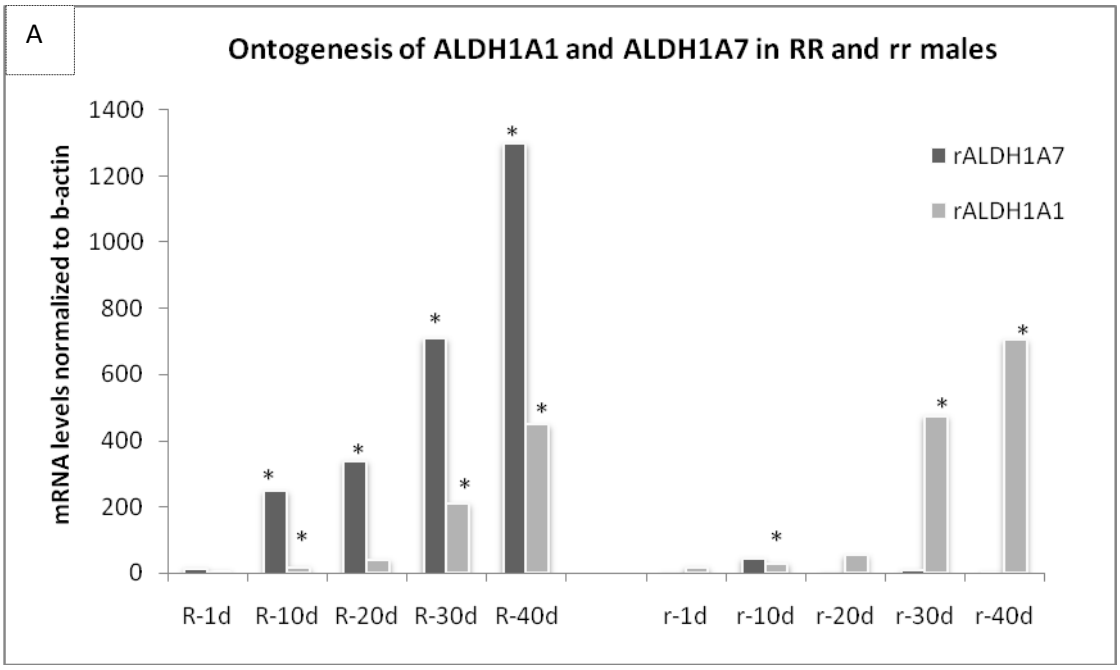


Figure 12A. Ontogenesis of ALDH1A1 and ALDH1A7 in **RR** and **rr** male rats aged 1, 10, 20, 30 and 40 days old. * sample vs 1d, $p<0.05$.

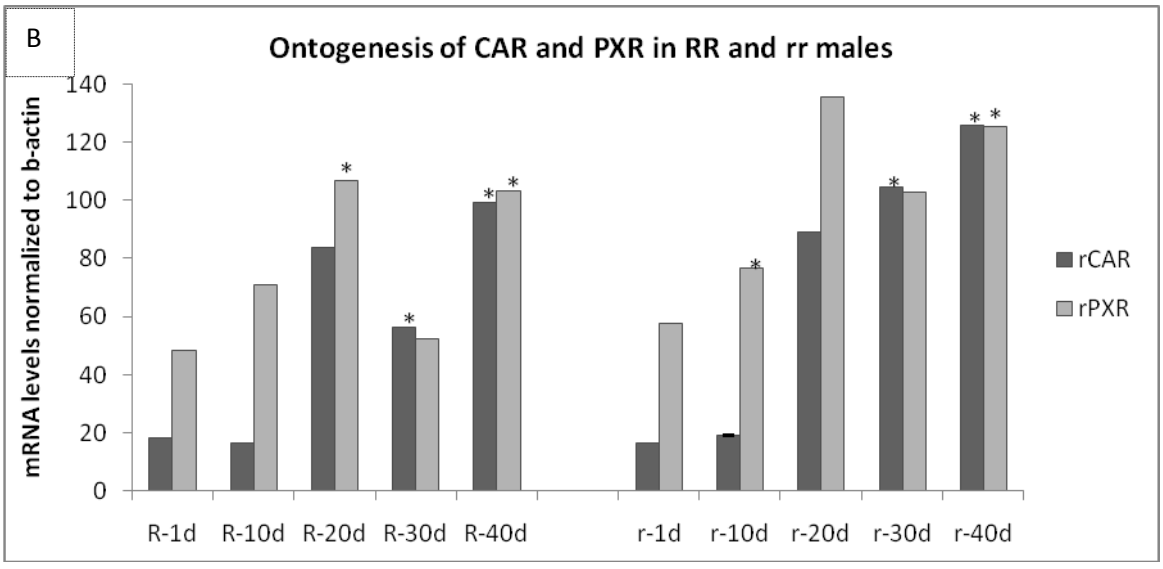


Figure 12B. Ontogenesis of CAR and PXR in **RR** and **rr** male rats aged 1, 10, 20, 30 and 40 days old. * sample vs 1d, $p<0.05$.

In vitro study of PB response based on primary hepatocyte cultures

Thus far our research was based on *in vivo* studies of the impact of drug administration on **RR** and **rr** rats, and the differential induction of ALDH1A1 and ALDH1A7 genes. On top of that, we decided to proceed in isolation of rat primary hepatocytes and primary culture development, in order to create an equivalent model *in vitro*.

Primary hepatocytes were isolated by **RR** and **rr** male rats and cultured for 24 hours. Cells were examined under microscope and snapshots were taken (Figure 13). After that point, some cells were treated for 6 hours with 1mM PB, while untreated cells were cultured in fresh medium and used as controls.

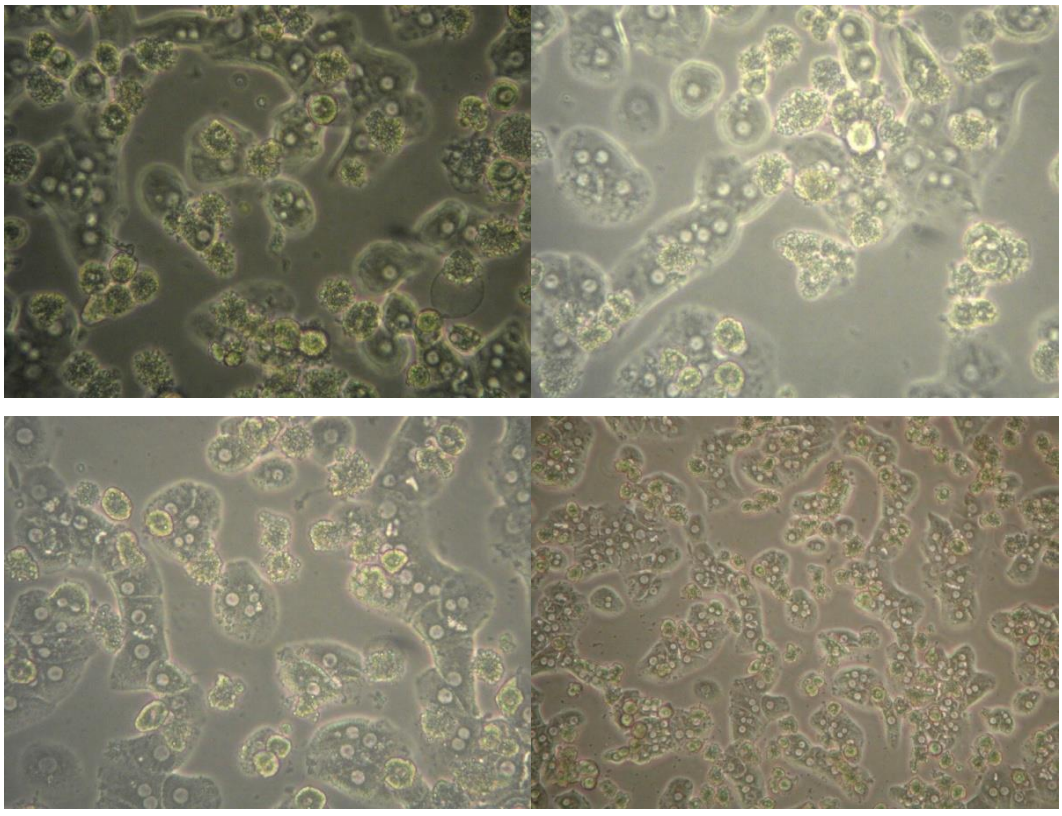


Figure 13. Rat primary hepatocytes, cultured for 24 hours into William's E medium in Petri dishes covered with collagene. Snapshots taken from 2 different Petri dishes.

The absence of mRNA expression of ALDH1A7 in **rr** rats, was further verified by the non-expression in **rr** primary hepatocytes, while in **RR** cells the administration of PB produced a significant increase. ALDH1A1 mRNA levels

appeared to be higher in **RR** compared to **rr** cells, both in controls and cells cultured in medium after addition of phenobarbital. However, differences in the induction of ALDH1A1 after PB administration did not appear to be statistically significant. On the other hand, the comparison of ALDH1A7 and ALDH1A1 between the strains, of either controls or PB-treated rats revealed substantially higher levels of induction in **RR** rats, in all cases (marked with **) (Fig. 14).

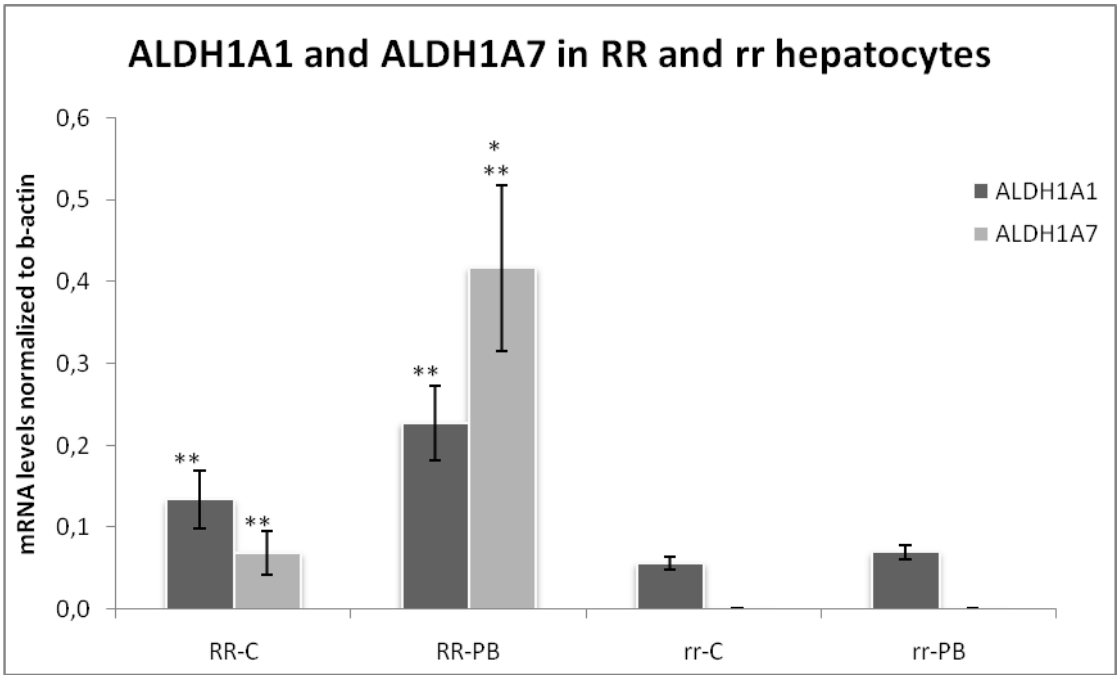


Figure 14. ALDH1A1 and ALDH1A7 expression in **RR** and **rr** rat primary hepatocytes, after treatment with 1mM PB for 6 hours. * control vs PB-treated cells, ** **RR** vs **rr** cells, $p<0.05$.

There is a remarkable difference between the two groups of cells, on basal expression levels of the receptor CAR. Control cells isolated from **RR** rats exhibited higher CAR mRNA levels in comparison to the corresponding levels in control cells from **rr** rats. Phenobarbital administration for 6 hours revealed a decreasing trend of CAR mRNA levels in both groups of cells, but not in a statistically significant way (Fig.15).

Of greater scale and statistically important were the differences observed in PXR mRNA levels between the strains, not only at control conditions, but also after the administration of 1mM PB for 6 hours. Primary hepatocytes from **RR** rats showed higher expression of the receptor than at **rr** rats, regardless the PB

administration. 6 hours of PB treatment had a slight, though notable, positive effect in PXR mRNA induction only in **rr** rats (Fig. 15).

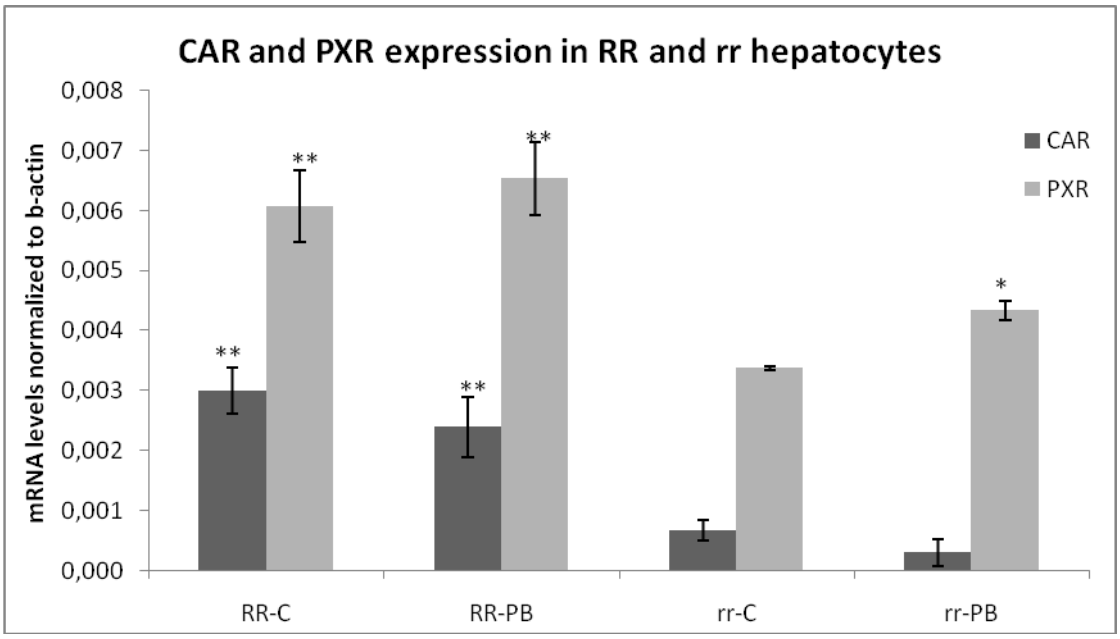


Figure 15. CAR and PXR expression in **RR** and **rr** rat primary hepatocytes, after treatment with 1mM PB for 6 hours. * control vs PB-treated cells, ** **RR** vs **rr** cells, $p<0.05$.

On the whole, there are clear differences in basal expression levels of nuclear receptors CAR and PXR between primary hepatocyte cultures derived from the two rat strains, which may be indicative of the differential expression of the two ALDH1A iso-enzymes (that has also been observed between **RR** and **rr** rats), and the potential involvement of the receptors in regulation of these genes.

Genomic DNA (gDNA) isolation from RR and rr rat strains and cloning of ALDH1A7 promoter regions into plasmid vectors

The remarkable difference in the levels of ALDHs induction between the two strains, especially the absence of ALDH1A7 expression in **rr** rats, had set as the main goal of this project to analyze the sequences of the promoter region of ALDH1A7 gene in **RR** and **rr**, respectively, in order to identify differences in significant binding sites. Such elements may be targets for CAR and PXR, and binding of the nuclear receptors in these positions may play an important role in regulating the expression of the gene.

For this purpose, gDNA from each strain was isolated, and ALDH1A7 promoter region (about 10 kbp) was sequenced and cloned into pGL3-Basic plasmid vectors. The vectors contained the luciferase gene downstream of a multiple cloning site (MCS) and a gene responsible for resistance to the antibiotic ampicillin.

Alignment of **RR**-ALDH1A7 and **rr**-ALDH1A7 promoter sequences showed that there were minor differences between the above sequences (such as deletions of a few base pairs or point mutations), while there was a region of 165 bps missing from rALDH1A7 promoter region (around -2200 bps upstream of TSS), which captured our interest and posed questions regarding the differential regulation of the gene in **RR** and **rr** rats, particularly after PB administration (Figure 16). Altogether, **rr**-ALDH1A7 promoter sequence has 149 bps less that **RR**-ALDH1A7.

Likewise, we aligned each of the promoter sequences with the corresponding one which is registered in databases for the *Rattus norvegicus*. Markedly, the comparison revealed that **RR**-ALDH1A7 is 98% identical to the registered promoter region, whereas **rr**-ALDH1A7 has no difference (100% identical).

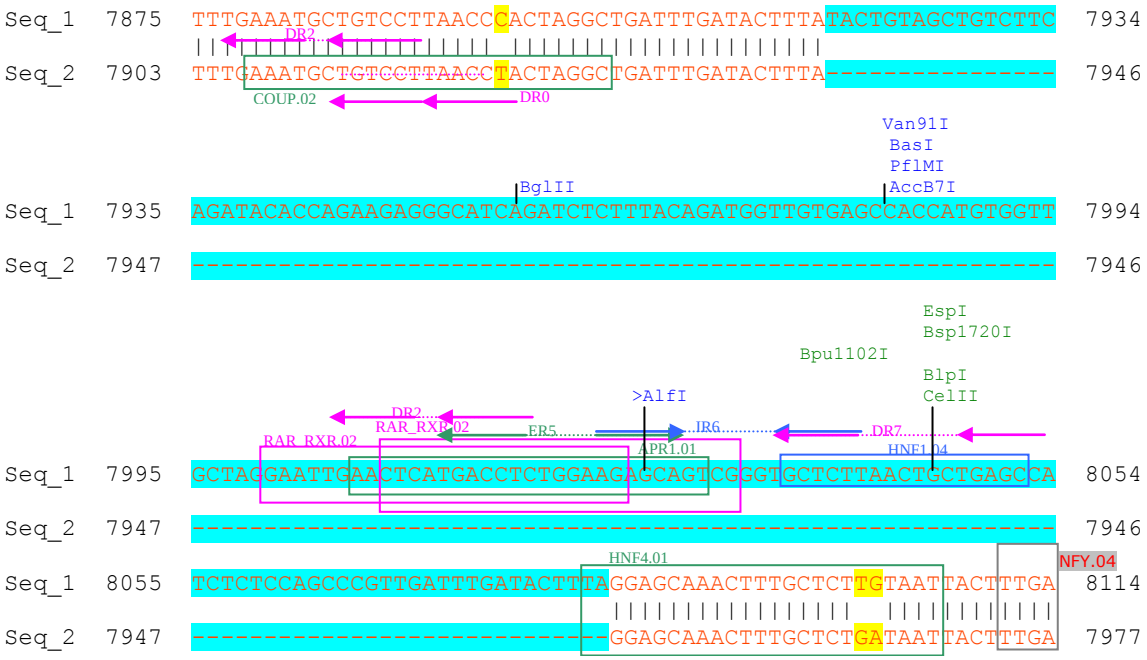


Figure 16. Visualization of the aligned ALDH1A7 promoter sequences from RR and rr rats. The selected region depicts the 165 bp long sequence (shown in blue) that is absent from rr promoter sequence. Seq_1: **RR**-ALDH1A7, Seq21: **rr**-ALDH1A7.

Based on the above information, we confined our study to the region from -2700 bps to +1 (TSS). For that purpose, specific primers were designed, and promoter fragments were amplified and cloned into plasmid vectors, as described in the respective section of materials and methods and shown in figures 4, 5, 6.

Figure 17 depicts an example of enhancement of the deletion fragments by PCR by use of the above-mentioned primers, and verification of the correct product by electrophoresis in agarose gel. Then, the products were purified with columns, by use of Cycle-Pure Spin kit (Omega-biotek, E.Z.N.A), for the subsequent cloning process.

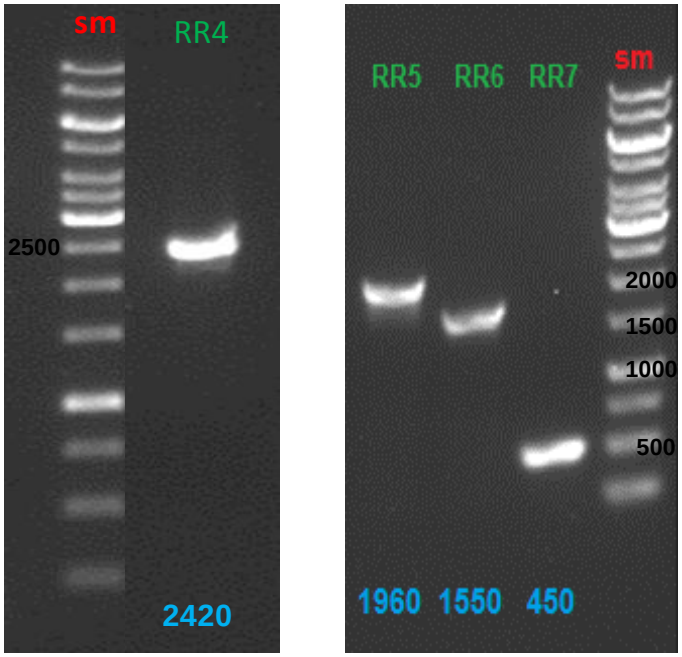


Figure 17. Verification of amplified deletion fragments of **RR**-ALDH1A7 promoter, based on their size after electrophoresis in agarose gel.

We went on to digestion of both the amplified products and the plasmid vector, and ligation reactions resulted in insertion of the deletion fragments into vector, as previously presented in figure 6.

Afterwards, bacteria cells (*Escherichia coli*, XL-10 competent or DH5a) were transformed with the constructs and grown into medium supplemented with ampicillin. Some of the colonies were picked by chance, from which plasmid DNA was extracted and digested with appropriate restriction enzymes, that produced fractions of DNA only in case of successful cloning, since the recognition site was included only within the deletion fragments. The products of the digestion reactions were electrophorized and positive colonies were selected for sequencing (Fig. 18). The correct constructs were further used in reporter gene assays.

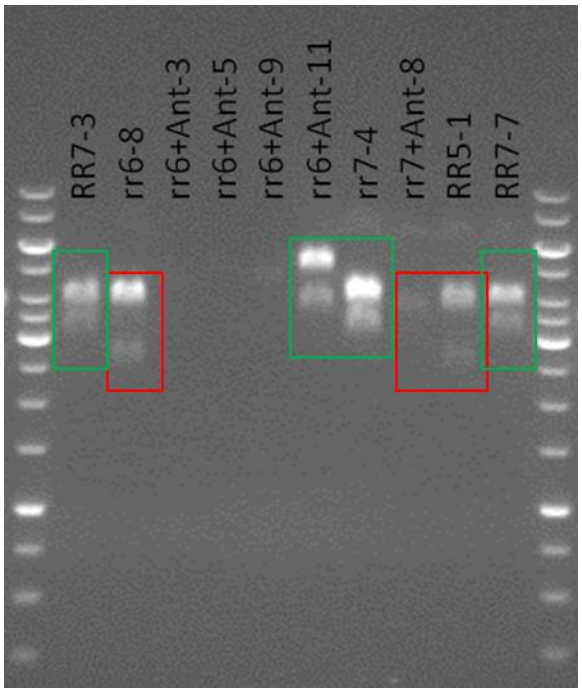


Figure 18. Electrophoresis of plasmid DNA isolated by transformed bacteria, after double digestion with restriction enzymes. Colonies in green box are positive (correct size of the two pDNA fractions), while in red are negative samples (pDNA of the vector alone, without insertion of any deletion constructs).

Apart from the double digestion with enzymes that created pDNA fractions of specific size, we also verified the colonies of bacteria that were transformed with the deletion constructs by performing colony PCR. Two pairs of primers were used (pGL3-Basic forward with RR7/rr7 reverse and RR7-rr7 forward with pGL3-Basic reverse) for amplification of selected colonies. Amplification was feasible only in samples containing pDNA with an insertion of ALDH1A7 deletion fragment, and the products were electrophorized in agarose gel (Fig. 19).

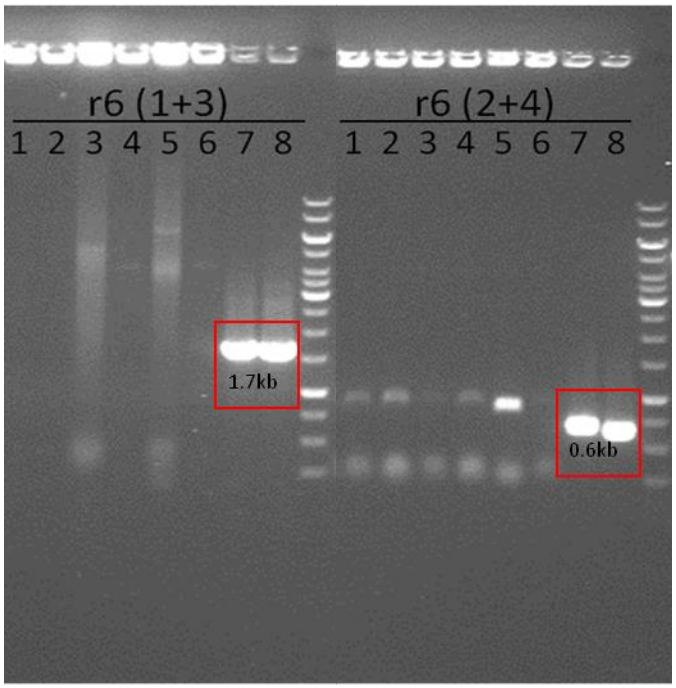


Figure 19. Electrophoresis of colony PCR products, after amplification with different pairs of primers. Into red boxes are two of the colonies that contained a deletion fragment, thus the amplification successfully lead to product.

Reporter gene assays with RR- and rr-ALDH1A7 promoter constructs

Four deletion fragments from **RR**-ALDH1A7 promoter and two from **rr**-ALDH1A7 promoter were cloned into expression vector carrying the luciferase gene. Activation of the gene was measured in each case and compared to the activity of empty vector.

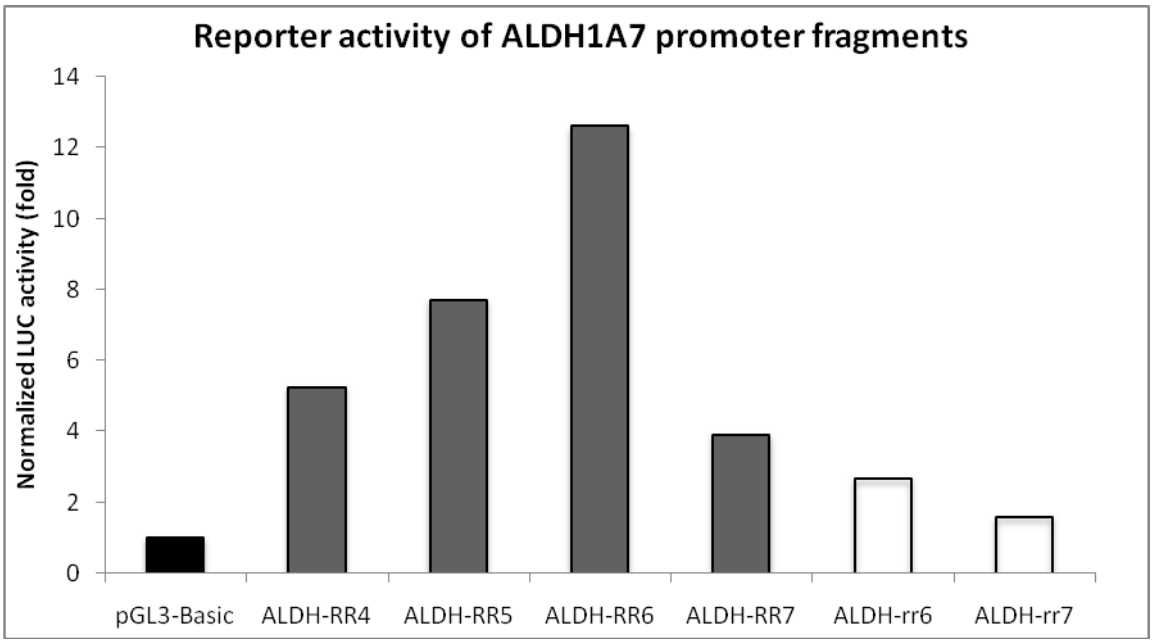


Figure 20. Measurement of Luciferase gene basal activation from ALDH1A7 promoter fragments.

Figure 20 indicates that the promoter activity of the longer ALDH1A7 construct from the ***RR*** strain (RR4) was about 5-fold higher than the promoter-less pGL3-Basic construct, and it increased up to 12.5-fold (RR6) upon deletion of sequences between -2667 and -1566, which may contain suppressive elements. In silico analysis using algorithms for prediction of presumable binding sites for nuclear receptors and other transcription factors augmented this speculation; two successive repressor sites were found upstream from RR6, within RR5 (also included in RR4). Further deletion of sequences between -1566 and -452 reduced the ALDH1A7 promoter activity to about 4-fold above control levels (RR7). Inspection of the promoter sequence between -1566 and -452 suggests that ALDH1A7 promoter may be activated by TFs, such as CCAAT binding factors,

Ccaat/Enhancer Binding Proteins, AP1 (Activating protein 1) and interestingly, an activator-, mediator-binding factor core promoter element, called **XCPE.**

Notably, the ALDH1A7 promoter from the ***rr*** strain shows much weaker activity with rr6 (3.2-fold) and rr7 (< 1.5-fold) construct. This indicates that the sequence differences in regions -1566 and -452 (for *RR* strain) or -1550 and -437 (for *rr* strain) to +5 between the ***RR*** and ***rr*** strains impair DNA binding and/or activation by hepatic TFs present in C3A cells. Alignment and comparison of the promoter sequences from the two strains revealed a single sequence difference; a deletion of 16 base pairs, upstream and close to predicted TATA box, could possibly respond to BRE site (TFIIB recognition element), which might be essential for the recruitment of RNA Polymerase II (Fig. 21). Even though we have not yet identified the TF(s) responsible to ALDH1A7 activation, the data strongly suggest that the poor basal and induced expression of ALDH mRNA in the ***rr*** rat liver (Fig. 21) is caused by such disruptive mutations of the ***rr*** proximal promoter.

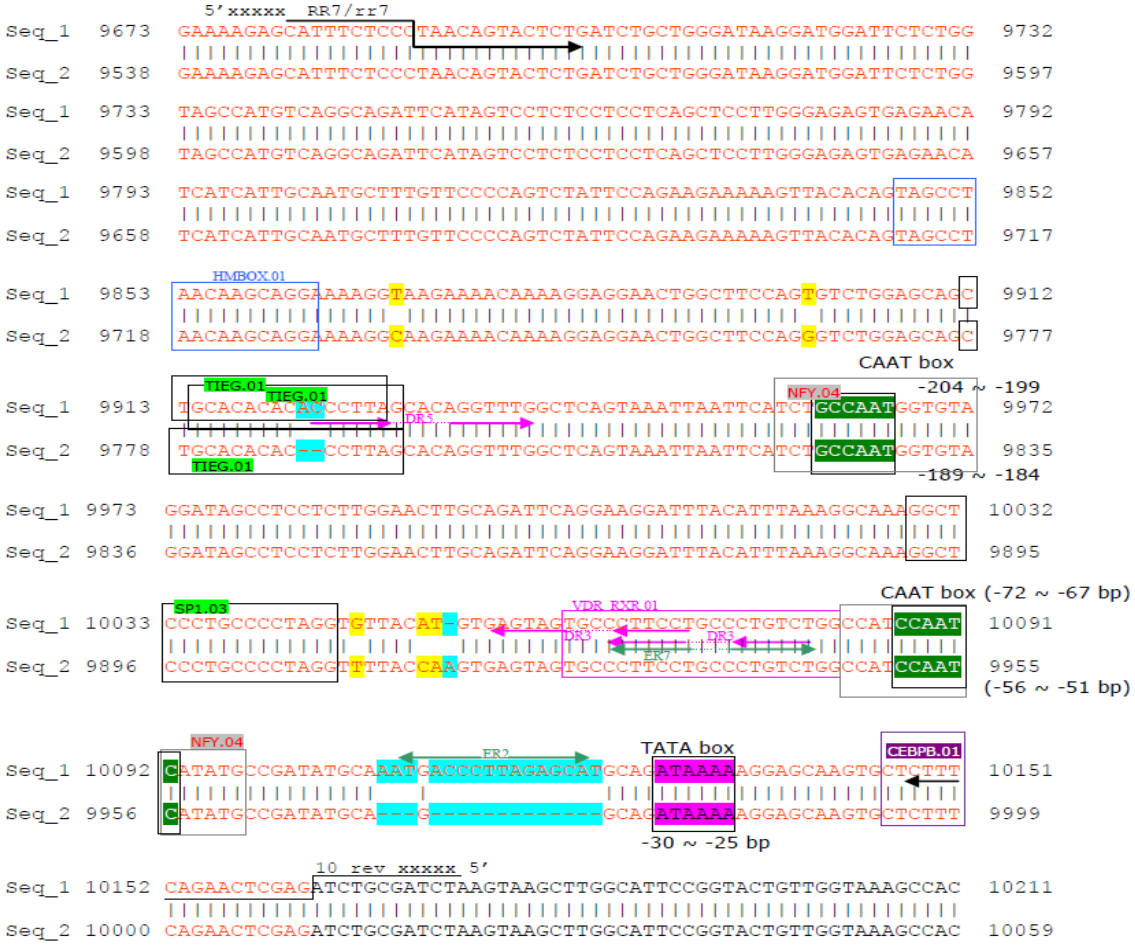


Figure 21. Visualization of the aligned ALDH1A7 promoter sequences from RR and rr rats. The selected region depicts the proximal promoter, where the only difference between the two sequences (16 bp long) is shown in blue. Seq_1: **RR**-ALDH1A7, Seq21: **rr**-ALDH1A7.

In co-transfection assays of hepatoma cells with the above ALDH1A7-constructs or empty pGL3-Basic vector and either an empty NR-expression vector or full-length mCAR- or mPXR- constructs, and the addition of vehicle control or NR ligands, a similar pattern in gene transcription was observed in all cases. This finding prompted us to assume that there is no participation of CAR/PXR within this region of -2667 to +5 that was included in deletion fragments studied (Fig. 22). Likewise, in experiments with hCAR- or hPXR- constructs and selective ligands (CITCO and rifampicin, respectively), no up-regulation of the gene was detected in presence of the NRs and their inducers (data not shown).

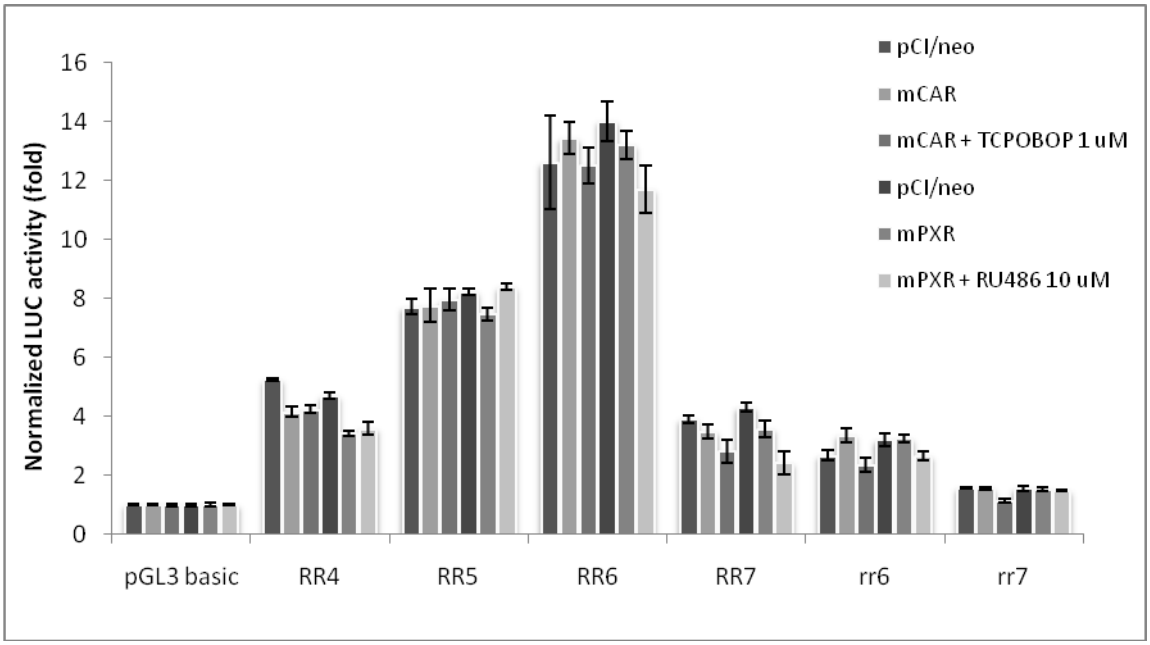


Figure 22. Measurement of Luciferase gene activation in co-transfection assays of the ALDH1A7-constructs and control or NR-expression vectors.

On the contrary, co-transfection of the cells with full-length constructs of **RR**-ALDH1A7 and **rr**-ALDH1A7 and the same mCAR- or mPXR- constructs and their ligands, revealed a tremendous mCAR-driven activation of the **RR**-ALDH1A7 construct, which was further upregulated after treatment of cells with TCPOBOP. Lower, but worth-mentioning positive regulation of **RR**-ALDH1A7 transcription was detected in co-transfection with mPXR, which was also raised after addition of RU486. Remarkably, the presence of nuclear receptors had no effect in transcription regulation in **rr**-ALDH1A7, compared to the empty NR-vehicle (Fig. 23). Alike experiments with human NR-constructs and their activators indicated a similar regulation pattern (data not shown).

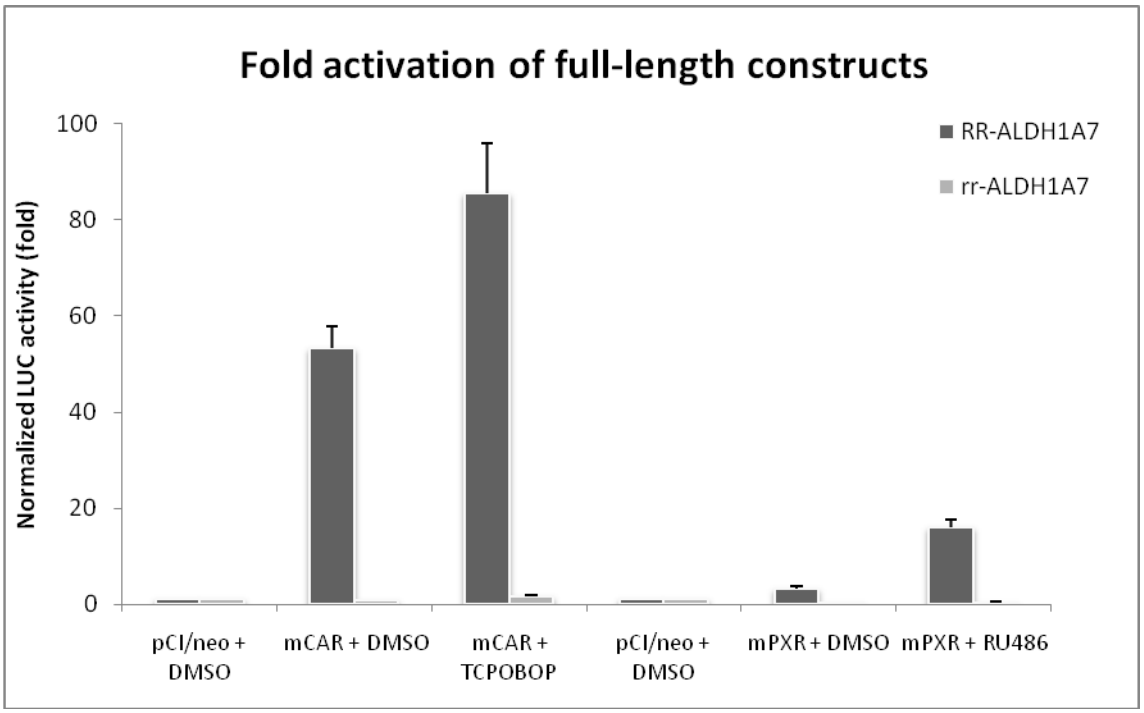


Figure 23. Measurement of Luciferase gene activation in co-transfection assays of the full-length **RR**-ALDH1A7 and **rr**-ALDH1A7 constructs with control or NR-expression vectors, and DMSO or selective ligands.

The results gained from the deletion fragments and the full-length constructs suggest that **RR**-ALDH1A7 is an active promoter, while **rr**-ALDH1A7 exhibited very low activity. Moreover, CAR/PXR-dependent response apparently lies upstream from -2667 (or deletion fragment 4).

Chromatin Immunoprecipitation (ChIP) assays on RR-ALDH1A7 and rr-ALDH1A7 promoter sequences

In order to shed more light on the differential regulation of ALDH1A7 expression between **RR** and **rr** rats, and elucidate whether nuclear receptors CAR and PXR are important mediators of this process or the structural alterations between the strains are responsible for the dramatic discrepancy, we studied the binding affinity of nuclear receptors and the enzyme polymerase II, which is the master regulator of transcription.

Figure 24 shows the binding affinity of polymerase II on the proximal promoter (-213 to -16) of ALDH1A7 in both strains, on chromatin extracted from animals either untreated or treated with PB. Polymerase II could barely bind on proximal promoter of **rr**-ALDH1A7 under any conditions, whereas in **RR** animals the affinity of binding was high, and markedly elevated in rats treated with the drug. This result lent strong support to our previous finding that **RR**-ALDH1A7 is an active promoter, while **rr**-ALD1HA7 exhibits only low levels of activation.

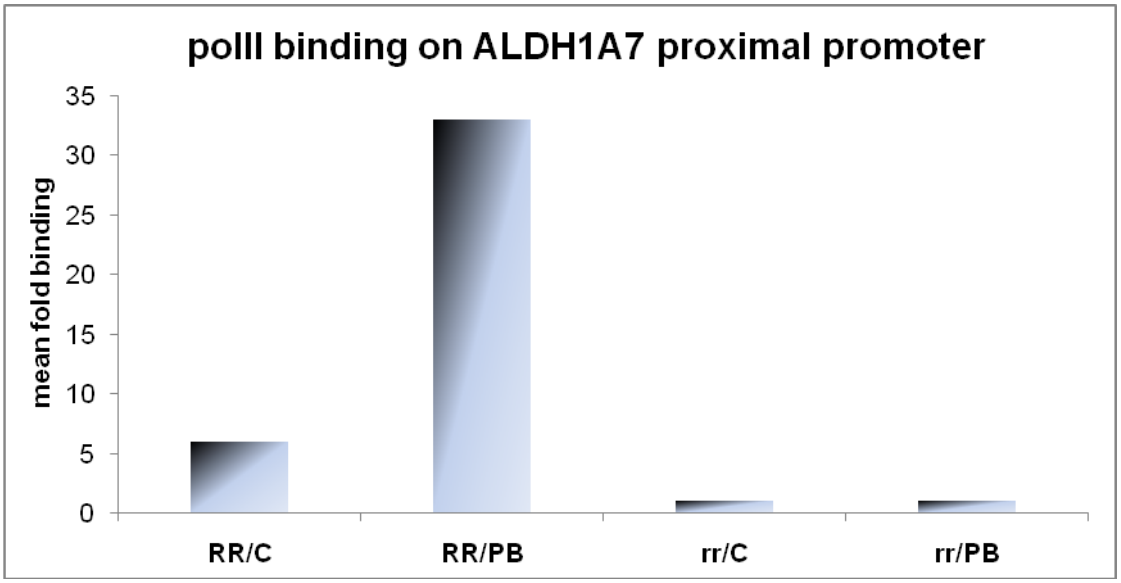


Figure 24. Polymerase II binding affinity on **RR**-ALDH1A7 and **rr**-ALDH1A7 proximal promoters.

Subsequently, our studies were aimed at resolving the CAR-/PXR-dependent response of **RR**-ALDH1A7. For that purpose, we chose to search for binding sites of CAR on the promoter sequence upstream from -2267 (fragment 4 at reporter gene assays), since CAR receptor was found to induce transcription of the gene at a greater extend, especially under activation by the selective agonist. RXRα is another nuclear receptor that forms heterodimers with CAR and bind to specific sequences on the promoter of a gene, usually acting as enhancers of the transcription. Binding of the receptor was detected at three of the putative sites studied.

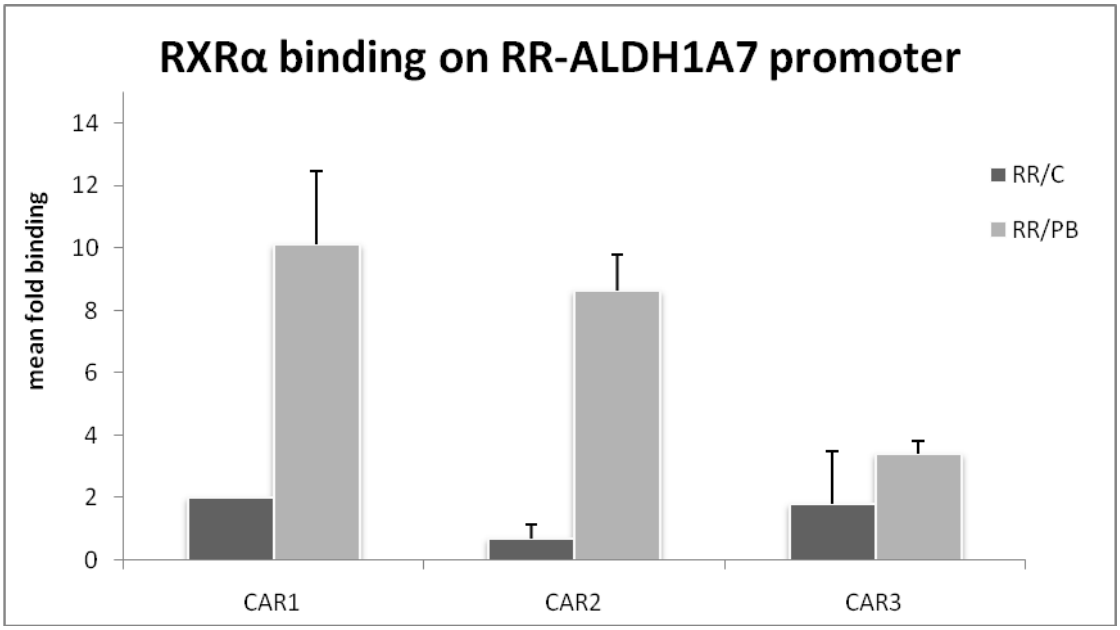


Figure 25. RXRα binding affinity on **RR**-ALDH1A7 promoter, at putative CAR/RXRα binding sites.

In figure 25, binding affinity of RXRα is depicted both in control state, and after PB administration. Based on the results, binding was detected at sites -3050 (CAR1), -3300 (CAR2) and -5100 (CAR3), and the fold induction after PB was respectively 5-, 13- and 2-times, higher compared to control (Fig. 26). Thus, CAR receptor could potentially be a substantial regulator of **RR**-ALDH1A7 gene transcription, regarding the heightened levels of activation after PB administration.

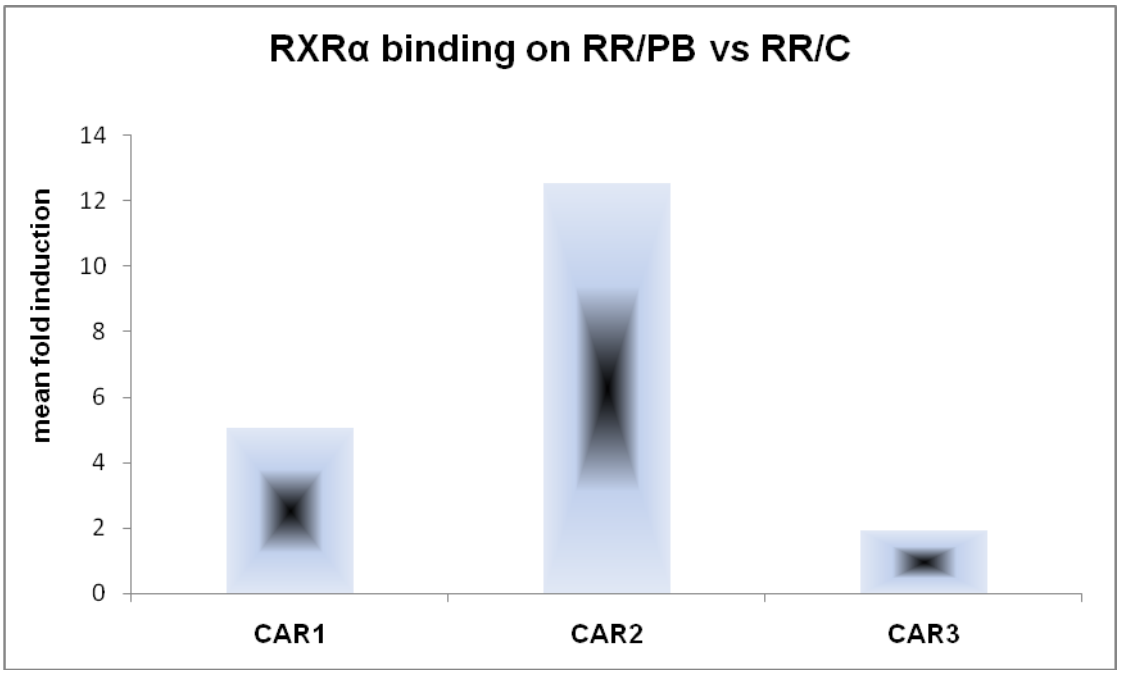


Figure 26. Fold induction of RXRα binding on **RR**-ALDH1A7 promoter after PB administration, at putative CAR/RXRα binding sites.

Measurement of ALDH1As' activity in RR and rr rats.

The animal model used in this study was a breed of Wistar/Af/Han/Mol/Kuo/Io male rats. Animals had been selected based on the induction of ALDHs genes expression after PB administration, and reproduced by inbreeding. Two rat strains had been created and designated **RR** (dominant – Responsive) or **rr** (recessive – non-responsive), considering their response to PB treatment.

Our aim was to certify the difference between the two strains regarding their potential to metabolize aldehyde dehydrogenases after administration of drugs, such as PB or PCN.

In untreated rats, same enzymatic efficacy of ALDH1As was detected in both strains. However, efficacy levels were found nine times higher in **RR** rats treated with PB; likewise, four times higher efficacy levels were detected after PCN administration. As expected, no alteration of enzymatic efficacy was observed in **rr** rats after administration of the drugs, compared to untreated rats (Fig. 27). These results confirm the variation between the strains, regarding the genes that control ALDH1As expression after treatment with PB or other agonists.

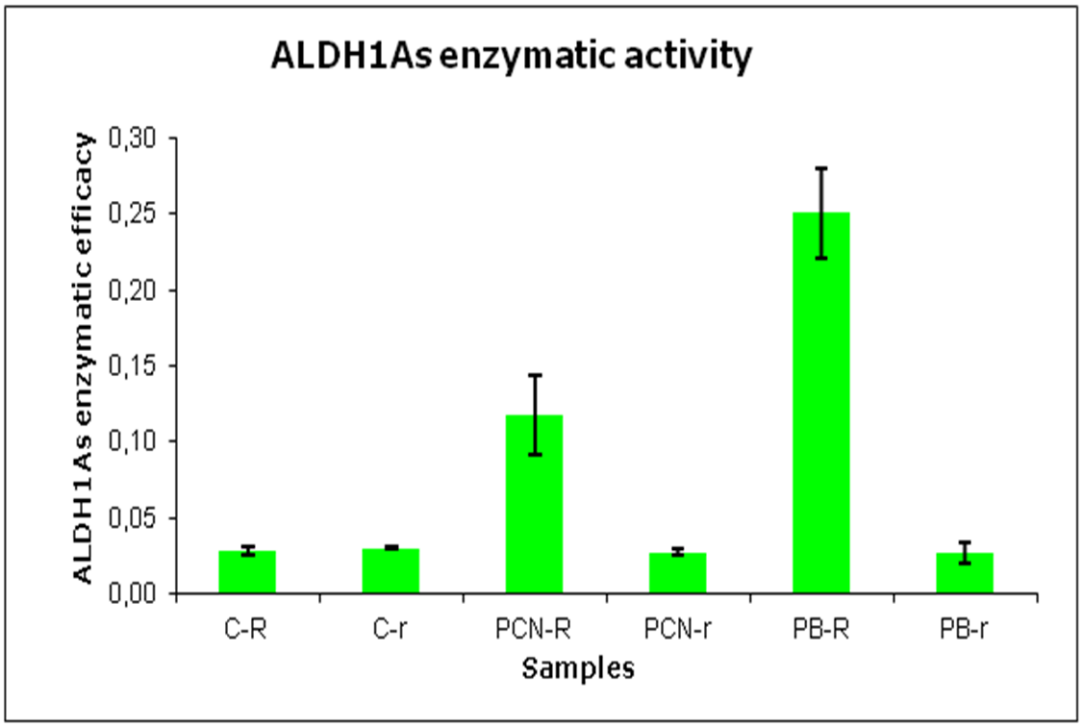


Figure 27. Measurement of ALDH1As efficacy in control animals and rats treated with PB or PCN.

DISCUSSION

The ALDH superfamily of genes has excited the interest of many researchers during the last decade, since there is evidence of functionality and participation of these genes in numerous mechanisms besides detoxification of the organism, including cellular growth and survival, and involvement in various types of disorders and diseases [Westerlund et al, 2005; Jackson et al, 2011]. In the same time, many studies are directed in the key role that nuclear receptors seem to possess in expression regulation of many genes, and therefore the implication of these transcription factors in important biological mechanisms [Goodwin and Moore, 2004].

With these concerns in mind, and utilizing the unique experimental model of Wistar/Af/Han/Mol/Kuo/Io rats that exhibit a characteristic genetic differentiation regarding the ALDHs expression, especially after PB administration, we focused on the expression of ALDH1A1 and ALDH1A7 genes. ALDH1A7 is also characterized as ALDH-PB, since this ALDH1A1-isoform is induced by PB [Kathmann et al, 2000; Kathmann and Lipsky, 1997]. Despite the great similarity in their amino acid sequence, the two isozymes exhibit differential expression patterns and activation [Marselos et al., 1987; Pappas et al., 1998; Hsu et al., 1999;]. Both of them are important drug metabolizing enzymes and PB or other PB-like chemicals are well-studied for their impact on such enzymes, therefore we addressed two questions: 1) whether CAR and the close relative PXR nuclear receptors are somehow implicated in transcription regulation, since CAR is activated mainly by PB, and also the two receptors share common targets [Li and Wang, 2010]; and 2) if the differential induction of ALDH1As between the two rat strains could be attributed to the presumable implication of the receptors, or whether other factors, such as promoter sequence alterations are responsible for the unequal expression levels and enzymatic activity observed in **RR** and **rr** rats.

Our studies on hepatic ALDH1As expression patterns in **RR** and **rr** rats, and the inductive effects of PB (selective agonist for CAR) and PCN (selective activator of PXR) have revealed that protein levels were comparatively higher in **RR** rats, and administration of agonists resulted in up-regulation of the genes, with a greater

impact on **RR** strain. Although the use of ALDH1/2 antibody in protein immuno-precipitation studies could not lead to discrimination between ALDH1A1 and ALDH1A7 expression patterns, the results shed light on the apparent difference in protein levels between the two rat strains, especially after the administration of drugs. ALDH1As levels were higher in the cytosol of **RR** liver cells than the respective samples from **rr** rats, and were further elevated after PB or PCN treatment. The effects of agonists were studied in experiments of multiple dose treatment and single-dose administration for 6, 12 and 24 hours. PB administration led to raised protein levels in the cytosol of treated animals, within 12 to 24 hours after last injection. Moreover, the effect of PB on ALDH1As expression was dose dependent, when 1-, 2- or 3-dose treated samples were compared. 4 doses of PCN had a similar inductive effect on ALDH1As, though to a lesser extent. A single dose of PCN could only slightly up-regulate ALDH1As in **RR** rats, 24 hours after treatment. Alike ALDH1As expression pattern was observed in samples from **rr** rats, however protein levels were lower that the respective from **RR** rats, either in basal or induced state. These findings imply that both PB and PCN trigger the expression of ALDH1As in hepatic tissue, with PB being the most effective. As expected, the two rat strains substantially differ in expression of ALDH1As, and the protein levels were significantly lower in **rr** rats, hence we studied the respective mRNA levels of the afore-mentioned samples.

The remarkable difference of ALDH1As levels was proved to be attributed in the absence of ALDH1A7 expression in **rr** rat strain. In **RR** rats, ALDH1A7 is expressed in control state, and a tremendous rise in mRNA levels (100 times higher compared to control) was detected already within 6 hours of PB treatment, which lasted even after 24 hours. Similarly, but in a lower scale, PCN up-regulated ALDH1A7 expression in **RR** rats. On the other hand, mRNA levels of ALDH1A1 were higher in **rr** rats, compared to corresponding levels in **RR** strain. Administration of both drugs further induced gene transcription, though PB showed a markedly higher up-regulation effect. Interestingly, the transcription up-regulation was found to differ between the strains after PB administration. In **RR** rats, significantly elevated mRNA levels were detected already after 6 hours, while PB triggered mRNA transcription after 24 hours in **rr** rats. This may be indicative

of a possible implication of transcription factors activated by PB, which potentially lead to differential induction of gene expression.

The differences marked in time- and dose- response experiments, regarding the expression of ALDH1A1 and ALDH1A7, were further supported by studying the expression of these genes during ontogenesis in neonates, as well as *in vitro* using primary hepatocytes. Primary hepatocyte cultures is a good model for in vivo studies of CAR-mediated gene transcription upon xenobiotic induction of the receptor, in contrast to liver-derived immortilized cells, such as HepG2, CAR translocation into nucleus is not PB-responsive but rather spontaneous [Muangmoonchai et al., 2001; Kanno and Inouye, 2008]. In both cases of primary hepatocytes and liver from neonates, we confirmed that ALDH1A7 expression is impaired in liver cells derived from **rr** rats. Higher levels of ALDH1A1 expression were observed in **rr** liver tissue from neonates, however transcription of the gene in **rr** primary hepatocytes was found to be lower than in cells from **RR** rat liver.

Unequivocally, there should be an outstanding mechanism for the differential gene regulation conducted in each strain, and our findings of augmented transcription upon PB and PCN administration indicate that nuclear receptors CAR and PXR presumably play a substantial role in this process. Based on our results, the only alteration in nuclear receptors' mRNA levels observed was after administration of PB, and only during the first 6 hours, after which mRNA levels were back to normal. Interestingly, the enrichment of mRNA levels of both receptors was prominent only in **RR** rats, raising additional thoughts regarding the sensitivity of transcription regulation in **rr** rats.

However, the most critical step in turning on gene expression is the translocation of the receptors from the cytoplasm, where they are physiologically sequestered, into nucleus. We have shown that PB effectively translocated CAR, even when administered in a single dose, and the translocation was carried out even after 1 hour of treatment, which is consistent with the rapid elevation of CAR protein levels into nucleus that was observed in studies on mouse livers treated with PB [Kawamoto et al., 1999; Zelko and Negishi, 2000, Li et al., 2007]. PB effect on CAR translocation was both time- and dose dependent (also supported by Li et al., 2007), and also in accordance to time- and dose-dependent up-regulation of

ALDH1/2 expression, prompting us to speculate the possibility of CAR's involvement in ALDHs regulation. Similarly, PXR accumulated into the nuclear compartment after treatment with PCN, but the receptor's shuttling seemed to be affected also by PB. However, PB activity on PXR was very low, as reported also in other studies [Xiong et al., 2002; Bell and Michalopoulos, 2006]. In contrast, PCN could not demonstrate the same impact on CAR (also shown by Smirlis at al., 2001). Despite the rapid nuclear accumulation of the receptors, and especially that of CAR after PB treatment, elevated ALDH1/2 protein levels were detected in the cytosolic compartment a few hours later. A possible explanation for this could be a delay in CAR-RXRα heterodimer formation and binding to regulatory sites, in order to transactivate gene expression [Li et al., 2007], and then some time is needed by the moment the mRNA is synthesized until translation of the protein.

The nuclear translocation of the receptors was found to be aligned to the up-regulation of ALDH1As in all cases of drug administration, hence fostered our notion of NRs implication in the genes' transcription regulation. In particular, CAR was nominated as a strong mediator of ALDH1As' induction, while PXR exhibited a similar, though milder activation potential of ALDH1As' expression. Despite nuclear receptors' translocation being a crucial step for regulation of gene expression, the presence on the receptors in the nuclear compartment does not assure up-regulation of a target gene. In fact, low CAR protein levels were detected by immunoblotting in control samples from both strains, and this finding is in agreement with other studies [Hosseinpour et al., 2006; Li et al., 2007]. Therefore, the activation potential of the receptors after their translocation into nucleus was asserted by measuring mRNA levels of the most well-studied target genes, CYP2B for CAR and CYP3A for PXR, upon treatment with their selective agonists CAR and PXR, respectively. PB had a warranted great impact on CYP2B1 expression, while also triggered CYP3A1 gene's induction, though the inductive effect was less striking. On the other hand, PCN highly up-regulated CYP3A1 expression via PXR, whereas CYP2B1 was also induced in low levels; however, a single dose of PCN was not sufficient enough to mediate transcription of the gene, and this finding was correlated to ineffective translocation of CAR receptor. Our results are consistent with those extrapolated by Smirlis and her colleagues (2001) from experiments on primary hepatocytes treated with the same inducers. Moreover, the

fact that no discernible difference in expression levels of both target genes was observed between the two rat strains lends strong support to the notion of PB's selective effect on triggering ALDHs expression [Dunn et al., 1989; Pappas et al., 1998 and 2001].

Based on the tremendous difference of ALDH1A7 expression between the strains and the discernible effect of drugs in transcription regulation, we focused on promoter regions of **RR**-ALDH1A7 and **rr**-ALDH1A7 in order to fully elucidate the complete absence of ALDH1A7 expression in **rr** rats, which was in contrast to high basal and even higher drug-induced enzymatic levels produced in **RR** strain. In reporter gene assays with deletion fragments containing the proximal promoter of the gene (-452 to +15), luciferase gene was induced 4 times higher compared to control in **RR** rats, while **rr** promoter barely activated gene transcription (1.5 times higher than control vehicle). Moreover, longer deletion fragments (-1566 to +15), exhibited even greater activation (12.5 times) of transcription in the case of promoter derived from **RR** strain, suggesting that there might lie binding sites for transcription factors that serve as activators. On the contrary, the respective fragment from **rr**-ALDH1A7 promoter conferred low activation (3.2 times), implying that ALDH1A7 transcription in **rr** rats is probably impaired for some reason. Luciferase assays with **RR**-ALDH1A7 deletion fragments about 2700 bp long have revealed that suppressive elements potentially lie from -2667 to -1566, displaying a negative regulation on gene transcription. Indeed, according to algorithms predicting putative binding sites for transcription factors and other important regulatory elements, two successive repressor sites were found on **RR**-ALDH1A7 promoter within the afore-mentioned region.

Co-transfection assays of the above deletion fragments with vectors expressing the full-length mCAR and mPXR, either upon administration of their activators or not, intimated no implication of the receptors in gene activation controlled by the selected promoter fragments. We then proceeded with co-transfection assays of mCAR and mPXR constructs and the full-length **RR**-ALDH1A7 and **rr**-ALDH1A7 constructs. Notably, reporter gene expression was greatly activated under control of the 10 kb-long **RR**-ALDH1A7 promoter in co-transfection with mCAR construct, and the activation was even higher after administration of TCPOBOP (selective agonist for mCAR). Co-transfection of the

same **RR** promoter fragment with mPXR indicated similar, though much lower, inductive effect, which was elevated after administration of RU486 (selective agonist for mPXR). As expected, **rr**-ALDH1A7 constructs were proved to be inactive regardless the presence of receptors and their agonists, and this finding converged well together with all our previous findings of impaired transcription of ALDH1A7 in **rr** rats.

Hitherto, our findings demonstrated that ALDH1A7 gene expression is induced only in **RR** rats, and any participation of CAR and/or PXR receptors is triggered via promoter sequences that lie upstream from -2667 on the ALDH1A7 promoter. It was therefore pertinent to study the binding potential of polymerase II and CAR/PXR on promoter sequences of the ALDH1A7 gene from both rat strains utilizing ChIP immuno-precipitation assays. The finding of **RR**-ALDH1A7 being an active promoter in contrast to the negligible activation capacity of **rr**-ALDH1A7 was confirmed by strong binding of polymerase II only on the proximal promoter from **RR** rats, and the tremendous activation when animals were treated with PB. Our efforts on assessing putative binding sites of CAR receptor proved to be unsuccessful due to the lack of high-quality CAR antibodies for chromatin immunoprecipitation [also reported by Molnár et al., 2013], therefore we studied the binding of CAR and PXR's partner in the functional heterodimer, RXRα, using a specific antibody for indirect examination of binding of the receptors to their cognate sequences, as performed also by Ohno and his colleagues (2014). No binding was detected in any of the promoters within the region from -2667 to +15, which was correlated to the longer of the deletion fragments tested in reporter gene assays, hence we were prompted to study upstream in the distal promoter region. Among a few selected putative binding sites, binding affinity of RXRα was essentially elevated in three sites, with the strongest binding being recorded in position -3300 upstream from TSS, in the **RR**-ALDH1A7 promoter.

It should be noted that our experiments were performed only with the use of male **RR** and **rr** rats, since in females the response to PB resembles that of males, but ALDHs activity is much lower compared to males [Marselos, 1976]. It was further shown that CAR protein expression is much greater in male Wistar-Kyoto (WKY) rats than in females, which subsequently results in higher induction of CYP2B mRNA levels in males WKY [Yoshinari et al., 2001]. The poor PB

response and the low induction of CYP2B expression in females was related to extremely low nuclear levels of CAR, although the cytosolic levels were same regardless the sex, lending strong support on the notion that nuclear translocation of CAR may be essential for target-gene induction.

On the whole, the remarkable difference in ALDH1As expression which is exhibited between **RR** and **rr** strains of the Wistar/Af/Han/Mol/Kuo/Io rats is attributed to the very weak expression of ALDH1A7 gene in **rr** rats, and our interpretation regarding the impaired induction of the gene was supported by low levels of mRNA or protein expression in all cases of basal and drug-mediated transcription. The structural differences revealed after sequencing and alignment of ALDH1A7 promoter region from the two strains, and the unknown promoter sequence of the inducible form of the gene (**RR**-ALDH1A7) could be now provided in databases for new studies, since the currently available represents the promoter region of the inactive **rr**-ALDH1A7. Furthermore, expression of ALDH1A7 in **RR** rats is significantly up-regulated upon PB or PCN administration, and CAR receptor is pointed out as a substantial regulator of **RR**-ALDH1A7, by acting as an enhancer of the drug-mediated transcription of the gene.

Some future perspectives of the present work would be the study of the effects of mutations on binding sites of polII, CAR1, CAR2, CAR3 on **RR**-ALDH1A7 promoter sequence, and the verification of their substantial role in gene activation and up-regulation after PB treatment. Also, additional binding assays with other transcription factors, such as HNF4, will shed more light on ALDH1A7 gene regulation. A promising thought is the development of specific ALDH1A7 antibody, since there is no such product available in the global market. Last, but not least, the unique experimental model of RR rats, which exists in our laboratory, should not be neglected, but rather promoted for studies on drug metabolism, as the differential induction of ALDH1A7 could potentially be related to other mechanisms of detoxification, as well.

SUMMARY

The magnitude of the genes belonging to the ALDH superfamily in organism's welfare and especially their protection against xenobiotics, as well as the accumulating evidence that indicate involvement of nuclear receptors in regulation of many genes including ALDHs, has excited our interest in unraveling the potential role of CAR and PXR in mediation/regulation of ALDH1As subfamily of genes, utilizing the unique experimental model of Wistar/Af/Han/Mol/Kuo/Io rats. Specifically, the two rat strains used in this study were either responsive (**RR**) or non-responsive (**rr**) to induction of ALDH1A genes after administration of PB (Phenobarbital) or other PB-type inducers.

The apparent difference of ALDH1As induction between the two strains, both at basal levels and after treatment with PB or PCN, which was detected with protein immunoprecipitation experiments, was further investigated at mRNA levels of expression. The results revealed that the remarkable difference was attributed to the complete absence of ALDH1A7 expression in **rr** strain. Furthermore, a striking up-regulation of the gene was observed in **RR** rats in the presence the above drugs, which act as selective agonists for the nuclear receptors CAR and PXR. Concurrently, expression of ALDH1A1 gene was only slightly higher in **rr** rats, compared to respective expression levels in **RR** animals. Supportive evidence to our findings was displayed in experiments of ontogenesis with liver samples derived from neonates and corresponding *in vitro* studies in primary hepatocyte cultures.

The nuclear translocation of CAR and PXR upon treatment with their selective agonists was evident in both rat strains and also time- and dose-dependent, however their potential gene activation had to be further tested by studying the induction of their well-studied target genes; CYP2B1 and CYP3A1, respectively.

Focusing on the tremendous difference of ALDH1A7 expression between the strains and the uncontest effect of drugs in transcription regulation, we performed reporter gene assays with various deletion constructs of **RR**-ALDH1A7 and **rr**-ALDH1A7 promoters, as well as chromatin immunoprecipitation assays on the same promoter regions. Interestingly, our results indicated **RR**-ALDH1A7 as an active promoter highly-upregulated in response to PB administration, which is in strong contrast to the barely active **rr**-ALDH1A7. Although the proximal promoter

is the essential region for turning on gene transcription, we nominated a region of the **RR**-ALDH1A7 promoter between -1566 to -452,which demonstrated highest activation potential.

CAR was emerged as a substantial regulator of **RR**-ALDH1A7 gene activation, especially when PB or other CAR-activators were administered. In **RR**-ALDH1A7, CAR was found to bind to the distal promoter of the gene, serving as enhancer of gene expression, and presumably PXR may also be able to bind, since the receptors are promiscuous to their binding to targets and cross-talk to each other.

ΠΕΡΙΛΗΨΗ ΣΤΑ ΕΛΛΗΝΙΚΑ

Η σπουδαιότητα των ενζύμων που ανήκουν στην υπερ-οικογένεια των αλδεϋδικών αφυδρογονασών (ALDHs) στην ευζωία των οργανισμών και κυρίως στην προστασία τους από ξενοβιοτικούς παράγοντες, καθώς και η συσσώρευση στοιχείων που υποδηλώνουν την εμπλοκή πυρηνικών υποδοχέων στην ρύθμιση της έκφρασης πολλών γονιδίων, συμπεριλαμβανομένων των αλδεϋδικών αφυδρογονασών, κέντρισε το ενδιαφέρον μας να αποκαλύψουμε τον πιθανό ρόλο των υποδοχέων CAR και PXR στην επαγωγή/ρύθμιση των ALDHs της υπο-οικογένειας 1A, αξιοποιώντας το ιδιαίτερο πειραματικό μοντέλο των επίμυων του γένους Wistar/Af/Han/Mol/Kuo/Io. Συγκεκριμένα, τα δυο στελέχη των επίμυων που χρησιμοποιήθηκαν στην παρούσα μελέτη χαρακτηρίζονται ως αποκρινόμενα (**RR**) ή μη-αποκρινόμενα (**rr**) ως προς την επαγωγή των γονιδίων ALDH1A, μετά την χορήγηση φαινοβαρβιτάλης (PB) ή άλλων PB τύπου επαγωγέων.

Η προφανής διαφορά στην επαγωγή των ALDH1As μεταξύ των δύο στελεχών, που ανιχνεύτηκε με πειράματα μελέτης των πρωτεϊνών, τόσο σε βασικά επίπεδα έκφρασης όσο και μετά τη χορήγηση φαινοβαρβιτάλης (PB) ή καρβονιτριλίου της πρεγνενολόνης (PCN), διερευνήθηκε περαιτέρω με μελέτη των επιπέδων παραγωγής mRNA. Τα αποτελέσματα αποκάλυψαν ότι η αξιοσημείωτη διαφορά οφείλεται στην παντελή έλλειψη έκφρασης του γονιδίου ALDH1A7 στο στέλεχος **rr**. Επίσης, παρατηρήθηκε εντυπωσιακή αυξο-ρύθμιση στους **RR** επίμυες, ιδιαίτερα παρουσία των παραπάνω φαρμάκων, τα οποία δρουν ως εκλεκτικοί αγωνιστές των πυρηνικών υποδοχέων CAR και PXR. Παράλληλα, η έκφραση του γονιδίου ALDH1A1 βρέθηκε λίγο υψηλότερη στους **rr** επίμυες, συγκριτικά με τα αντίστοιχα επίπεδα στο **RR** στέλεχος. Αποτελέσματα που υποστηρίζουν τα παραπάνω στοιχεία προέκυψαν και από πειράματα οντογένεσης που πραγματοποιήθηκαν σε ήπαρ από νεογέννητους επίμυες, αλλά και σε αντίστοιχη *in vitro* μελέτη πρωτογενών καλλιεργειών ηπατοκυττάρων.

Η μετακίνηση των υποδοχέων CAR και PXR στον πυρήνα των κυττάρων, μετά την χορήγηση των εκλεκτικών αγωνιστών τους ήταν εμφανής και στα δύο στελέχη των επίμυων, και επίσης ήταν χρονο- και δόσο-εξαρτώμενη. Ωστόσο, η ικανότητά τους να ενεργοποιούν την έκφραση γονιδίων μελετήθηκε περαιτέρω, με

μέτρηση της επαγωγής γνωστών γονιδίων στόχων τους: των κυτοχρωμάτων CYP2B1 και CYP3A1, αντίστοιχα.

Εστιάζοντας στην σημαντικά υψηλή διαφορά μεταξύ των δύο στελεχών ως προς την έκφραση της ALDH1A7 και την αδιαμφισβήτητη επίδραση των παραπάνω φαρμάκων στην ρύθμιση της έκφρασης, πραγματοποιήσαμε δοκιμασίες μέτρησης γονιδίου αναφοράς, χρησιμοποιώντας διάφορα τμήματα των υποκινητών του γονιδίου, προερχόμενα από τα δύο στελέχη (**RR**-ALDH1A7 και **rr**-ALDH1A7). Επιπλέον, πραγματοποιήθηκαν δοκιμασίες ανοσοκαθίζησης της χρωματίνης στα παραπάνω τμήματα των υποκινητών. Ιδιαίτερα ενδιαφέρον ήταν το εύρημα ότι ο υποκινητής **RR**-ALDH1A7 είναι ενεργός και μάλιστα η ενεργότητά του αυξάνεται δραστικά σε περίπτωση χορήγησης PB, γεγονός το οποίο είναι σε πλήρη αντίθεση με την ελάχιστη ενεργότητα που επέδειξε ο υποκινητής **rr**-ALDH1A7. Παρ' όλο που ο η περιοχή του υποκινητή που βρίσκεται κοντά στην αρχή του γονιδίου είναι η πιο σημαντική για την ενεργοποίηση της έκφρασης, βρέθηκε ότι η περιοχή του υποκινητή **RR**-ALDH1A7 που βρίσκεται μεταξύ -1566 και -452 είναι υπεύθυνη για την μέγιστη ενεργότητα.

Ο πυρηνικός υποδοχέας CAR αναδεικνύεται ως κύριος ρυθμιστής της έκφρασης του γονιδίου **RR**-ALDH1A7, ιδιαίτερα μετά την χορήγηση PB ή άλλων αγωνιστών του CAR. Ειδικότερα στο γονίδιο **RR**-ALDH1A7 ο CAR βρέθηκε ότι προσδένενται στον υποκινητή μακριά από την θέση έναρξης της μεταγραφής, δρώντας κυρίως ως ενισχυτής της έκφρασης του γονιδίου, και πιθανότατα και ο PXR να δύναται να προσδεθεί, δεδομένου ότι οι δύο υποδοχείς συχνά ανταλλάσουν τις θέσεις πρόσδεσής τους στα γονίδια-στόχους και αλληλεπιδρούν μεταξύ τους.

REFERENCES

Alnouti Y and Klaassen C.D. **Tissue distribution, ontogeny, and regulation of aldehyde dehydrogenase (Aldh) enzymes mRNA by prototypical microsomal enzyme inducers in mice**. *Toxicol. Sciences*. 101(1): 51-64, 2008

Aranda A., and Pascual A. **Nuclear hormone receptors and gene expression**. *Physiol. Rev*. 81: 1269-1304, 2001

Bailey I., Gibson G.G., Plant K., Graham M., Plant N. **A PXR-mediated negative feedback loop attenuates the expression of CYP3A in response to the PXR agonist pregnenalone-16α-carbonitrile.** *PLoS ONE* 6(2): e16703, 2011

Bell A.W. and Michalopoulos G.K. **Phenobarbital regulates nuclear expression of HNF-4α in mouse and rat hepatocytes independent of CAR and PXR.** *Hepatology*44 (1): 186-194, 2006

Bing Y., Zhu S., Jiang K., Dong G., Li J., Yang Z., Yue J. **Reduction of thyroid hormones triggers down-regulation of hepatic CYP2B through nuclear receptors CAR and TR in a rat model of acute stroke.** *Biochemical Pharmacology* 67: 636-649, 2014

Black W.J., Stagos D., Marchitti S.A., Nebert D.W., Tipton K.F., Bairoch A., Vasiliou V. **Human aldehyde dehydrogenase genes: Alternatively spliced transcriptional variants and their suggested nomenclature**. *Pharmacogen. Genomics*. 19: 893-902, 2009

Bookout A.L., Jeong Y., Downes M., Yu R.T., Evans R.M., Mangelsdorf D.J. **Anatomic profiling of nuclear receptor expression reveals a hierarchical transcriptional network**. *Cell*126: 789-799, 2006

Carnahan V.E., Redinbo M.R. **Structure and function of the human nuclear xenobiotic receptor PXR**. *Curr. Drug Metab.* 6: 357-367, 2005

Chang T.K.H. **Activation of pregnane X receptor (PXR) and constitutive androstane receptor (CAR) by herbal medicines**. *The AAPS Journal* 11 (3), 2006

Chang T.K., and Waxman D.J. **Synthetic drugs and natural products as modulators of constitutive androstane receptor (CAR) and pregnane X receptor (PXR)**. *Drug Metab. Rev.* 38: 51-73, 2006

Chen Y., Tang Y., Guo C., Wang J., Boral D., Nie D. **Nuclear receptors in the multidrug resistance through the regulation of drug-metabolizing enzymes and drug transporters.** *Biochem. Pharmacol.* 83: 1112‑1126, 2012

Chirulli V., Longo V., Marini S., Mazzaccaro A., Fiorio R., Gervasi P.G. **CAR and PXR expression and inducibility of CYP2B and CYP3A activities in rat and rabbit lungs**. *Life Sciences* 76: 2535-2546, 2005

Choi H.S., Chung M., Tzameli I., Simha D., Lee Y.K., Seol W., and Moore D.D. **Differential transactivation by two isoforms of the orphan nuclear hormone receptor CAR**. *Jour. Biol. Chem.* 272: 23565-23571, 1997

Daskalopoulos E.P., Malliou F., Rentesi G., Marselos M., Lang M.A., and Konstandi M. **Stress is a critical player in CYP3A, CYP2C, and CYP2D regulation: role of adrenergic receptor signaling pathways.** *Am J Physiol Endocrinol Metab* 303:E40–E54, 2012

Deitrich R.A. **Genetic aspects of increase in rat liver aldehyde dehydrogenase induced by phenobarbital**. *Science*. 173: 334-336, 1971

Dekeyser J.G., and Omiecinski C.J. **Constitutive androstane receptor.** In*Comprehensive Toxicology:Vol –Cellular and Molecular Toxicology, 2nd ed.,* Elsevier, New York, NY. 169-181, 2010

Dunn T.J., Koleske A.J., Lindahl R., Pitot H.C. **Phenobarbital-inducible aldehyde dehydrogenase in the rat. cDNA sequence and regulation of the mRNA by phenobarbital in responsive rats.** *J Biol Chem* 264:13057-13065, 1989

Forman B.M., Tzameli I., Choi H.S., Chen J., Simha D., Seol W., Evans R.M., and Moore D.D. **Androstane metabolites bind to and deactivate the nuclear receptor CAR-beta**. *Natur*,395:612-615, 1998

Goodwin B, Hodgson E, Liddle C. **The orphan human pregnane X receptor mediates the transcriptional activation of *CYP3A4* by rifampicin through a distal enhancer module.** *Mol. Pharmacol.* 56(6): 1329-1339, 1999

Goodwin B., and Moore J.T. **CAR: detailing new models**. *Trends in Phar. Sci.*,25(8):437-441, 2004

Goodwin B., Moore L.B., Stolts C.M., McKee D.D., and Kliewer S.A. **Regulation of the human CYP2B6 gene by the nuclear pregnane X receptor**. *Mol. Pharmacol.* 60:427-431, 2001

Guo D., Sarkar J., Suino-Powell K., Xu Y., Matsumoto K., Jia Y., Yu S., Khare S., Haldar K., Sambasiva Rao M., Foreman J.E., Monga S.P.S., Peters J.M., Xu H.E., and Reddy J. **Induction of nuclear translocation of constitutive androstane receptor by peroxisome proliferator-activated receptor α synthetic ligands in mouse liver**. *Jour. Biol.Chem.*282(50): 36766-36776, 2007

Hariparsad N., Chu X., Yabut J., Labhart P., Hartley DP., Dai X., Evers R. **Identification of pregnane-X-receptor target genes and coactivator and corepressor binding to promoter elements in human hepatocytes**. *Nucleic Acids Res.* 37 (4), 1160-1173, 2009

Handschin C., Meyer U.A. **Induction of drug metabolism: the role of nuclear receptors**. *Pharmacol. Rev.* 55, 649-673, 2003

Honkakoski P., Jääskeläinen I., Kortelahti M., and Urtti A. **A novel drug-regulated gene expression system based on the nuclear receptor Constitutive Androstane Receptor (CAR)**. *Pharm. Research.* 18(2), 2001

Honkakoski P., and Negishi M. **Protein serine/threonine phosphatase inhibitors suppress phenobarbital-induced Cyp2b10 gene transcription in mouse primary hepatocytes**. *Biochem. J.* 330: 889-895, 1998

Honkakoski P., and Negishi M. **Regulation of cytochrome P450 (*CYP*) genes by nuclear receptors**. *Biochem. J.* 347: 321-337, 2000

Honkakoski P., Moore R., Gynther J., and Negishi M. **Characterization of phenobarbital-inducible mouse *cyp2b10* gene transcription in primary hepatocytes**. *Jour. Biol. Chem.* 271: 9746-9753, 1996

Honkakoski P., Moore R., Washburn K.A., and Negishi M. **Activation by diverse xenochemicals of the 51-base pair Phenobarbital-Responsive Enhancer Module in the CYP2B10 gene**. *Mol. Pharmac.* 53: 597-601, 1998

Honkakoski P, Sueyoshi T, Negishi M. **Drug-activated nuclear receptors CAR and PXR**. *Ann Med* 35: 172-182, 2003

Honkakoski P, Zelko I., Sueyoshi T., and Negishi M. **The nuclear orphan receptor CAR-retinoid X receptor heterodimer activates the phenobarbital responsive enhancer module of the CYP2B gene**. *Mol. Cell. Biol.* 18: 5652-5658, 1998c

Hosseinpour F., Moore R.,Negishi M., Sueyoshi T. **Serine 202 regulates the nuclear translocation of constitutive active/androstane receptor.** *Mol. Pharmacol.* 69: 1095-1102, 2006

Hsu L.C., Chang W-C., Hoffmann I., and Duester G. **Molecular analysis of two closely related mouse aldehyde dehydrogenase genes: identification of a role for Aldh1, but not Aldh-pb, in the biosynthesis of retinoic acid**. *Biochem. J.* 339: 387-395, 1999

Imai J., Yamazoe Y., and Yoshinari K. **Novel cell-based reporter assay system using epitope-tagged protein for the identification of agonistic ligands of constitutive androstane receptor (CAR).** *Drug Metab. Pharmacokinet.* 28(4): 290–298, 2013

Jackson B., Brocker C., Thompson D.C., Black W., Vasiliou K.,Nebert D.N., Vasiliou V. **Update on the aldehyde dehydrogenase gene (ALDH) superfamily**. *Human Genomics*. 5(4): 283–303, 2011

Joannard F., Galisteo M., Corcos L., Guillouzo A., Lagadic-Gossmann D. **Regulation of phenobarbital-induction of CYP2B and CYP3A genes in rat cultured hepatocytes: involvement of several serine/threonine protein kinases and phosphatase.** *Cell Biol. Toxicol.* 16: 325–337, 2000

Kanno Y. and Inouye Y. **Molecular basis of intracellular localization of the Constitutive Androstane Receptor (CAR).** *Journal of Health Science,*54 (3): 261–266, 2008

Kanno Y., Suzuki M., Nakahama T., and Inouye Y. **Characterization of nuclear localization signals and cytoplasmic retention region in the nuclear receptor CAR.** *Bioch.et Bioph. Acta* 1745: 215–222, 2005

Kathmann E.C., and Lipsky J.J. **Cloning of a cDNA Encoding a Constitutively Expressed Rat Liver Cytosolic Aldehyde Dehydrogenase**. *Biochem. and Biophys. Res. Com.*236: 527–531, 1997

Kathmann E.C., Naylor S., and Lipsky J.J. **Rat liver constitutive and Phenobarbital-inducible cytosolic aldehyde dehydrogenases are highly homologous proteins that function as distinct isozymes**. *Biochemistry*. 39: 11170–11176, 2000

Kawamoto T., Sueyoshi T., Zelko I., Moore R., Washburn K., and Negishi M. **Phenobarbital-responsive nuclear translocation of the receptor CAR in induction of the CYP2B gene**. *Mol and Cell Biol.* 19 (9): 6318–6322, 1999

Kawamura A., Yoshida Y., Kimura N., Oda H., and Kakinuma A. **Phosphorylation/dephosphorylation steps are crucial for the induction of CYP2B1 and CYP2B2 gene expression by PB**. *Biochem.Biophys. Res. Commun.* 264: 530–536, 1999

Kawana K., Ikuta T., Kobayashi Y., Gotoh O., Takeda K., and Kawajiri K. **Molecular mechanism of nuclear receptor of an orphan nuclear receptor, SXR**. *Molec. Pharmacol.* 63: 524–531, 2003

Klaunig J.E., Goldblatt P.J., Hinton D.E., Lipsky M.M., Chacko J., and Trump B.F. **Mouse liver cell culture. I. Hepatocyte isolation.** *In Vitro* 17:913–925, 1981

Kliewer S.A., Moore J.T., Wade L., Staudinger J.L., Watson M.A., Jones S.A., McKee D.D., Oliver B.B., Wilson T.M., Zettersto R.H., Perlmann T., and Lehmann J.M. **An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway.** *Cell*92, 73–82, 1998

Kobayashi K., Sueyoshi T., Inoue K., Moore R., and Negishi M. **Cytoplasmic accumulation of the nuclear receptor CAR by a tetratricopeptide repeat protein in HepG2 cells**. *Mol. Pharmacol.*64:1069-1075, 2003

Konno Y., Kodama S., Moore R., Kamiya N, and Negishi M. **Nuclear xenobiotic receptor Pregnane X Receptor locks corepressor silencing mediator for**

retinoin and thyroid hormone receptors (SMRT) onto the *CYP24A1* promoter to attenuate vitamin D_3 activation. *Mol. Pharmacol.*75(2): 265-271, 2009

Konno Y., Negishi M., and Kodama S. **The roles of nuclear receptors CAR and PXR in hepatic energy metabolism**. *Drug Metab. Pharmacokinet.*23: 8-13, 2008

Konstandi M., Kostakis D., Harkitis P., Marselos M., Johnson E.O., Adamidis K., and Lang M.A. **Role of adrenoceptor-linked signaling pathways in the regulation of *CYP1A1* gene expression.** *Biochem Pharmacol* 69:277–287, 2005

Kretschmer X., Baldwin W. **CAR and PXR: xenosensors of endocrine disrupters?** *Chemico-Biological Interactions*155: 111-128, 2005

Küblbeck J, Jyrkkärinne J, Poso A, Turpeinen M, Sippl W., Honkakoski P, Windshügel B. **Discovery of substituted sulfonamides and thiazolidin-4-one derivatives as agonists of human constitutive androstane receptor.** *Biochem. Pharmacol*. 76(10): 1288-1297, 2008

Küblbeck J, Jyrkkärinne J, Molnàr F., Kuningas T., Patel J., Windshügel B.,Nevalainen T., Laitinen T., Sippl W., Poso A, and Honkakoski P.**New in vitro tools to study human constitutive androstane receptor (CAR) biology: Discovery and comparison of human CAR inverse agonists.** *Mol. Pharmaceutics*8(6): 2424-2433, 2011

LeCluyse E.L. **Pregnane X receptor: molecular basis for species differences in CYP3A induction by xenobiotics.** *Chemico-Biological Interactions* 134, 283-289, 2001

Lehmann J.M., McKee D.D., Watson M.A., Wilson T.M., Moore J.T., and Kliewer S.A. **The human orphan nuclear receptor PXR is activated by compounds that regulate CYP3A4 gene expression and cause drug interactions.** *J. Clin. Invest.* 102 (5), 1016-1023, 1998

Li L., Chen T., Stanton J.D., Sueyoshi T., Negishi M., Wang H. **The peripheral benzodiazepine receptor ligand 1-(2-chlorophenyl-methylpropyl)-3-isoquinoline-carboxamide is a novel antagonist of human constitutive androstane receptor.** *Mol. Pharmacol.* 74: 443-453, 2008

Li C-C., Lii C-K., Liu K-L., Yang J-J., Chen H-W. **DHA down-regulates phenobarbital-induced cytochrome P450 2B1 gene expression in rat primary hepatocytes by attenuating CAR translocation.** *Toxicol. And Applied Pharmacol.* 225: 329-336, 2007

Li H. and Wang H. **Activation of xenobiotic receptors: Driving into the nucleus.** *Expert Opin Drug Metab Toxicol*. 6(4): 409-426, 2010

LinJ.H. **CYP induction-mediated drug interactions:in vitro assessment and clinical applications.** *Pharm. Res.* 23: 1089-1116, 2006

Maglich J.M., Parks D.J., Moore L.B., Collins J.L., Goodwin B., Billin A.N., Stoltz C.A., Kliewer S.A., Lambert M.H., Willson T.M. and Moore J.T. **Identification of a novel human constitutive androstane receptor (CAR) agonist and its use in the identification of CAR target genes.** *J. Biol. Chem*. 278: 17277-17283, 2003

Mäkinen J., Frank C., Jyrkkärinne J., Gynther J., Carlberg C.,and Honkakoski P. **Modulation of mouse and human phenobarbital-responsive enhancer module by nuclear receptors.** *Mol. Pharmacol.* 62 (2): 366-378, 2002

Mäkinen J., Reinisalo M., Niemi K., Viitala P., Jyrkkärinne J., Chung H., Pelkonen O. and Honkakoski P. **Dual action of oestrogens on the mouse constitutive androstane receptor.** *Biochem. J*. 376: 465-472, 2003

Mangelsdorf D.J., Thummel C., Beato M., Herrlich P., Schütz G., Umesono K., Blumberg B., Kastner P., Mark M., Chambon P., Evans R.M. **The nuclear receptor superfamily: the second decade.** *Cell*. 83: 835-839, 1995

Marselos M. **Genetic variation of drug-metabolizing enzymes in the Wistar rat.** *Acta pharmacol. et toxicol.* 39: 186-197, 1976

Marselos M., Strom S.C., and Michalopoulos G. **Effect of phenobarbital and 3-methylcholanthrene on aldehyde dehydrogenase activity in cultures of HepG2 cells and normal human hepatocytes.** *Chem.-Biol. Inter*. 62: 75-88, 1987

Matic M., Nakhel S., Lehnert A.M., Polly P., Clarke S.J., Robertson G.R. **A novel approach to investigate the subcellular distribution of nuclear receptors in vivo.** *Nucl. Recept. Signal* 7:e004, 2009

Matic M., Mahns A., Tsoli M., Corradin A., Polly P., and Robertson G.R. **Pregnane X receptor: promiscuous regulator of detoxification pathways.** *Int. Jour. Biol. Chem.* 39:478-483, 2007

Miao J., Fang S., Bae Y., Kemper J.K. **Functional inhibitory cross-talk between constitutive androstane receptor and hepatic nuclear factor-4 in hepatic lipid/glucose metabolismis mediated by competition for binding to the DR1 mootif and to the common coactivators, GRIP-1 and PGC-1 alpha.** *Jour. Biol. Chem.* 281: 14537-14546, 2006

Meyer U.A., and Hoffmann K. **Phenobarbital-mediated changes in gene expression in the liver.** *Drug Metab. Rev.* 31: 365-373, 1999

Moore J.T., Moore L.B., Maglich J.M., and Kliewer S.A. **Functional and structural comparison of PXR and CAR.** *Biochim. Biophys. Acta* 1619: 235-238, 2003

Moore L.B., Parks D.J., Jones S.A., Bledsoe R.K., Consler T.G., Stimmel J.B., Goodwin B., Liddle C., Blanchard S.G., Willson T.M., Collins J.L., and Kliewer S.A. **Orphan nuclear receptors constitutive androstane receptor and pregnane X receptor share xenobiotic and steroid ligands.** *Jour. Biol. Chem.* 275: 15122-15127, 2000

Moreau A., Vilarem M.J., Maurel P., Pascussi J.M. **Xenoreceptors CAR and PXR activation and sequences on lipid metabolism, glucose homeostasis, and inflammatory response.** *Mol. Pharm.* 5: 35-41, 2008

Muangmoonchai R., Smirlis D., Wong S-C., Edwards M., Phillips I.R., and Shephard E.A. **Xenobiotic induction of cytochrome P450 2B1 (CYP2B1) is mediated by the orphan nuclear receptor constitutive androstane receptor (CAR) and requires steroid co-activator 1 (SRC-1) and the transcription factor Sp1.** *Biochem. J.* 355: 71-78, 2001

Mutoh S., Osabe M., Inoue K., Moore R., Pedersen L., Perera L., Rebolloso Y., Sueyoshi T., and Negishi M. **Dephosphorylation of threonine 36 is required for nuclear translocation and activation of human xenobiotic receptor CAR (NR1I3).** *Jour. Biol. Chem.* 284: 34785-34792, 2009

Negishi M. and Honkakoski P. **Induction of drug metabolism by nuclear receptor CAR: molecular mechanisms and implications for drug research.** *Eur. Jour. Of Pharm. Sci.* 11: 259-264, 2000

Numazawa S., Shindo S., Maruyama K., Chibana F., Kawahara Y., Ashino T., Tanaka S., Yoshida T.**Impaired nuclear translocation of CAR in hepatic preneoplastic lesions: Association with an attenuated CYP2B induction by phenobarbital.** *FEBS Letters.* 579: 3560-3564, 2005

Omiecinski C.J., Coslo D.M., Chen T., Laurenzana E.M., and Peffer R.C. **Multi-species analyses of direct activators of the constitutive androstane receptor.** *Toxicol. Sci.* 123(2): 550-562, 2011

Ohno M., Kanayama T., Moore R., Ray M., Negishi M. **The roles of co-chaperone CCRP/DNAJC7 in Cyp2b10 gene activation and steatosis development in mouse livers.** *PLoS ONE.* 9 (12): e115663, 2014

Pappas P., Stephanou P., Karamanakos P., Vasiliou V., Marselos M. **Phenobarbital inducibility and differences in protein expression of an animal model.** *Chem Biol Inter.* 130-132: 275-283, 2001

Pappas P., Stephanou P., Vasiliou V., Marselos M. **Zoxazolamine-induced paralysis in two rat substrains: differences in hepatic drug metabolism.** *Eur J Drug Metab Pharmacokinet* 23: 461-467, 1998

Pascussi J.M., Coniat M.B.L., Maurel P., Vilarem M.J. **Transcripitonal analysis of the orphan nuclear receptor constitutive androstane receptor (NR1I3) gene promoter: identification of a distal glucocorticoid response element.** *Mol. Endocrinol.* 17: 42-55, 2003

Pustylnyak V.O., Gulyaeva L.F Lyakhovich V.V. **CAR expression and inducibility of CYP2B genes in liver of rats treated with PB-like inducers.** *Toxicology* 216: 147-154, 2005

Pustylnyak V.O., Gulyaeva L.F Lyakhovich V.V. **Induction of cytochrome P4502B: role of regulatory elements and nuclear receptors.** *Biochemistry (Moscow)* 72: 608-617, 2007

Pustylnyak V., Pivovarova E., Slynko N., Gulyaeva L., Lyakhovich V. **Species-specific induction of CYP2B by 2,4,6-tryphenyldioxane-1,3 (TPD).** *Life Sciences* 85: 815-821, 2009

Pustylnyak V., Yarushkin A., Kachaylo E., Slynko N.,Lyakhovich V., Gulyaeva L. **Effect of several analogs of 2,4,6-triphenyldioxane-1,3 on constitutiveandrostane receptor activation.** *Chemico-Biol. Inter.* 192:177–183, 2011

Reschly E.J., and Krasowski M.D. **Evolution and function of the NR1I nuclear hormone receptor subfamily (VDR, PXR, and CAR) with respect to**

metabolism of xenobiotics and endogenous compounds. *Curr. Drug Metab.* 7: 349-365, 2006

Shindo S., Numazawa S., Yoshida T. **A physiological role of AMP-activated protein kinase in phenobarbital-mediated constitutive androstane receptor activation and CYP2B induction.** *Biochem. J.* 401: 735-741, 2007

Sidhu J.S., and Omiecinski C.J. **An okadaic acid-sensitive pathway involved in the phenobarbital-mediated induction of CYP2B gene expression in primary rat hepatocyte cultures.** *Journ. Pharmac. Exp. Ther.* 282: 1122-1129, 1997

Smirlis D., Muangmoonchai R., Edwards M., Phillips I.R., and Shephard E.A. **Orphan receptor promiscuity in the induction of cytochromes P450 by xenobiotics.** *The Journal of Biol. Chem.* 276(16): 12822-12826, 2001

Sophos N.A., Pappa A., Ziegler T.L., Vasiliou V. **Aldehyde dehydrogenase gene superfamily: the 2000 update.** *Chem.-Biol. Inter*. (130-132): 323-337, 2001

Squires E.J., Sueyoshi T., and Negishi M. **Cytoplasmic localization of pregnane X receptor and ligand-dependent nuclear translocation in mouse liver.** *Jour. Biol. Chem.* 279: 49307-49314, 2004

Sueyoshi T., and Negishi M. **Phenobarbital response elements of cytochrome P450 genes and nuclear receptors.** *Annu. Rev. Pharmacol. Toxicol*. 41: 123-143, 2001

Sueyoshi T., Kawamoto T., Zelko I., Honkakoski P., and Negishi M. **The repressed nuclear receptor CAR responds to phenobarbital in activating the human CYP2B6 gene.** *Jour. Biol. Chem.* 274: 6043-6046, 1999

Swales K., Negishi M. **CAR, driving into the future.** *Mol. Endocrinol.* 18: 1589-1598, 2004

Timsit Y.E., Negishi M. **CAR and PXR: the xenobiotic-sensing receptors.** *Steroids* 72: 231-246, 2007

Tirona R.G., Leake B.F., Podust L.M., and Kim R.B. **Identification of amino acids in rat pregnane X receptor that determine species-specific activation.** *Mol. Pharmacol.* 65(1): 36-44, 2004

Tolson A.H. and Wang H. **Regulation of drug-metabolizing enzymes by xenobiotic receptors: PXR and CAR.** *Advanced Drug Delivery Reviews*. 62: 1238-1249, 2010

Tompkins L.N., Wallace A.D. **Mechanisms of cytochrome P450 induction.** *Jour. Bioch. And Mol. Toxicol.*21: 176-181, 2007

Tzameli I., Pissios P., Schuetz E.G., and Moore D.D. **The xenobiotic compound1,4-bis[2-(3,5-dichloropyridyloxy)]benzeneis an agonist ligand for the nuclear receptor CAR.** *Mol. Cell. Biol.*20: 2951-2958, 2000

Wada T., Gao J., Xie W. **PXR and CAR in energy metabolism.** *Trends Endocrinol Metab* 20 (6): 273-279, 2009

Wang H., Faucette S., Sueyoshi T., Moore R., Ferguson S., Negishi M., and LeCluyse E.L. **A novel distal enhancer module regulated by pregnane X receptor/constitutive androstane receptor is essential for the maximal induction of CYP2B6 gene expression.** *Jour. Biol. Chem.* 278: 14146-14152, 2003

Wang H., Li H., Moore L.B., Jonhson M.D. Maglich J.M., Goodwin B., Ittoop O.R., Wisely B., Creech K., Parks D.J., Collins J.L., Willson T.M., Kalpana G.V., Venkatesh M., Xie W., Cho S.Y., Roboz J., Redinbo M., Moore J.T., Mani S. **The phytoestrogen coumestrol is a naturally occurring antagonist of the human pregnane X receptor.** *Mol. Endocrinol.* 22(4): 838-857, 2008

Wang H., and Negishi M. **Transcriptional regulation of cytochrome p450 2B genes by nuclear receptors.** *Curr. Drug Metab.* 4: 515-525, 2003

Waxman D.J., and Azaroff L. **Phenobarbital induction of cytochrome P-450 gene expression.** *Biochem. J.*281: 577-592, 1992

Westerlund M., Galter D., Carmine A., Olson L. **Tissue- and species specific expression patterns of class I, III and IV Adh and Aldh1 mRNAs in rodent embryos.** *Cell Tissue Res*. 322: 227-236, 2005

Xia J., and Kemper B. **Structural determinants of CAR required for its GRIP1-mediated nuclear accumulation.** *J. Biol. Chem.* 280: 7285-7293, 2005

Xia J., and Kemper B. **Subcellular trafficking signals of constitutive androstane receptor: Evidence for a nuclear export signal in the DNA-binding domain.** *Drug Metab. And Disp.* 35 (9): 1489-1494, 2007

Xiong H., Yoshinari K., Brouwer K.L.R., and Negishi Masahiko. **Role of constitutive androstane receptor in the in vivo induction of Mrp3 and CYP2B1/2 by phenobarbital.** *Drug Metab. And Disp.* 30(8): 918-923, 2002

Yamamoto Y., Kawamoto T., Negishi M. **The role of the nuclear receptor CAR as a coordinate regulator of hepatic gene expression in defense against chemical toxicity.** *Arch. Biochem. Biophys.*. 409: 207-211, 2003

Yamamoto Y., Moore R., Goldsworthy T.L., Negishi M., and Maronpot R.R. **The orphan nuclear receptor constitutive active/androstane receptor is essential for liver tumor promotion by phenobarbital in mice.** *Cancer Res.* 64: 7197-7200, 2004

Yoon M., Madden M.C., and Barton H.A. **Developmental expression of aldehyde dehydrogenase in rat: a comparison of liver and lung development.** *Toxic. Sci.* 89(2): 386-398, 2006

Yoshinari K., Kobayashi K., Moore R., Kawamoto T., and Negishi M. **Identification of the nuclear receptor CAR:HSP90 complex in mouse liver and recruitment of protein phosphatase 2A in response to phenobarbital**. *FEBS Lett.*.548:17-20, 2003

Yoshinari K., Sueyoshi T., Moore R., and Negishi M. **Nuclear receptor CAR as a regulatory factor for the sexually dimorphic induction of CYP2B1 gene by phenobarbital in rat livers.** *Mol. Pharmacol.* 59(2): 278-284, 2001

Zelko I. and Negishi M. **Phenobarbital-elicited activation of nuclear receptor CAR in induction of cytochrome P450 genes.** *Biochem. And Biophys. Res. Commun.*277:1-6, 2000

Zhou C., Poulton E.J., Grun F.,Bammler T.K., Blumberg B., Thummel K.E., Eaton D.L. **The dietary isothiocyanate sulforaphane is an antagonist of the human steroid and xenobiotic nuclear receptor.** *Mol. Pharmacol.* 71(1):1220-229, 2007