UNIVERZITA KARLOVA V PRAZE

FARMACEUTICKÁ FAKULTA V HRADCI KRÁLOVÉ

Katedra farmakologie a toxikolgie

RIGORÓZNÍ PRÁCE

STANOVENÍ ANTHOKYANIDINŮ JAKO POTENCIÁLNÍCH LIGANDŮ LIDSKÉHO KONSTITUTIVNÍHO ANDROSTANOVÉHO RECEPTORU (hCAR) *IN VITRO*

Vedoucí rigorózní práce:

Doc. PharmDr. Petr Pávek, Ph.D.

Hradec Králové, 2013

Mgr. Dagmar Škoricová

CHARLES UNIVERSITY IN PRAGUE

FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ

Department of Pharmacology and Toxicology

RIGOROSUM THESIS

INVESTIGATION OF ANTHOCYANIDINS AS THE PUTATIVE LIGANDS OF THE HUMAN CONSTITUTIVE ANDROSTANE RECEPTOR (hCAR) *IN VITRO*

Supervisor of the thesis:

Doc. PharmDr. Petr Pávek, Ph.D.

Hradec Králové, 2013

Mgr. Dagmar Škoricová

PROHLÁŠENÍ

Prohlašuji, že tato práce je mým původním autorským dílem, které jsem vypracovala samostatně. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury a jsou v práci řádně citovány. Práce nebyla využita k získání jiného nebo stejného titulu.

DECLARATION

I declare that this work is my original author work, which I had developed by myself. All literature and other sources, which I had used, are given in the list of used literature and they are quoted in text regularly. The work has not been used for achievement of identical or any other academic degree.

Hradec Králové, 16.12. 2013 .....

Dagmar Škoricová

ACKNOWLEDGEMENT

I take this opportunity to sincerely express my deepest gratitude to the supervisor of this thesis, Doc. PharmDr. Petr Pávek, Ph.D. His abundant help, invaluable support, guidance and encouragement provide a great inspiration to my future life and professional career.

Dagmar Škoricová

ABSTRAKT

Univerzita Karlova v Praze

Farmaceutická fakulta v Hradci Králové

Katedra farmakologie a toxikologie

Kandidát: **Mgr. Dagmar Škoricová**

Školitel: **Doc. PharmDr. Petr Pávek, Ph.D.**

Název rigorózní práce: **Stanovení anthokyanidinů jako potenciálních ligandů lidského konstitutivního androstanového receptoru (hCAR) *in vitro***

Anthokyanidiny jsou přírodní flavonoidní sloučeniny vyskytující se u některých druhů ovoce a plodů, které reprezentují důležitou součást zdravé stravy. Nedávné studie naznačují, že vedle neodmyslitelných pozitivních vlivů na lidské zdraví jsou flavonoidy schopné aktivovat xenosensory, a tím regulovat široké spektrum enzymů zapojených do biotransformace léčiv, což může být spojeno se zvýšeným rizikem interakcí mezi potravou a léky.

Lidský konstitutivní androstanový receptor (hCAR) je xenosensor a nukleární receptor, který reguluje aktivitu různých jaterních a mimojaterních biotransformačních enzymů, zvláště enzymů ze superrodiny CYP.

V této rigorózní práci jsme použili molekulárně-biologické metody Gene reporter assay a Two-hybrid assay ke zjištění schopnosti šesti různých anthokyanidinů (cyanidin, delfinidin, malvidin, pelargonidin, peonidin a petunidin) aktivovat hCAR receptor *in vitro*. Zjistili jsme, že žádný ze zkoumaných anthokyanidinů neaktivoval hCAR statisticky signifikantně.

ABSTRACT

Charles University in Prague

Faculty of Pharmacy in Hradec Králové

Department of Pharmacology and Toxicology

Candidate: **Mgr. Dagmar Škoricová**

Supervisors: **Doc. PharmDr. Petr Pávek, Ph.D.**

Title of rigorosum thesis: **Investigating of anthocyanidins as the putative ligands of the human constitutive androstane receptor (hCAR) *in vitro***

Anthocyanidins are natural flavonoid compounds occurring in various fruits and berries, which represent an important part of the normal healthy diet. In addition to their doubtless health benefits, recent studies have suggested that flavonoids are able to activate xenosensors and therefore regulate a wide range of xenobiotic enzymes involved in the biotransformation of drugs, which might be associated with the increased risk of drug-food interaction.

Human constitutive androstane receptor (hCAR) is a xenosensor and a nuclear receptor, which regulates the activity of various hepatic and extrahepatic biotransformation enzymes, especially from the CYP superfamily.

In this thesis we used the molecular biology methods - Gene reporter assay and Two-hybrid assay to examine the ability of 6 diverse anthocyanidins, cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin to activate the hCAR receptor *in vitro*. We found, that none of the examined anthocyanidins has activated hCAR in the statistically significant matter.

TABLE OF CONTENTS

1 ABBREVIATIONS

ABC	ATP-binding cassette
AD	activation domain
AF	activation function
AhR	aryl hydrocarbon receptor
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BBB	blood–brain barrier
BCRP	breast cancer resistant protein
BD	binding domain
CAR	constitutive androstane receptor
CCRP	cytoplasmic CAR retention protein
cDNA	cyclic DNA
CITCO	6-(4-chlorophenyl) imidazo[2,1-b][1,3]thiazole-5-carbaldehyde-O-(3,4-dichlorobenzyl) oxime
CNS	central nervous system
Cy	cyanidin
CYP	cytochrome P450
DBD	DNA binding domain
Dp	delphinidin
DME	drug-metabolizing enzyme

DMEM	Dulbecco´s modified eagle medium
DMSO	dimethylsulfoxid
DNA	deoxyribonucleic acid
EH	epoxide hydrolase
FBS	fetal bovine serum
FMO	flavin-containing monooxydase
FXR	farnesoid X receptor
GST	glutathione S-transferase
hCAR	human constitutive androstane receptor
hPXR	human pregnane X receptor
Hsp90	human heat shock protein 90
LBD	ligand binding domain
Mv	malvidin
mCAR	mouse constitutive androstane receptor
MCP-1	tumor necrosis factor-alfa-induced monocyte chemoattractant protein 1
MDR	multidrug resistance
MDR1	multidrug resistance-associated protein 1
mRNA	messenger RNA
MT	methyltransferase
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NAT	N-acetyltransferase

NES	nuclear export signal
NLS	nuclear localization signal
NQO	NADPH-quinone oxidoreductase
NR	nuclear receptor
OATP	organic anion transport protein
Opti-MEM	medium with reduced serum
P-gp	P-glycoprotein
PB	phenobarbital
PBRE	phenobarbital-response element
PBREM	phenobarbital-responsive enhancer module
PBS	Phosphate buffered saline
Pg	pelargonidin
PK11195	1-(2-chlorophenyl-N-methylpropyl)-3-isoquinoline-carboxamide
Pn	peonidin
PPP1R16A	membrane-associated subunit of protein phosphatase
Pt	petunidin
PXR	pregnane X receptor
RXR	retinoid X receptor
SULT	sulfotransferase
TCPOBOP	1,4-bis[2-(3,5-dichlorpyridyloxy)]benzene
TPR	tetratricopeptide repeat
UAS	upstream activation sequence

UDP	uridine diphosphate
UGT	uridine-5’-diphosphate glucuronosyl transferase
XME	xenobiotic-metabolizing enzyme
XRE	xenobiotic-responsive element
XREM	xenobiotic responsive element

2 THEORETICAL PART

2.1 INTRODUCTION

Permanent exposure and accumulation of xenobiotics within the body can have severe consequences and profoundly affect human health. With the years of evolution, living organisms have developed various means to protect themselves from the threat represented by xenobiotics and this ability has become essential to survival (Nebert and Dieter 2000).

Hepatic xenobiotic metabolizing enzymes of cytochrome P450 (CYP) family play major role in the overall metabolism of endobiotics and xenobiotics in humans. In fact, CYP2B6 and CYP2C enzymes are involved in the metabolism of approximately 20% of all xenobiotics. Furthermore, CYP3A4 alone can metabolize not only 50–60% of clinically used drugs, but it is also essential to the metabolism of an extensive range of endogenous substrates, including bile acid and steroid hormones (Chen et al. 2012).

Activation or inhibition of enzymes from CYP family may therefore severely affect metabolism of a number of clinically used drugs and lead to increased risk of food-drug and drug-drug interactions. As the regulation of most CYP genes is influenced by a variety of ligand-activated nuclear receptors including constitutive androstane receptor (CAR), it is highly important to understand the mechanism of action of CAR and to investigate its putative endogenous and exogenous ligands.

2.2 XENOBIOTICS

Xenobiotics are chemical compounds that are found in and organism, but are normally not produced or not expected to be present in it, thus they are foreign to an organism's normal biochemistry. Those include not only drugs, but also environmental pollutants, dietary supplements, and food additives. These compounds can be often harmless or represent a toxic threat to an organism, but either way they are treated by the body as foreign (Idle and Gonzalez 2007).

Xenobiotics can be eliminated unchanged, but the vast majority utilize endogenous mechanisms such as enzymatic and/or conjugation reactions that facilitate their elimination. They use processes that are also involved in the metabolism and transport of endogenous compounds such as bilirubin, lipids, and steroids (Johnson et al. 2012). Xenobiotic biotransformation does not always result to harmless metabolites, but can actually produce pharmacologically active or even more toxic substance than the original compound (Chen et al. 2012).

2.3 BIOTRANSFORMATION REACTIONS

Biotransformation reactions generally lead to increasing of water solubility of hydrophobic xenobiotic compounds in order to facilitate their elimination. Biotransformation reactions are commonly divided into three broad reaction categories: phase I, phase II and phase III reactions (Figure 1).

Phase I reactions include a multistep process leading to the eventual excretion of the biotransformation products. Phase I reactions can occur as oxidation reactions, where an atom of oxygen is added to the structure of the chemical, reduction reactions, where hydrogen atoms are added, or hydrolysis reactions, where a molecule of water is incorporated, often resulting into breaking of the chemical structure.

Phase II reactions include conjugation reactions, where an endogenous molecule, such as sugar or amino acid, is added on to the foreign chemical. The products of this reaction are usually highly water soluble and thus can be rapidly eliminated in the urine or bile.

Finally, phase III reactions refer to the processes by which transporter proteins facilitate the transport and elimination of chemically modified and conjugated xenobiotics across biological membranes.

Because most xenobiotics that undergo phase I and phase II reactions within a cell lack sufficient lipid solubility to diffuse across cell membranes, it is necessary for these water-soluble metabolites to be actively transported across the cell membrane (Eaton et al. 2008).

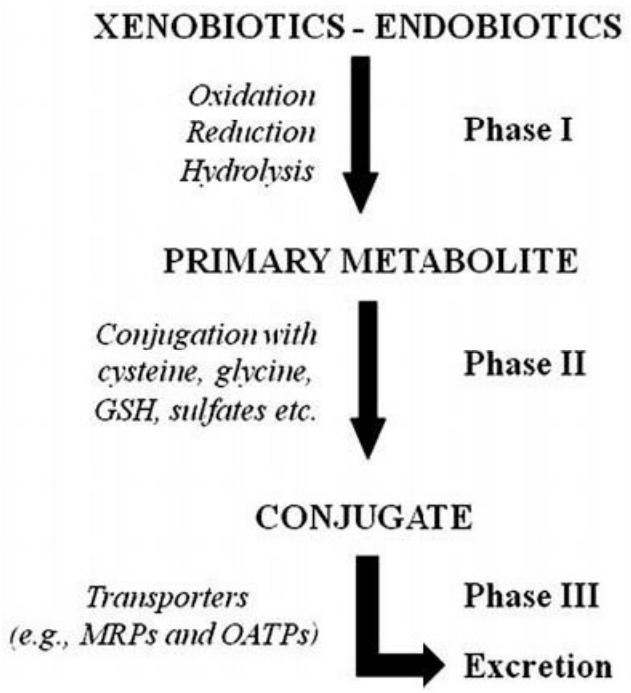


Figure 1 Schematic illustration of the three phases of biotransformation of xenobiotics and endobiotics (di Masi et al. 2009). Phase I reactions give rise to primary metabolites with broken primary structure. Phase II reactions refer to the conjugation reactions with endogenous molecule leading to highly increased water solubility. Phase III reactions leads to actual excretion of the endogenous or exogenous molecule from the organism

2.4 ENZYMES

Xenobiotic or Drug metabolizing enzymes (XME/DME) are involved in the biotransformation reactions and are found in most tissues in the body with the highest levels located in the tissues of the gastrointestinal tract (liver, small and large intestines).

The phase I oxidation reactions are carried out exclusively by CYPs, flavin-containing monooxygenases (FMO), and epoxide hydrolases (EH), while the phase II enzymes include several superfamilies of conjugating enzymes. Among those of most importance belong the glutathione-S-transferases (GST), UDP-glucuronosyltransferases (UGT), sulfotransferases (SULT), N-acetyltransferases (NAT), and methyltransferases (MT) (Figure 2). These conjugation reactions usually require the substrate to contain oxygen, nitrogen and sulfur atoms in the molecule that serve as acceptor sites for a hydrophilic moiety, such as glutathione, glucuronic acid, sulfate, or an acetyl group, that is covalently conjugated to an acceptor site on the molecule (Gonzalez et al. 2006).

ENZYMES	REACTIONS
Phase 1 "oxygenases"	
Cytochrome P450s (P450 or CYP)	C and O oxidation, dealkylation, others
Flavin-containing monooxygenases (FMO)	N, S, and P oxidation
Epoxide hydrolases (mEH, sEH)	Hydrolysis of epoxides
Phase 2 "transferases"	
Sulfotransferases (SULT)	Addition of sulfate
UDP-glucuronosyltransferases (UGT)	Addition of glucuronic acid
Glutathione-S-transferases (GST)	Addition of glutathione
N-acetyltransferases (NAT)	Addition of acetyl group
Methyltransferases (MT)	Addition of methyl group
Other enzymes	
Alcohol dehydrogenases	Reduction of alcohols
Aldehyde dehydrogenases	Reduction of aldehydes
NADPH-quinone oxidoreductase (NQO)	Reduction of quinones

Figure 2 Overview of the XME involved in all three phases of biotransformation reactions (Gonzalez et al. 2006).
mEH and sEH - microsomal and soluble epoxide hydrolase;
UDP - uridine diphosphate;
NADPH - reduced nicotinamide adenine dinucleotide phosphate.

2.4.1 CYPs

Among the phase I DMEs, the cytochrome P450s superfamily (CYPs; P450s) plays the major role in the overall metabolism of lipophilic compounds (Pascussi et al. 2008). In cells CYPs are localized predominantly in the endoplasmatic reticulum and inner mitochondrial membrane. Their expression and activity is influenced by a combination of exogenous and endogenous physiological and patophysiological factors, toxicants and drugs, as well as by genetic factors or hormonal regulation of the organism which results into enormous variation of interindividual drug response (Dvorak and Pavek 2010). It has also been known that the regulation of most CYP genes is influenced by various ligand-activated nuclear receptors (Pavek and Dvorak 2008).

2.5 NUCLEAR RECEPTORS

Nuclear receptors (NRs) constitute a superfamily of phylogenetically related proteins encoded in humans by 48 genes. These proteins are intracellular ligand-activated transcription factors that bind to specific regulatory domains of DNA and directly regulate the expression of target genes involved in number of processes like reproduction, development and general metabolism (Zhang et al. 2003). They include endocrine receptors (receptors for steroid and thyroid hormones and for active metabolites of vitamin A and D), orphan nuclear receptors and so called adopted receptors. Orphan receptors share sequence identity with NRs, but their regulatory ligands have not yet been found. The class of adopted receptors refer to the receptors initially classified as orphan with the ligands subsequently detected (Kachaylo et al. 2011).

NRs share a common, characteristic structure consisting of a highly conserved central DNA-binding domain (DBD) and a less conserved ligand-binding domain (LBD). The DBD regions recognize receptor specific xenobiotic or hormone response elements and guide the receptors to the promoter regions of target genes. In case that NRs are activated under the influence of xenobiotics, this sequence is called xenobiotic responsive element (XRE) (Kachaylo et al. 2011). The LBD region is highly flexible and

possesses subdomains for receptor dimerization, nuclear translocation and binding of nuclear receptor co-regulators (Whitfield et al. 1999).

NRs are able to recognize a variety of structurally diverse compounds and they play an important role in the regulation of wide range of drug metabolizing enzymes (Chen and Nie 2009). NRs of most importance to xenobiotic metabolism and drug therapy are aryl hydrocarbon receptor (AhR), the pregnane X receptor (PXR), and the constitutive androstane receptor (CAR), although the role of other NRs is still under investigations (Dvorak et al. 2008). The main function of these xenosensors is to detect xenobiotics in the cells and coordinate the expression of genes encoding the most appropriate series of XME to inactivate and/or eliminate these compounds (Pascussi et al. 2008).

2.5.1 Aryl hydrocarbon receptor (AhR)

Aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor predominantly expressed in the cytoplasm. It is activated by both polyaromatic hydrocarbons and digoxin (Guenthner and Nebert 1977) and it is involved in the regulation of metabolism of wide scale of xenobiotics, pollutants, drugs and contaminants. Following its discovery in 1977, various exogenous ligands (i.e. polyaromatic amines, polychlorinated aromatic compounds, flavonoids, resveratrol, omeprazole, lansoprazole) and endogenous ligands (bilirubin, biliverdin, indole, tryptofane derivates, eicosanoids, etc.) were uncovered (Dvorak and Pavek 2010). Recently, the AhR has been reported to cross-talk with glucocorticoid receptor during xenobiotic response, which suggests its major role in glucocorticoid induced regulation (Dvorak et al. 2008).

2.5.2 Pregnane X receptor (PXR)

Another member of the NR superfamily, the pregnane X receptor (NR1I2), is primarily expressed in the liver and intestine (Lehmann et al. 1998). In the non-exposed cell it is localized in the nucleus and it was found to respond not only to endogenous pregnanes, but it is also able to bind to a wide range of structurally distinct endogenous and exogenous compounds (Honkakoski et al. 2003).

It has been shown to coordinate the detoxification of endobiotics and xenobiotics by modulating the expression of DMEs, including CYP3A, CYP2B6, and various members of the UGTs superfamily, as well as drug efflux transporters, including P-gp and MRP2. In addition, PXR has been shown to be involved in the regulation of cholesterol homeostasis and bile acid metabolism, and, possibly, in the development of some cancers. This extreme flexibility in ligand recognition and target gene activation of PXR enable it to serve as a xenobiotic sensor for diverse drugs (Chen et al. 2012).

2.5.3 Constitutive androstane receptor (CAR)

The nuclear constitutive androstane receptor (NR1I3) was discovered in 1994 as the receptor MB67 based on its ability to exhibit a high level of basal transcriptional activity that activates expression of reporter gene constructs in the absence of ligand stimulation (Baes et al. 1994). This basal transcriptional activity distinguishes CAR from typical NRs. However, it was subsequently found to be activated also in the presence of a variety of endogenous and exogenous ligands. It is abundantly expressed in the liver, intestine and kidneys (Lehmann et al. 1998), but it was also found to be expressed in small amount in brain and heart (Kachaylo et al. 2011).

Much like PXR, CAR functions as a xenobiotic sensor and regulates a wide spectrum of hepatic and intestinal genes involved in xenobiotic metabolism and transport (Dvorak et al. 2008). Furthermore, CAR was found to cross-talk with PXR during xenobiotic response by binding to the similar xenobiotic responsive elements in their target gene promoters and by regulating overlapping set of xenobiotic metabolizing enzymes (Honkakoski et al. 2003). This include among others various CYPs (i.e. CYP3A4, CYP2B6, CYP2Cs and CYP2A6), phase 2 enzymes (UDP-glucuronosyl transferases - UGTs, glutathione-S-transferases – GST and sulfotransferases - SULTs) and a number of drug and endobiotic transporters (Tolson and Wang). This suggests a highly important physiological role of CAR on xenobiotic metabolism, detoxification and excretion. In addition, CAR has a major influence on various physiological functions, such as gluconeogenesis, lipid metabolism, bilirubin and bile acids excretion, hormonal regulation etc. (Kachaylo et al. 2011) (Figure 3), which makes it an attractive

therapeutic target for treatment of dyslipidemia, obesity or type 2 diabetes (Jiang and Xie 2013).

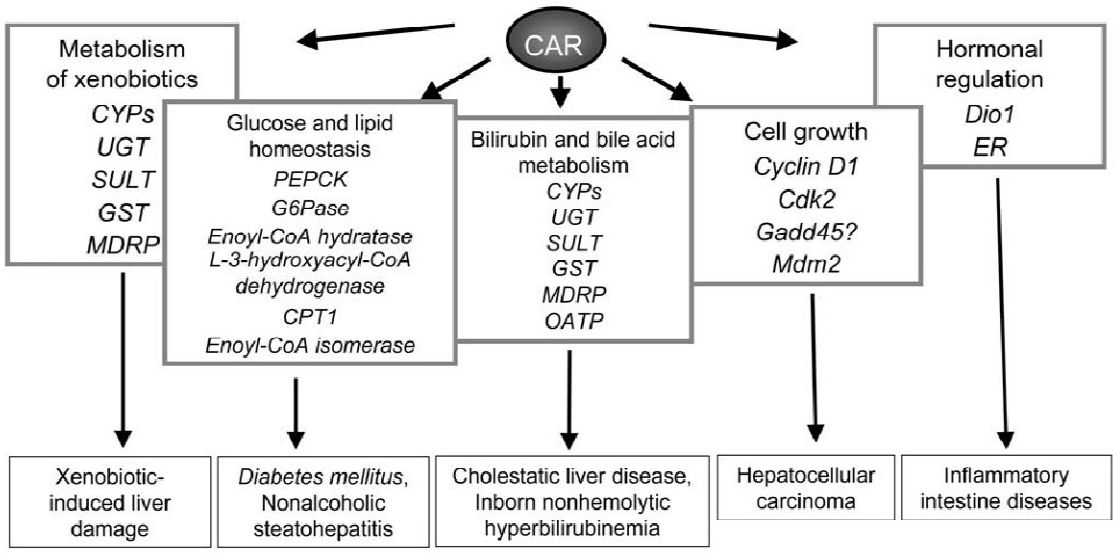


Figure 3 Patophysiological role of CAR. Target genes of CAR include members of all three phases of xenobiotic transformations and enzymes, such as CYPs, UDP-glucuronosyltransferase, sulfotransferase etc., which makes CAR a potential target for treatment of many pathological states (Auerbach et al. 2007; Kachaylo et al. 2011)

Inactivated receptor resides in the cytoplasm, attached to a complex comprised of tetratricopeptide repeat (TPR), cytoplasmic CAR retention protein (CCRP), heat shock protein (hsp90), and PPP1R16A (the membrane-associated subunit of protein phosphatase). This complex inhibits CAR transport through the nuclear membrane (Kachaylo et al. 2011). Ligand dependent or independent activation dissociates CAR from this cytoplasmic complex and translocates it into the nucleus, where CAR induces specific CAR-expression genes (Chen et al. 2012).

2.5.3.1 Structure of CAR

The structure of CAR is similar to the majority of nuclear receptors. The two main structural domains (DBD and LBD) determine its functions (Kachaylo et al. 2011). Moreover, as in other NR, there are two transcriptional activation domains at the

terminals - the activation function 1 (AF-1) in the N-terminal domain, and the activation function 2 (AF-2) in the C-terminal portion of the LBD (Pavek and Dvorak 2008) (Figure 4).

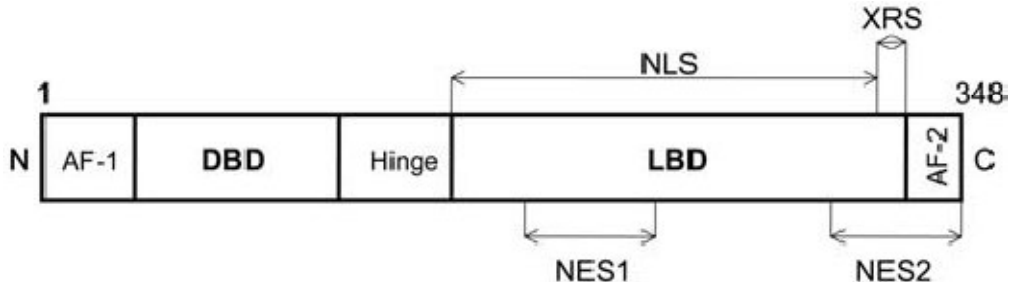


Figure 4 Schematic structure of human CAR

The AF-1 is responsible for the tissue corresponding specific response of the receptor and in CAR is this domain constitutively active in order to ensure its ligand independent activation of CAR. The AF-1 domain is able of conformational change after ligand binding to LBD region, therefore it is essential for the proper ligand-dependent receptor response. The nuclear localization signal (NLS) and nuclear export signal (NES) provide movement from cytoplasm to nucleus after activation. The hinge region is essential for interaction of the receptor with asymmetric sequences of DNA (Kachaylo et al. 2011).

2.5.3.2 CAR Ligands

Clearly, CAR has a capacity for binding a variety of ligands. Generally, ligands for the CAR are all small lipophilic molecules, which enables them to easily diffuse into cells. However, the species variability on response to individual ligands is highly specific, which suggests that rodent model systems do not reflect the xenobiotic response of humans.

The first class of CAR ligands including androstanol (5α-androstan-3α-ol) and androstenol (5α-androst-16en-3α-ol) was identified in 1998 and they were subsequently characterized as inverse agonists, as they repressed the constitutive activity of CAR in vitro (Forman et al. 1998). In 2003, CITCO was identified as the first selective hCAR activator by directly binding and activating hCAR. However, the main endogenous ligand with high affinity to CAR is still unknown. Therefore, CITCO remains

together with 1,4-bis[2-(3,5-dichlorpyridyloxy)]benzene (TCPOBOP) the only compounds that are reported to be able to specifically bind to mCAR and hCAR (di Masi et al. 2009) without repressing its constitutive activity. Recently, the steroid 5β-pregnane-3,20-dione, the pesticide 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene, and probably other endogenous compounds are reported to be putative agonists that activate gene expression when bound to CAR, but their mechanisms of action require further investigations (di Masi et al. 2009).

PK11195, antimycotic clotrimazole, and the antiemetic meclizine are known to be inverse agonists, while they inhibit gene activation by hCAR. However, meclizine preferentially activates mouse CAR, but inhibits gene induction by human CAR (Huang et al. 2004; Swales and Negishi 2004). PK11195 and androstanol may represent a chemical tool for distinguishing indirect activators from direct activators of hCAR, while the repression of the constitutive activity of hCAR with PK11195 could be effectively reactivated with the direct activator CITCO, but not with the indirect hCAR activator phenobarbital (PB) (Li et al. 2008) (Figure 5).

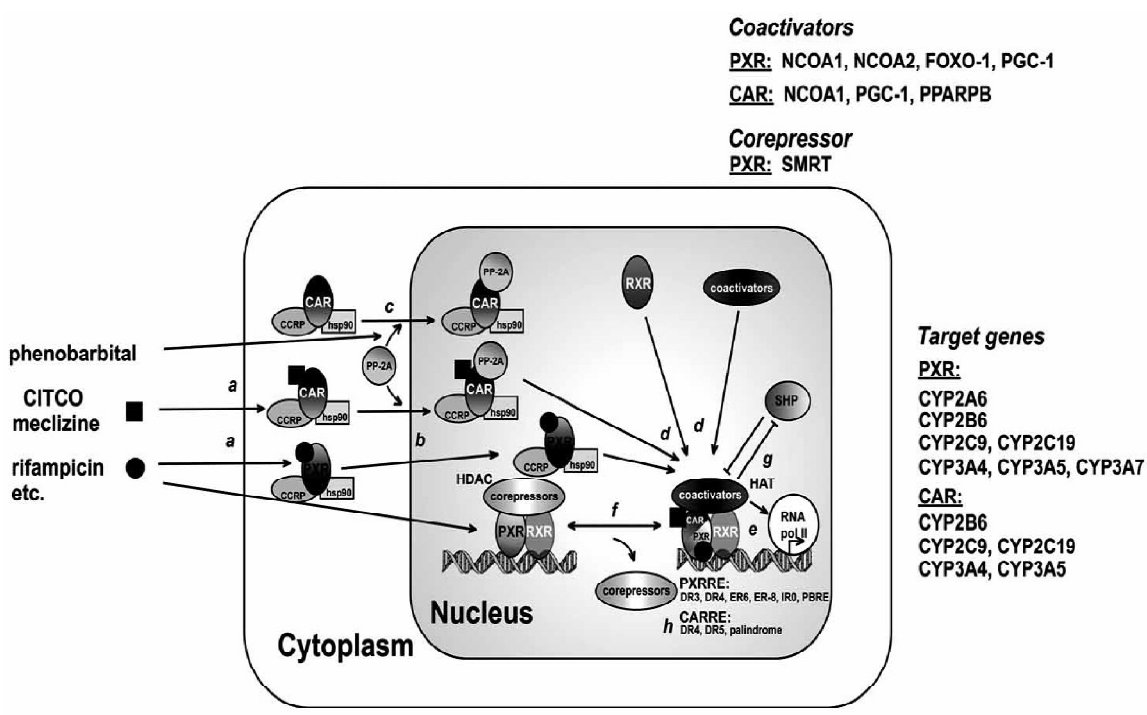


Figure 5 Schematic illustration of PXR and CAR activation mechanisms (Pavek and Dvorak 2008). CAR activation can be mediated directly or by indirect mechanisms while activation of PXR is purely ligand dependent.

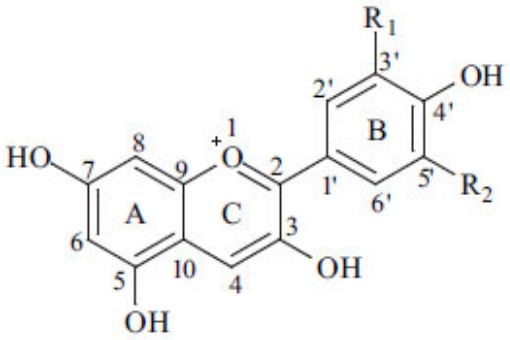
CAR and PXR receptors were also found to establish crosstalk with other nuclear receptors or transcription factors controlling signaling pathways that regulate the homeostasis of bile acids, lipids, glucose, inflammation, vitamins or hormones. These crosstalks are expected to modify the xenobiotic/drug disposition and toxicity and they provide molecular mechanisms to explain how physiopathological stimuli affect xenobiotic/drug disposition, and how xenobiotics/drugs interaction may affect physiological functions and generate toxic responses (Pascussi et al. 2008).

2.5.3.3 Anthocyanidins

Anthocyans (Greek anthos = flower and kyanos = blue) are flavanoic compounds that occur in plants and impart the bright red, blue and purple coloration on vegetables and fruits (Andersen et al. 2006). Over the recent years, numerous epidemiological studies have provided compelling evidence for their preventive and therapeutic benefits profoundly affecting human health (Kong et al. 2003).

Chemically, anthocyans are hydrophilic glycosylated, polyhydroxy or polymehoxy derivates of 2-phenylbenzopyrylium containing two benzoyl rings (A and B) separated by a heterocyclic ring (C) (Figure 6). Anthocyanins refer to a glycosidic-part, while anthocyanidins for the aglycon, responsible for the intense coloration (Cooke et al. 2005). However, in plans are anthocyans present exclusively as glycosidic compounds having glucose, galactose, rhamnose, xylose or arabinose attached to the aglycon. The number of hydroxyl groups and the number and nature of the attached sugar are responsible for the huge variety of anthocyans found in the nature so far, estimated for more than 500 (Hou et al. 2004).

However, there are only 6 anthocyanidins common in higher plants – cyanidin (Cy), delphinidin (Dp), malvidin (Mv), pelargonidin (Pg), peonidin (Pn) and petunidin (Pt) (for structures and colours, see Figure 6). The most widespread anthocyanin in nature is cyaniding 3-glucoside (Kong et al. 2003; Zhang et al. 2005).



Name	R1	R2	Color
Delphinidin	OH	OH	Bluish-red
Petunidin	OCH_3	H	Bluish-red
Cyanidin	OH	H	Orang-red
Pelargonidin	H	H	Orange
Peonidin	OCH_3	H	Orange-red
Malvidin	OCH_3	OCH_3	Bluish-red

Figure 6 Chemical structures of the major anthocyanidins of the higher plants. The most common sugar components of anthocyanins are glucose, galactose, rhamnose, xylose and arabinose, usually attached to the anthocyanidin molecule via C3 hydroxyl group in the ring C. The exact coloration depends on their solution's pH and the presence of chelating metal ions (Hou 2003; Cooke et al. 2005).

Anthocyanidins are natural pigments with hydrophilic character, which makes them interesting for their use as natural water-soluble colorants in food industry (Castaneda-Ovando et al. 2009). The anthocyanidin colours, intensity and relative instability are resulting from their chemical structure and characteristics and are easily affected by number of factors such as pH, storage temperature, concentration, light and oxygen exposure, the presence of solvents, enzymes etc. (Cabrita et al. 2000).

Anthocyanins are *in vivo* either directly absorbed from the gastrointestinal tract and distributed to the blood (McGhie and Walton 2007), or are first metabolized and hydrolyzed by the gut microflora, leading to anthocyanidins and phenolic acid metabolites (Tsuda et al. 1999).

Anthocyanidins carry a positive charge in the central ring structure in contrast to the other flavonoids and due to their hydrophilic character, they are unable to cross the cell membrane by passive diffusion. However, their intracellular activity indicates the obvious existence of specific membrane transporters in mammalian tissues (Hou et al. 2004).

2.5.3.4 Therapeutic effects of anthocyanidins

There is growing evidence that anthocyanidins are more potent as confounders of the therapeutic effect than their glycosidic compound. Moreover, anthocyanidins are able to elicit their activity in different human cell lines at even very low concentrations, at those attained in plasma after consumption of ordinary serving of berries (1nM) (McGhie and Walton 2007).

Anthocyanidins have been reported to be active in cardio- and neuroprotection, and as anti-cancer, anti-inflammatory, normo-lipidemic and normo-glicemic agents (Hou et al. 2004; Chong et al. 2010; Tarozzi et al. 2010; Kausar et al. 2012). Since oxidative stress underlies many chronic degenerative diseases, the common mechanism responsible for these different health-protecting effects might be associated with the strong free radical scavenging and antioxidant properties of anthocyanidins derived from their phenolic structure (Hou et al. 2004; Bornsek et al. 2012). In animal models, anthocyanins and anthocyanidins have demonstrated cancer chemopreventive properties of breast, skin, oeasophageal, lung, oral and gastrointestinal carcinogenesis (Thomasset et al. 2009). However, the cancer chemoprevention by anthocyanidins is mediated not only by their strong antioxidant properties, but they were found to be directly involved in the anticancerogenesis and in the apoptosis induction of tumor cells (Hou 2003). In addition, they confound many signaling events involved in oncogene expression, cellular transformation, cell-cycle regulation, metastasis and angiogenesis through their effects on a myriad of signaling molecules, including DNA repair genes and transcription factors (Konczak and Zhang 2004).

In addition, *in vitro* and animal studies have shown a protection against cardiovascular diseases and chronic inflammation after moderate consumption of

anthocyanidins, which is mediated through the decrease of tumor necrosis factor-alfa-induced monocyte chemoattractant protein 1 (MCP-1) production (Garcia-Alonso et al. 2004; Toufektsian et al. 2008). MCP-1 is a chemokine that recruits macrophages to infection or inflammation sites, whose modulation of expression is likely to make a significant contribution to the treatment of atherosclerosis in the future (Garcia-Alonso et al. 2009).

2.5.4 CAR/PXR and multidrug resistance

Multidrug resistance (MDR) is a phenomenon characterized by decreased intracellular drug retention and changed therapy response and is one of the primary factors that lead to cancer therapy failure (Leonessa and Clarke 2003). As MDR is a complex process involving a large scale of enzymes and transporters, it is therefore highly important to develop multi-targeted strategies to overcome the induction of MDR.

NR xenosensors, such as PXR and CAR have been found to be expressed in elevated levels in breast, prostate, intestinal, colon and endometrial cancer cells, therefore it is speculated, that CAR and PXR might enhance tumorgenesis. Furthermore, CAR/PXR have been found to be activated by most chemotherapeutic agents and co-administered drugs and thus it is speculated that these receptors may play important roles in cancer MDR (Chen et al. 2012).

Efflux transporters of the ATP-binding cassette (ABC) gene family are localized in the apical membranes of blood–brain barriers (BBB) endothelial cells, enterocytes and hepatocytes (Eisenblatter et al. 2003). They main function is to determine the drug distribution to and from the central nervous system (CNS) and intestine and modulate the hepatobiliary excretion (Kusuhara and Sugiyama 2002). They mediate the active extrusion of many xenobiotics (nutrients, toxins, drugs) and their metabolites back into the capillary lumen and intestine. Moreover, transporters have been implicated in drug interactions that may possibly increase the likelihood of adverse effects (Dreiseitel et al. 2009) .

Among the ABC efflux transporters are the multidrug resistance protein 1 (MDR1, P-glycoprotein) and the breast cancer resistance protein (BCRP), both prominently expressed not only in organs important for absorption (small intestine), distribution (placenta and BBB) and elimination (liver, kidney, small intestine) of xenobiotics (Gottesman et al. 1996), but they were also found in several tumors (Robey et al. 2007).

Recent studies also demonstrated that dietary flavonoids significantly activated hCAR, raising the possibility that dietary anthocyanidins are also able to activate CAR to stimulate detoxification and energy expenditure (Yao et al. 2010). In addition, distinct affinities of anthocyanins and anthocyanidins were found for the human efflux transporter BCRP and MDR1, which suggests that anthocyans are able to interfere with transport and the pharmacokinetics of other substrates of ABC transporters (Dreiseitel et al. 2009).

3 AIM OF THE WORK

The aim of this thesis is to examine the ability of 6 different anthocyanidins (cyanidin, delphinidin, malvidin, pelargonidin, peonidin a petunidin) to activate human constitutive receptor (hCAR) *in vitro* using the gene reporter assay and the assembly two-hybrid assay method, which may contribute to understanding of the underlying mechanisms of the therapeutic effects of anthocyanidins *in vivo*.

4 MATERIALS

4.1 Cells

HepG2 cell line

The cells were obtained from The European Collection of Cell Cultures (ECACC)

4.2 Plasmids

pPBREM(CYP2B6)-lucP – luciferase reporter gene construct (Gene Script)

pRL-TK – expression vector for Renilla luciferase (Promega, Madison, WI)

pCR3-CAR – king gift from Dr. Negishi

phCAR-c/BIND (Act) – generated in our laboratory, originally generated by Kaoru Kobayashi et al. 2010

phCAR-N/BIND – generated in our laboratory, originally generated by Kaoru Kobayashi et al. 2010

pGAL4-UAS (Promega, Madison, Wi)

4.3 Materials

Carbon dioxide tank (Air Liquid Medical GmbH; Düsseldorf, GE)

Coverslips (Medite GmbH; Brugdorf, GE)

Falcon tubes 15 ml, 50 ml (Sarstedt AG & Co.; Nümbrecht, GE)

Glass pipettes (Axon Labortechnik GmbH; Kaiserslautern, GE)

Microtube 1.5 ml (Sarstedt AG & Co.; Nümbrecht, GE)

Pipette (Gilson, Inc.; Middleton, WI, USA)

Pipette boy – neoAccupette (Wager & Munz GmbH; München, GE)

Pipette tips (Greiner bio-one GmbH; Frickenhausen, GE)

Tissue culture dishes (Becton Dickinson Labware; Le Pon de Claix, France)

4.4 Instruments

Centrifuge (Kendro Laboratory Products GmbH; Osterode, GE)

CO_2 Incubator (Thermo Fisher Scientific Inc.; Egelsbach, GE)

Hood (Thermo Electron LED GmbH; Langenselbold, GE)

Mikroskope Leica DM LS2 (Leica Microsystems GmbH; Wetzlar, GE)

Nanodrop (Peqlab Biotechnologie GmbH; Erlangen; GE)

Thermomixer (Eppendorf AG; Hamburg, GE)

Vortex machine (IKA® Werke GmbH & Co. KG; Staufen, GE)

Luminometer (Tecan, USA)

4.5 Chemicals

All used chemicals were in the highest purity.

Androstanol (Sigma-Aldrich St.Louis, MO)

Anthocyanidins: Cyanidin (Extrasynthese, France)
Delfinidin (Extrasynthese, France)
Malvidin (Extrasynthese, France)
Pelargonidin (Extrasynthese, France)
Peonidin (Extrasynthese, France)
Petunidin (Extrasynthese, France)

CITCO (Biomol, Plymouth Meeting, PA)

DMSO (Sigma-Aldrich St. Louis, MO)

Dual-Luciferase Reporter Assay System (Promega, Madison, WI)

Ethanol 99% (Merck KGaA; Darmstadt, GE)

FBS (PAA, Pasching, Austria)

Lipofectamine 2000 (Invitrogen GmbH, Carlsbad, CA)

PK11195 (Sigma-Aldrich St. Louis, MO)

Stop and Glo Buffer (Promega, Madison, WI)

4.6 Enzymes

Trypsin (Sigma-Aldrich Chemie GmbH; Steinheim, GE)

4.7 Mediums

DMEM (Gibco, Invitrogen GmbH; Carlsbad, CA)

Opti-MEM (Promega, Madison, WI)

4.8 Solutions

Table 1 Stock solutions

TBS 10x:	24.23 g Tris
	0.06 g NaCl
	mix in 800 ml ddH_2O
	adjust to pH 7.6 with HCl
	add 1000 ml ddH_2O

PBS 10x:	80.0 g NaCl
	2.0 g KCl
	18 g $Na_2HPO_4.2H_2O$
	2.4 g KH_2PO_4
	adjust to pH 7.4 with HCl
	add up 1000 ml with ddH_2O

4.9 Software

Endnote X1

GraphPad Prism 6.01

XFluor4

Microsoft Office

5 METHODS

5.1 Cell culture of HepG2 cells

HepG2 epithelial cell line has been isolated from a liver biopsy of a 15 year old male Caucasian, with a well differentiated hepatocellular carcinoma. The cells secrete a variety of major plasma proteins and have been grown successfully in large scale cultivation systems. Hepatitis B virus antigens have not been detected. The cells respond to stimulation with human growth hormone.

5.1.1 Cell culture cultivation

The cells were cultivated and maintained according to the manufacturer's instructions, in antibiotic-free Dubecco's modified Eagle media nutrient mixture (DMEM) supplemented with 2mM glutamine, 1% nonessential amino acids and 10% FBS. The cells were grown in the medium for 3 days at 37°C in an atmosphere of 5 % CO_2. The vitality of the culture was checked every day under microscope and the medium was replaced by the fresh one. The passaging of the cells was done depending on the culture density every 3-4 days (at the confluence of 70-80%).

5.1.2 Replacing of the medium

The old medium was removed and the cells were washed with 3 ml of PBS. Afterwards, cells were enzymatically digested with 1 ml of the 0.25% trypsin and incubated for 1 minute at 37°C. The cells were subsequently mechanically dissociated with glass pipette in order to detach the culture from the flask wall. The digestion was stopped by adding 10% FBS and fresh DMEM.

On the third day, achieving the confluence of 80-90%, the medium was replaced and 160 µl of the cell solution (approximate concentration of 30.000 cells/well) was carefully inoculated into a 48-well plate and let for incubation at 37°C in an atmosphere of 5 % CO_2 for 24 hours. On the day of transfection, the medium was replaced with the fresh one directly in the well plate shortly before the experiment.

5.2 Gene reporter assay

A gene reporter assay is a widely used method for study of gene expression, intracellular signalization and receptor or transcription factor activity, in cells or organisms.

A reporter gene (reporter) is a gene that is attached to a regulatory sequence of another gene of interest and inserted in the plasmid to bacteria, cell culture or organisms. Expression of reporters is always bound with a characteristic measurable signal that serves as an indicator of expression of the gene of interest. We used a gene for Firefly luciferase as a reporter gene, which is transcripted into mRNA together with the regulatory elements of the plasmid. The expression of the reporter gene is detected enzymatically or based on chemiluminescence after transcription and translation of the Luciferase reporter gene.

In a dual-luciferase assay, a control plasmid containing a Renilla luciferase is co-transfected together with the reporter plasmid. This plasmid uses a constitutively active promoter and provides a reference to compare results against, as two individual reporter enzymes are measured in one complex. This reduces error due to experimental variations such as cell handling or transfection effectivity, therefore, a dual-luciferase reporter assay enables more reliable interpretation of experimental analysis.

In this thesis, we used Dual-Luciferase® Reporter Assay System from Promega, where the activity of a Firefly luciferase and Renilla luciferase is measured in one single solution.

5.2.1 Reporters

The luciferase reporter assay is based on the interaction of the enzyme luciferase with a luminescent substrate luciferin, which releases light by the process of bioluminescence. The firefly luciferase, from Photinus pyralis, is the most commonly used bioluminescent reporter due to both sensitivity and convenience of the enzyme assay and due to the tight coupling of protein synthesis with enzyme activity. It is a

monomeric enzyme that catalyzes a two-step oxidation reaction to yield green to yellow light. Upon mixing with substrates, firefly luciferase produces an initial burst of light that decays over about 15 seconds to a low level of sustained luminescence. The gene encoding firefly luciferase (*luc*) is a cDNA and does not require any post-translational modifications. This means it is available as a mature enzyme directly upon translation from its mRNA. The amount of firefly luciferase is dependent on the activity of the regulatory element being studied (Figure 7).

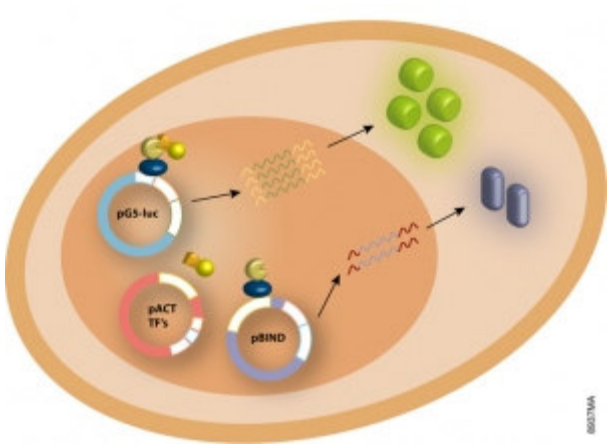


Figure 7 Illustration of the cell, with two reporter plasmids with chemiluminescent signals (http://promega.wordpress.com/).

The other commonly used luciferase is Renilla luciferase from Renilla reniformis, a coelenterate that creates bright green flashes upon tactile stimulation. Renilla luciferase is a monomeric enzyme that catalyzes the oxidation of coelenterazine to yield coelenteramide and blue light (Figure 8). Like firefly luciferase, the reporter gene for Renilla is a cDNA called Rluc that needs no post-translational modifications for the enzymatic activity as well. The production of Renilla luciferase is independent of experimental regulatory element activity and serves also as an indicator of a transfection efficiency.

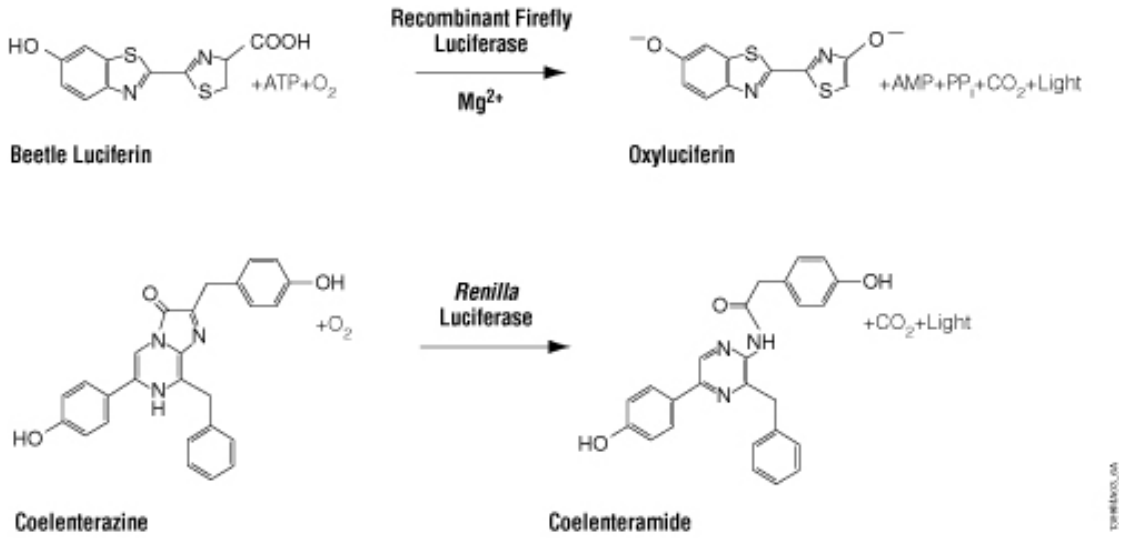


Figure 8 Schematic bioluminescent reactions of Firefly and Renilla luciferase with their respective substrates, to yield light (www.promega.com).

5.2.2 Plasmid solutions

pRL-TK – expression vector for *Renilla* luciferase (control reporter)

pPBREM(CYP2B6)-lucP – Reporter gene construct containing the regulatory sequence of hCAR (from CYP2B6 gene), viral SV40 promoter with binding domain for DNA Polymerase II and basal transcription factors, and reporter gene for firefly luciferase. PBRE region (Phenobarbital-responsive enhancer region) of the CYP2B6 gene is a 51-base-pair enhancer element that mediates induction of hCAR expression.

pCR3-CAR – expression plasmid for nuclear receptor CAR

5.2.3 Transfection procedure

Transfection is a process of delivering nucleic acids into eukaryotic cells, which can be accomplished by a variety of methods. In this experiment, we used the lipid based transfection using liposomes as the transfection agents. Liposomes are colloid particles composed of cationic lipid bilayer that create complex with negatively charged nucleic acid, which possess the ability to fuse with the cell membrane and deposit their cargo inside of the cell.

We used Lipofectamin 2000 for the incorporation of the genes of interest into the cells. Lipofectamin 2000 is used for transfection of nucleic acids into eukaryotes and it is able to achieve high efficiencies. The complex DNA-Lipofectamin is directly incubated with cells in the medium. The ratio of DNA (ng):Lipofectamin 2000 (µl) is set by the manufacturer of 1:2 to 1:3. For the transfection the confluence of 90-95% is recommended in order to achieve the best efficacy of the experiments. It is recommended to add Opti-MEM/Reduced Serum Medium to the Lipofectamin 2000 solution in order to prevent the complex of DNA with FBS.

For the transfection of DNA, Lipofectamine complex was prepared by mixing the Opti-MEM solution with Lipofectamin 2000. Subsequently, the previously calculated DNA amount was combined with the diluted Lipofectamine and incubated for 20 minutes at the room temperature.

We used 50 ng of pRL-TK plasmid, 100 ng of hCAR expression vector and 200 ng of pPBREM(CYP2B6)-lucP reporter gene plasmid for each well. As a negative control, 1‰DMSO in DMEM was used.

The DNA-Lipofectamine complex was added into cell solution with fresh medium located into 48-well plate at the amount of 60 µl to each well. The cells were incubated overnight at 37 °C in the atmosphere of 5 % CO_2 .

5.2.4 Treatment of the cells

The next day media containing the DNA-Lipofectamine complexes were removed and the cells in each well were treated with 150 µl of previously prepared treating solution composed of testing compounds in DMEM and let incubated overnight at 37°C in the atmosphere of 5 % CO_2. The experiment was carried out under subdued light to minimize photo degradation of the test agents.

After 24 hours, the medium was removed and the cells were washed once with previously heated PBS. 100 µl of the hypoosmotic Passive lysis buffer were added and the plate was let frozen. This procedure ensures a complete lysis of cells by creating crystals that penetrate through the cell walls.

5.2.5 Signal detection

40 µl of the lysate were put into 96-well plate and 40 µl of Luminol were added to start the chemiluminescent reaction. The amount chemiluminescence of Firefly luciferase was measured on plate luminometer Genios Plus (Tecan).

Subsequently, the Stop&Glo solution was added into Stop&Glo buffer in the ratio of 1:50 and 30 µl of this solution was added into each analyzed well. This creates a substrate for Renilla luciferase, which can be measured on the same luminometer.

5.3 Assembly two-hybrid assay

An assembly two-hybrid assay is a molecular biology method used to study protein-protein interaction, protein-DNA interaction, gene expression and its function in living cells and organisms. It is based on a fact that the DNA binding domain and the transactivation domain are separable in most transcription factors.

In simple two-hybrid assay is the transcription factor split into two separate fragments-the binding domain (BD), responsible for binding of a transcription factor to the upstream activation sequences (UAS) and activating domain (AD), essential for transcription of the reporter gene. The interaction between these two chimeric proteins, each containing one part of a transcription factor, results to an increase in transcription from a reporter gene, which can be detected enzymatically or based on chemiluminescence.

The difference between the assembly two-hybrid assay and the simple two-hybrid assay lies in the fact that in addition to splitting the transcription factor into two fragments is the receptor of interest spitted into two separate parts, whose successful assembly is required for a reaction between the studied substance and the receptor.

In our experiment, we used the GAL4 gene, encoding the yeast transcription activator protein Gal4, as a source of the DNA binding domain, which specifically binds to the UAS in the luciferase. Our gene of interest was placed under control of GAL4 UAS and therefore was activated only in the presence of Gal4.

As a source of the activating domain we used the herpes simplex virus activator protein VP16, which activates the promoter and therefore, in the presence of GAL4 UAS, initiates the luciferase reporter gene transcription (Figure 9). Both activating and binding domain were attached to one part of our receptor of interest - hCAR (ACT or BIND).

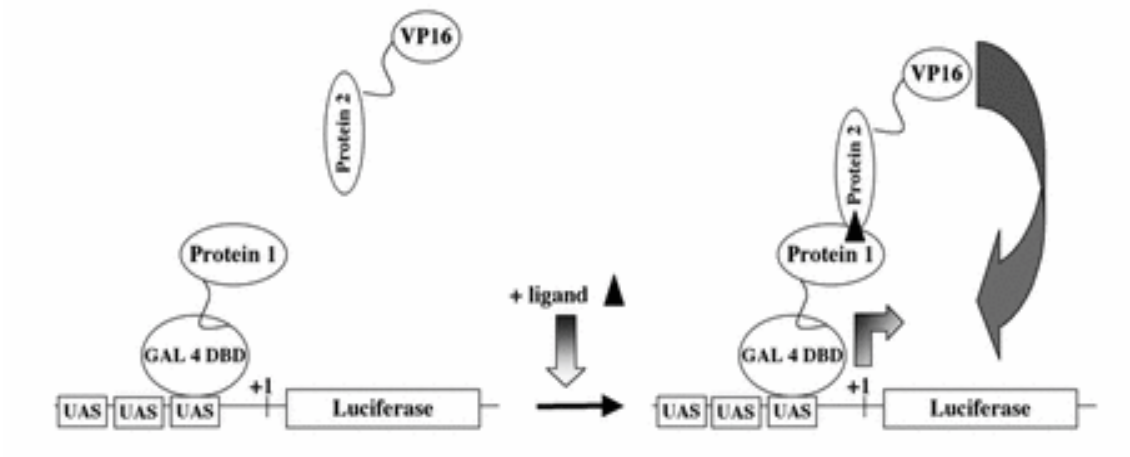


Figure 9 Schematic illustration of the two hybrid assay. Transcription of a reporter gene, luciferase, is activated upon interaction of protein 1 with protein 2 due to proximity of the VP16 activation domain to the promoter (Tyree and Klausing 2003).

5.3.1 Plasmid solutions

pRL-TK - expression vector for *Renilla* luciferase (control reporter)

phCAR-C/ACT - expression plasmid A for nuclear receptor CAR

phCAR-N/BIND - expression plasmid B for nuclear receptor CAR

pGAL4-UAS - the yeast transcription activator protein Gal4 with an upstream activation sequence

In our experiments we used assembly two hybrid assay in ligand binding setup using phCAR-C/ACT and phCAR-N/BIND plasmids (Kobayashi et al. 2010). In the assay, two parts of hCAR ligand binding domain are connected to form hCAR ligand binding domain if a tested compound is an hCAR ligand.

5.3.2 Transfection procedure

Transfection was performed as described previously. The cell confluence was around 90-95% and the ratio of DNA (ng):Lipofectamin 2000 (µl) was set to 1:2.

The DNA-Lipofectamine complex was added into cell solution with fresh medium located into 48-well plate at the amount of 60 µl to each well. The cells were incubated overnight at 37 °C in the atmosphere of 5 % CO_2 .

5.3.3 Treatment of the cells

The next day medium containing the DNA-Lipofectamine complexes was removed and the fresh solutions of tested anthocyanidins were prepared in DMEM for each experiment. They were diluted to the final 10 µM and 50 µM concentration in culture media immediately before adding to the cells.

Cells in each well were treated with 150 µl of the previously prepared treating solution and let incubated for overnight at 37 °C in the atmosphere of 5 % CO_2. The experiment was carried out under subdued light to minimize photo degradation of the test agents.

After 24 hours, the medium was removed and the cells were washed once with previously heated PBS. 100 µl of the hypoosmotic Passive lysis buffer was added and the plate was let frozen.

5.3.4 Signal detection

The Firefly luciferase and a control Renilla luciferase detection was performed as previously described using a plate luminometer Genios Plus (Tecan) to detect a chemiluminescence signal.

5.4 Statistical analysis

All data are presented as mean ± SEM. Statistical comparison of two groups was provided using Student's unpaired two-tailed t-test whereas multiple groups comparison by using one way analysis of variance (ANOVA) with Dunnett's posthoc

test. Vehicle (DMSO 1‰)-treated cells were used as reference groups. $P<0.05$ was considered to be statistically significant difference.

6 RESULTS

6.1 Induction of hCAR expression on the pPBREM(CYP2B6)-lucP reporter plasmid

First we used gene reporter method in order to first examine the ability of well known hCAR ligands to regulate its expression on the pPBREM(CYP2B60)-lucP reporter plasmid. Thus, after transfection procedure and 24 hour treatment of the cell culture of HepG2, the fluorescent signal was detected using luminometer and compared with signal intensity of the culture treated with the DMSO 1‰ alone, used as a negative control.

We observed an activation of hCAR after CITCO treatment, which was used as a positive control. PK11195 failed to activate receptor, whereas treatment with both PK11195/CITCO led to reactivation of the receptor compared to PK11195 alone (Figure 10).

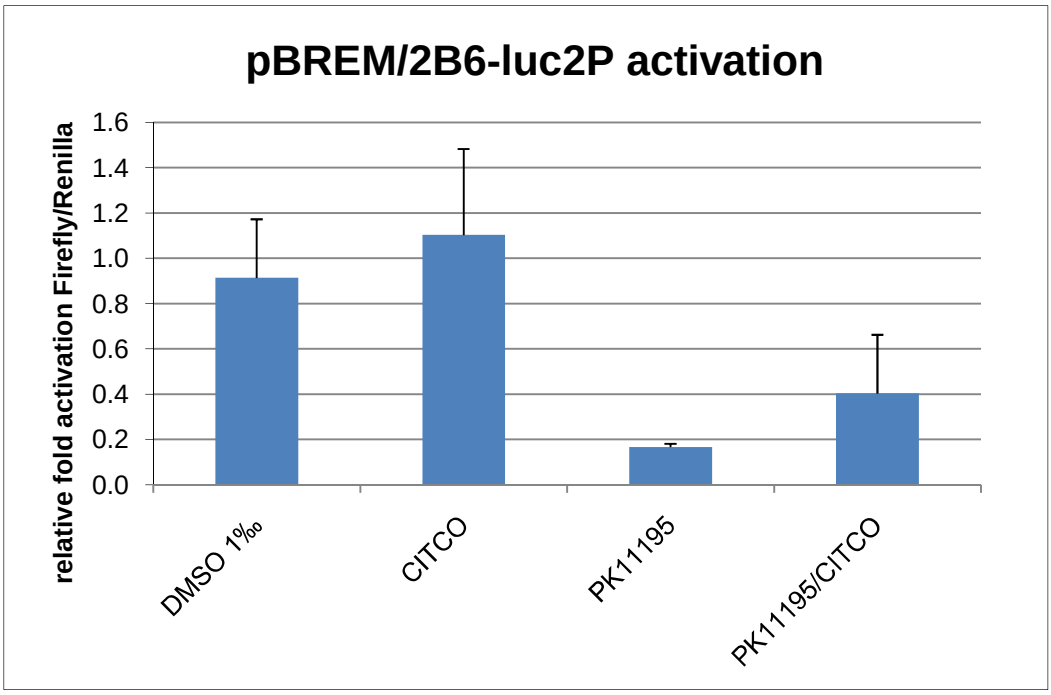


Figure 10 Induction of hCAR expression by well-known hCAR ligands on the pPBREM(CYP2B6)-lucP reporter plasmid in HepG2 cells detected by luminometer showing an increased transcriptional activity of hCAR after 24 hour CITCO exposure. PK11195 as an inverse agonist failed to activate hCAR whereas PK111195/CITCO treatment led to reactivation of hCAR. Vehicle treatment (DMSO 1‰) was used as a reference group. HepG2 cells were incubated for 24 hours with the solutions of testing compounds after previously performed transfection. Firefly luciferase activities were normalized to Renilla luciferase signal and were expressed relative to basal control levels.

Results of this experiment were not statistically examined, as they provided the informative overview of the function of the method on the pPBREM(CYP2B6)-lucP reporter plasmid. It serves as reference for a subsequent experiment on the pPBREM(CYP2B6)-lucP reporter plasmid with the testing compounds of interest - anthocyanidins.

6.2 Induction of hCAR by anthocyanidins

It has been reported that the antioxidative and therapeutic properties of anthocyanidins are associated with the activation of various hepatic enzymes and receptors responsible for the biotransformation of xenobiotics.

Firstly, we used a gene reporter assay method in order to study the ability of 6 different anthocyanidins in a concentration of 25 μM to activate hCAR. We used luminometer to specifically measure the activity of a reporter gene after transfection procedure and treatment of the HepG2 cell culture with 6 different anthocyanidins - cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin. Results of the experiment were subsequently evaluated using one way analysis of variance (ANOVA) with Dunnett's posthoc test.

We found out that all of the examined anthocyanidins failed to activate hCAR in the statistically significant matter after 24 hour treatment with anthocyanidins in contrast to DMSO 1‰ used as negative controls (Figure 11).

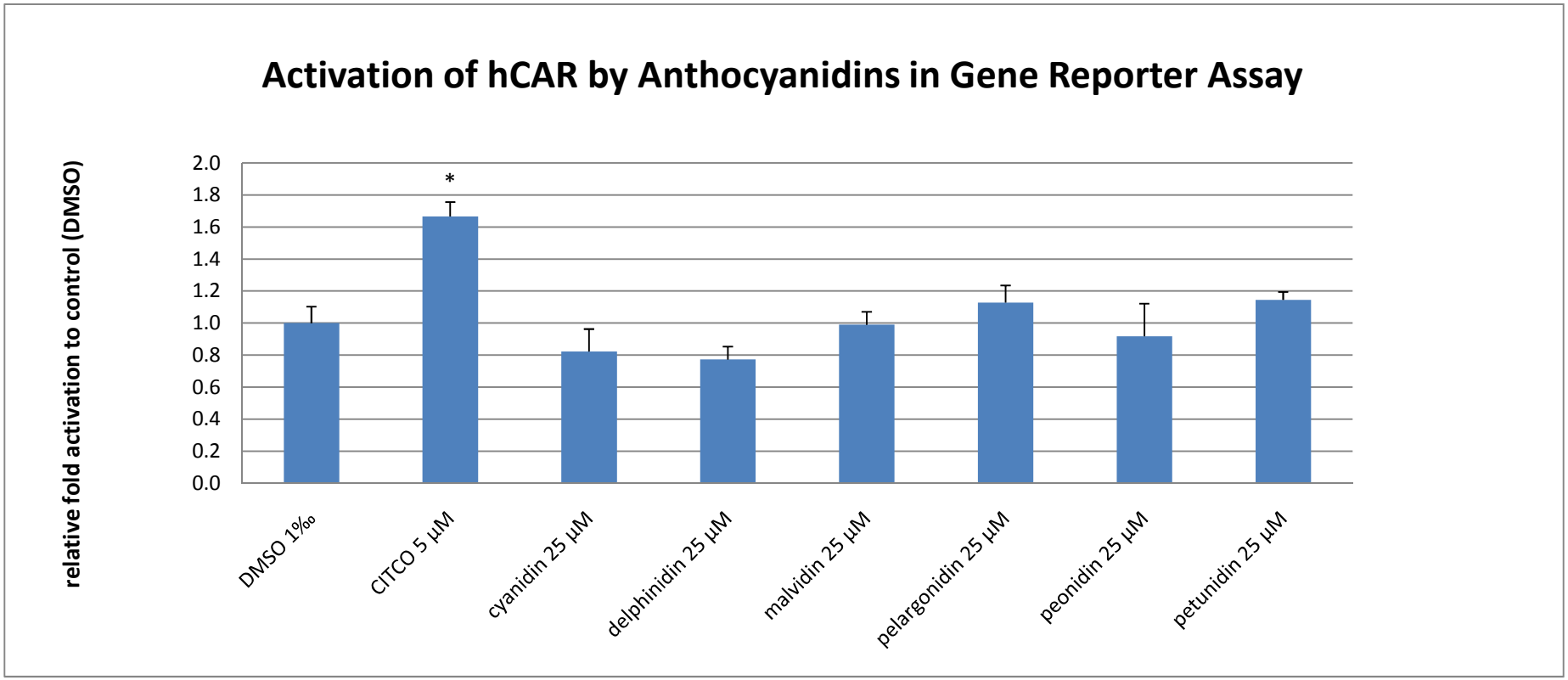


Figure 11 Quantification of Induction of hCAR expression by anthocyanidins in HepG2 cells in Gene Reporter Assay detected by luminometer showing statistically not significant changes in response after 6 different anthocyanidins exposure in the concentration of 25 µM compared to DMSO 1‰. Firefly luciferase activities were normalized to Renilla luciferase signal and were expressed relative to basal control levels, which were assigned a value of 1. * P < 0.05.

6.3 Activation of hCAR by anthocyanidins in assembly two hybrid Assay

In order to double prove the negative results of the previous experiment, we used another molecular biology method, assembly two hybrid assay, to examine the ability of anthocyanidins to activate hCAR. The two hybrid assembly assay may provide more accurate results compared to the gene reporter assay due to its higher sensitivity caused by the need of assembly of the hCAR receptor prior to the actual activation by the tested compounds.

After transfection procedure, we treated the HepG2 cell culture for 24 hours with anthocyanidins in a concentration of 10 µM and 50 µM in order to investigate the possible concentration dependent effects of anthocyanidins. Subsequently, the fluorescent signal was detected using luminometer and compared with signal intensity of the culture treated with the DMSO 1‰ alone, used as a negative control.

In line with our previous observation, we found out that all of the examined anthocyanidins, both in concentration of 10 µM and 50 µM failed to activate hCAR in the statistically significant matter after 24 hour treatment in contrast to DMSO 1‰ used as negative control (Figure 12).

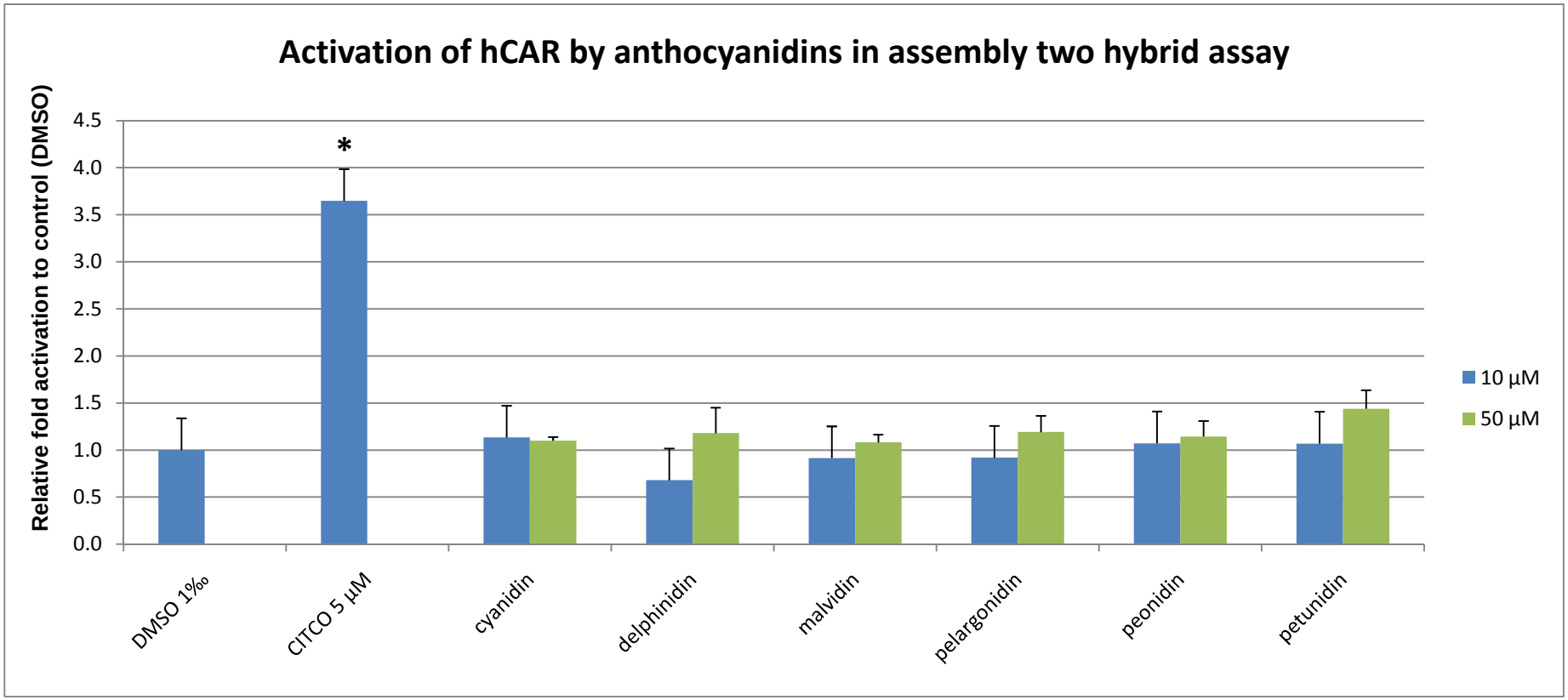


Figure 12 Quantification of Induction of hCAR expression by anthocyanidins in HepG2 cells in Assembly Two Hybrid Assay detected by luminometer showing statistically not significant changes in response after 6 different anthocyanidins exposure in 10 µM and 50 µM concentration compared to DMSO 1‰, used as a negative control. Firefly luciferase activities were normalized to Renilla luciferase signal and were expressed relative to basal control levels, which were assigned a value of 1. * $P < 0.05$.

7 DISCUSSION

In this thesis we aimed to investigate the ability of 6 different anthocyanidins to activate the hCAR *in vitro*. First we evaluated the validity of the gene reporter assay method on the CYP2B6 reporter gene construct containing the proximal PBREM. Therefore, we used well known hCAR ligands – CITCO, the hCAR agonist, the inverse agonist PK11195 and the combination of these substances and gene reporter assay was performed in order to detect changes in the expression of hCAR after transfection and treatment of the HepG2 cell culture with these substances.

In line with earlier observation, there was an activation of hCAR detected after CITCO treatment, which was used as a positive control. PK11195 failed to significantly activate receptor due to its inverse agonistic activity, while treatment with both PK11195/CITCO led to reactivation of the receptor compared to PK11195 alone (Figure 10). Although results of this experiment were not statistically examined, they demonstrate and prove the functionality of the method on the reporter plasmid. The gene reporter method analysis therefore revealed that the well-known hCAR ligands effectively activated hCAR on the pPBREM(CYP2B60)-lucP reporter plasmid, which indicated the possible use of the method for the subsequent experiments to study new putative hCAR ligands.

It has been found that a variety of dietary flavonoids activate hCAR (Yao et al. 2010). Its activation by a wide range of drugs and dietary compounds might represent the molecular basis for a class of harmful drug-drug and drug-food interactions. Furthermore, it is also known that delphinidin, cyanidin and their glucosides have higher intrinsic antioxidant activity than malvidin and its glucoside (Kahkonen and Heinonen 2003) suggesting the hypothesis that these anthocyanidins might also be more potent to activate hCAR in the statistically significant matter.

In agreement with this study, with gene reporter assay and assembly two hybrid assay we detected the changes of hCAR activity after 6 different anthocyanidins exposure-cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin. We

found out, that all of the examined anthocyanidins failed to activate hCAR in the statistically significant matter in contrast to DMSO 1‰, used as a negative control. These findings clearly suggest that none of the examined anthocyanidins (cyanidin, delphinidin, malvidin, pelargonidin, peonidin a petunidin) does mediate the hCAR activation.

Isolated anthocyanidins are known to be highly instable and very susceptible to degradation caused by their fenolic structure that is likely to be influenced by many factors including light exposure, temperature or pH changes (Cabrita et al. 2000; Giusti and Wrolstad 2003; Castaneda-Ovando et al. 2009). Even though all experiments in this thesis were performed under constant conditions subdue light, minor changes in the activity of anthocyanidins in our experiment cannot be excluded which might also led to a lower level of hCAR activation.

Moreover, most present studies have shown significantly higher anticancer effect of the anthocyanidins in the mixture form than the individual compounds (Kausar et al. 2012). These findings were based on the hypothesis that since most of the anthocyanidins are present as a complex mixture in berries, they might exhibit better effects in the mixture rather than when used individually. There were found significantly higher anticancer effects of the anthocyanidins in the mixture form than the individual compounds against both tumor cell proliferation and metastasis and in modulation of various molecular targets mediating the antiproliferative, antimetastatic and apoptotic effects (Kausar et al. 2012). It is therefore necessary to perform further investigations of anthocyanidins on the hCAR not only individually but as a complex solution in order to cut out the possible hCAR modulation with these substances. Though, as total anthocyanidin content of the berries varies in a wide range also with the type of berries, it is rather disputable to set up the optimal concentration of individual compounds in the solution.

Activation of hCAR might represent one of the factors corresponding to the development of drug-food and drug-drug interactions. However, the elucidation of physiological and pathophysiological functions of hCAR, its endogenous and exogenous ligands and a selective activation of hCAR-pathway and is highly important to provide

novel perspective in treatment of many pathological states including cancer, dyslipidemia or type 2 diabetes.

8 CONCLUSION

Anthocyanidins are common dietary compounds occurring in many fruits and berries in relatively high amounts that are receiving a great attention lately for their pharmacological properties. We found that 6 diverse anthocyanidins (cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin) failed to directly activate hCAR *in vitro,* which possibly also demonstrates that anthocyanidins do not elicit their positive health effects through modulation of hCAR activity. However, much work is still needed in order to fully understand the physiological and patophysiological role of hCAR and to elucidate the underlying mechanism of action of the anthocyanidins therapeutic properties *in vivo*.

9 REFERENCES

Andersen, O. M., Jordheim, M., in Andersen, O. M., Markham, K. R., (Eds.), Flavonoids Chemistry, Biochemistry and Applications, CRC Press, Taylor and Francis, Boca Raton 2006, pp. 471–551.

Auerbach, S., Dekeyser, J., Stoner, M., and Omiecinski, C. 2007. CAR2 Displays Unique Ligand Binding and RXRα Heterodimerization Characteristics. *Drug Metabolism Dispos* **35**(3): 428-439.

Baes, M., Gulick, T., Choi, H.S., Martinoli, M.G., Simha, D., and Moore, D.D. 1994. A NEW ORPHAN MEMBER OF THE NUCLEAR HORMONE-RECEPTOR SUPERFAMILY THAT INTERACTS WITH A SUBSET OF RETINOIC ACID RESPONSE ELEMENTS. *Mol Cell Biol* **14**(3): 1544-1552.

Bornsek, S.M., Ziberna, L., Polak, T., Vanzo, A., Ulrih, N.P., Abram, V., Tramer, F., and Passamonti, S. 2012. Bilberry and blueberry anthocyanins act as powerful intracellular antioxidants in mammalian cells. *Food Chemistry* **134**(4): 1878-1884.

Cabrita, L., Fossen, T., and Andersen, O.M. 2000. Colour and stability of the six common anthocyanidin 3-glucosides in aqueous solutions. *Food Chem* **68**(1): 101-107.

Castaneda-Ovando, A., Pacheco-Hernandez, M.D., Paez-Hernandez, M.E., Rodriguez, J.A., and Galan-Vidal, C.A. 2009. Chemical studies of anthocyanins: A review. *Food Chem* **113**(4): 859-871.

Cooke, D., Steward, W.P., Gescher, A.J., and Marczylo, T. 2005. Anthocyans from fruits and vegetables - Does bright colour signal cancer chemopreventive activity? *European Journal of Cancer* **41**(13): 1931-1940.

di Masi, A., De Marinis, E., Ascenzi, P., and Marino, M. 2009. Nuclear receptors CAR and PXR: Molecular, functional, and biomedical aspects. *Mol Asp Med* **30**(5): 297-343.

Dreiseitel, A., Oosterhuis, B., Vukman, K.V., Schreier, P., Oehme, A., Locher, S., Hajak, G., and Sand, P.G. 2009. Berry anthocyanins and anthocyanidins exhibit distinct affinities for the efflux transporters BCRP and MDR1. *British Journal of Pharmacology* **158**(8): 1942-1950.

Dvorak, Z. and Pavek, P. 2010. Regulation of drug-metabolizing cytochrome P450 enzymes by glucocorticoids. *Drug Metabolism Reviews* **42**(4): 621-635.

Dvorak, Z., Vrzal, R., Pavek, P., and Ulrichova, J. 2008. An evidence for regulatory cross-talk between aryl hydrocarbon receptor and glucocorticoid receptor in HepG2 cells. *Physiological Research* **57**(3): 427-435.

Eaton, D. L.; Gilbert, S. G. In Casarett and Doulls Toxicology: The Basic Science of Poisons; Klaassen, C. D., Ed.; McGraw Hill: New York, 2008; pp 18–44.

Eisenblatter, T., Huwel, S., and Galla, H.J. 2003. Characterisation of the brain multidrug resistance protein (BMDP/ABCG2/BCRP) expressed at the blood-brain barrier. *Brain Res* **971**(2): 221-231.

Forman, B.M., Tzameli, I., Choi, H.S., Chen, L., Simha, D., Seol, W., Evans, R.M., and Moore, D.D. 1998. Androstane metabolites bind to and deactivate the nuclear receptor CAR-beta. *Nature* **395**(6702): 612-615.

Garcia-Alonso, M., Minihane, A.M., Rimbach, G., Rivas-Gonzalo, J.C., and de Pascual-Teresa, S. 2009. Red wine anthocyanins are rapidly absorbed in humans and affect monocyte chemoattractant protein 1 levels and antioxidant capacity of plasma. *J Nutr Biochem* **20**(7): 521-529.

Garcia-Alonso, M., Rimbach, G., Rivas-Gonzalo, J.C., and De Pascual-Teresa, S. 2004. Antioxidant and cellular activities of anthocyanins and their corresponding vitisins A - Studies in platelets, monocytes, and human endothelial cells. *Journal of Agricultural and Food Chemistry* **52**(11): 3378-3384.

Giusti, M.M. and Wrolstad, R.E. 2003. Acylated anthocyanins from edible sources and their applications in food systems. *Biochem Eng J* **14**(3): 217-225.

Gonzalez, Frank J.; Robert H. Tukey (2006). "Drug Metabolism". In Laurence Brunton, John Lazo, Keith Parker (eds.). Goodman & Gilman's The Pharmacological Basis of Therapeutics (11th ed.). New York: McGraw-Hill. p. 71-90. ISBN 978-0-07-142280-2.

Gottesman, M.M., Pastan, I., and Ambudkar, S.V. 1996. P-glycoprotein and multidrug resistance. *Curr Opin Genet Dev* **6**(5): 610-617.

Guenthner, T.M. and Nebert, D.W. 1977. CYTOSOLIC RECEPTOR FOR ARYL HYDRO CARBON HYDROXYLASE EC-1.14.14.2 INDUCTION BY POLY CYCLIC AROMATIC COMPOUNDS EVIDENCE FOR STRUCTURAL AND REGULATORY VARIANTS AMONG ESTABLISHED CELL CULTURE LINES. *Journal of Biological Chemistry* **252**(24): 8981-8989.

Honkakoski, P., Sueyoshi, T., and Negishi, M. 2003. Drug-activated nuclear receptors CAR and PXR. *Ann Med* **35**(3): 172-182.

Hou, D.X. 2003. Potential mechanisms of cancer chemoprevention by anthocyanins. *Current Molecular Medicine* **3**(2): 149-159.

Hou, D.X., Fujii, M., Terahara, N., and Yoshimoto, M. 2004. Molecular mechanisms behind the chemopreventive effects of anthocyanidins. *J Biomed Biotechnol*(5): 321-325.

Huang, W.D., Zhang, J., Wei, P., Schrader, W.T., and Moore, D.D. 2004. Meclizine is an agonist ligand for mouse constitutive androstane receptor (CAR) and an inverse agonist for human CAR. *Molecular Endocrinology* **18**(10): 2402-2408.

Chen, Y., Tang, Y., Guo, C., Wang, J., Boral, D., and Nie, D. 2012. Nuclear receptors in the multidrug resistance through the regulation of drug-metabolizing enzymes and drug transporters. *Biochemical Pharmacology* **83**(8): 1112-1126.

Chen, Y.K. and Nie, D.T. 2009. Pregnane X Receptor and its Potential Role in Drug Resistance in Cancer Treatment. *Recent Patents Anti-Canc Drug Discov* **4**(1): 19-27.

Chong, M.F.F., Macdonald, R., and Lovegrove, J.A. 2010. Fruit polyphenols and CVD risk: a review of human intervention studies. *British Journal of Nutrition* **104**: S28-S39.

Idle, J.R. and Gonzalez, F.J. 2007. Metabolomics. *Cell Metab* **6**(5): 348-351.

Jiang, M.X. and Xie, W. 2013. Role of the constitutive androstane receptor in obesity and type 2 diabetes: a case study of the endobiotic function of a xenobiotic receptor. *Drug Metabolism Reviews* **45**(1): 156-163.

Johnson, C.H., Patterson, A.D., Idle, J.R., and Gonzalez, F.J. 2012. Xenobiotic Metabolomics: Major Impact on the Metabolome. in *Annual Review of Pharmacology and Toxicology, Vol 52*, pp. 37-56. Annual Reviews, Palo Alto.

Kahkonen, M.P. and Heinonen, M. 2003. Antioxidant activity of anthocyanins and their aglycons. *Journal of Agricultural and Food Chemistry* **51**(3): 628-633.

Kachaylo, E.M., Pustylnyak, V.O., Lyakhovich, V.V., and Gulyaeva, L.F. 2011. Constitutive androstane receptor (CAR) is a xenosensor and target for therapy. *Biochemistry-Moscow* **76**(10): 1087-1097.

Kausar, H., Jeyabalan, J., Aqil, F., Chabba, D., Sidana, J., Singh, I.P., and Gupta, R.C. 2012. Berry anthocyanidins synergistically suppress growth and invasive potential of human non-small-cell lung cancer cells. *Cancer Lett* **325**(1): 54-62.

Kobayashi, K., Saito, K., Takagi, S., and Chiba, K. 2010. Ligand-Dependent Assembly of Pregnane X Receptor, Constitutive Androstane Receptor and Liver X Receptor Is Applicable to Identify Ligands. *Drug Metabolism Letters* **4**(2): 88-94.

Konczak, I. and Zhang, W. 2004. Anthocyanins - More than nature's colours. *J Biomed Biotechnol*(5): 239-240.

Kong, J.M., Chia, L.S., Goh, N.K., Chia, T.F., and Brouillard, R. 2003. Analysis and biological activities of anthocyanins. *Phytochemistry* **64**(5): 923-933.

Kusuhara, H. and Sugiyama, Y. 2002. Role of transporters in the tissue-selective distribution and elimination of drugs: transporters in the liver, small intestine, brain and kidney. *J Control Release* **78**(1-3): 43-54.

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

Leonessa, F. and Clarke, R. 2003. ATP binding cassette transporters and drug resistance in breast cancer. *Endocrine-Related Cancer* **10**(1): 43-73.

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

McGhie, T.K. and Walton, M.C. 2007. The bioavailability and absorption of anthocyanins: Towards a better understanding. *Mol Nutr Food Res* **51**(6): 702-713.

Nebert, D.W. and Dieter, M.Z. 2000. The evolution of drug metabolism. *Pharmacology* **61**(3): 124-135.

Pascussi, J.-M., Gerbal-Chaloin, S., Duret, C., Daujat-Chavanieu, M., Vilarem, M.-J., and Maurel, P. 2008. The tangle of nuclear receptors that controls xenobiotic metabolism and transport: Crosstalk and consequences. in *Annual Review of Pharmacology and Toxicology*, pp. 1-32.

Pavek, P. and Dvorak, Z. 2008. Xenobiotic-induced transcriptional regulation of xenobiotic metabolizing enzymes of the cytochrome P450 superfamily in human extrahepatic tissues. *Current Drug Metabolism* **9**(2): 129-143.

Reporters – Figure 7 [internet]. Madison: Promega Corporation, 2013 [cited: 11.11.2013]. Available at: http://promega.wordpress.com/2010/04/16/extended-use-of-reporter-assays/

Reporters – Figure 8 [internet]. Madison: Promega Corporation, 2013 [cited: 11.11.2013]. Available at: http://www.promega.com/resources/pubhub/enotes/bioluminescent-reporter-genes/

Robey, R.W., Polgar, O., Deeken, J., To, K.W., and Bates, S.E. 2007. ABCG2: determining its relevance in clinical drug resistance. *Cancer Metastasis Rev* **26**(1): 39-57.

Swales, K. and Negishi, M. 2004. CAR, driving into the future. *Mol Endocrinol* **18**(7): 1589-1598.

Tarozzi, A., Morroni, F., Merlicco, A., Bolondi, C., Teti, G., Falconi, M., Cantelli-Forti, G., and Hrelia, P. 2010. Neuroprotective effects of cyanidin 3-O-glucopyranoside on amyloid beta (25-35) oligomer-induced toxicity. *Neuroscience Letters* **473**(2): 72-76.

Thomasset, S., Teller, N., Cai, H., Marko, D., Berry, D.P., Steward, W.P., and Gescher, A.J. 2009. Do anthocyanins and anthocyanidins, cancer chemopreventive pigments in the diet, merit development as potential drugs? *Cancer Chemother Pharmacol* **64**(1): 201-211.

Tolson, A.H. and Wang, H.B. Regulation of drug-metabolizing enzymes by xenobiotic receptors: PXR and CAR. *Advanced Drug Delivery Reviews* **62**(13): 1238-1249.

Toufektsian, M.C., Lorgeril, M., Nagy, N., Salen, P., Donati, M.B., Giordano, L., Mock, H.P., Peterek, S., Matros, A., Petroni, K., Pilu, R., Rotilio, D., Tonelli, C., de Leiris, J., Boucher, F., and Martins, C. 2008. Chronic dietary intake of plant-derived anthocyanins protects the rat heart against ischemia-reperfusion injury. *J Nutr* **138**(4): 747-752.

Tsuda, T., Horio, F., and Osawa, T. 1999. Absorption and metabolism of cyanidin 3-O-beta-D-glucoside in rats. *Febs Letters* **449**(2-3): 179-182.

Tyree, C. and Klausing, K. 2003. The Mammalian Two-Hybrid Assay for Detection of Coactivator-Nuclear Receptor Interactions. *Methods Mol Med*(85): 175-183.

Whitfield, G.K., Jurutka, P.W., Haussler, C.A., and Haussler, M.R. 1999. Steroid hormone receptors: Evolution, ligands, and molecular basis of biologic function. *Journal of Cellular Biochemistry*: 110-122.

Yao, R.Q., Yasuoka, A., Kamei, A., Kitagawa, Y., Tateishi, N., Tsuruoka, N., Kiso, Y., Sueyoshi, T., Negishi, M., Misaka, T., and Abe, K. 2010. Dietary Flavonoids Activate the Constitutive Androstane Receptor (CAR). *Journal of Agricultural and Food Chemistry* **58**(4): 2168-2173.

Zhang, Y.J., Vareed, S.K., and Nair, M.G. 2005. Human tumor cell growth inhibition by nontoxic anthocyanidins, the pigments in fruits and vegetables. *Life Sciences* **76**(13): 1465-1472.

Zhang, Y.Q., Kast-Woelbern, H.R., and Edwards, P.A. 2003. Natural structural variants of the nuclear receptor farnesoid X receptor affect transcriptional activation. *J Biol Chem* **278**(1): 104-110.