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REGULATION OF HUMAN CARBONYL REDUCTASE 3 (CBR3) EXPRESSION

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Doctoral thesis

Study programme: Biochemistry

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Supervisor: Prof. Ing. Vladimír Wsól, Ph.D.

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Declaration

Hereby I declare that this thesis is my original authorial work that I developed independently. All literature and other sources, which I used for the elaboration, are listed in the references and properly cited in the text. The work has not been submitted for the award of any other or same degree.

Prohlášení

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

Mgr. Petra Malátková

Abstract

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Title of Doctoral Thesis: **Regulation of Human Carbonyl Reductase 3 (CBR3) Expression**

The regulation of human carbonyl reductase 3 (CBR3) expression has been complete mystery until recently and is still not well understood. Because the transcriptional regulation of a gene is closely related to the function of encoded protein, the elucidation of the regulation of CBR3 might help to understand its physiological role which has not been elucidated up to the present. The promoter of CBR3 has been described in 2009. The CBR3 promoter contains several putative binding sites for various transcription factors. In 2010, we have shown that CBR3 is regulated *via* the Nrf2/ARE signaling pathway. This was the first study about the transcriptional regulation of CBR3. The involvement of Nrf2 in the regulation of CBR3 has been recently confirmed by another research group.

The functional antioxidant response element (ARE) is located at 2698 bp upstream of the translation initiation codon of CBR3 ($_{-2698}$ARE). However, the analysis of CBR3 promoter encompassing 2500 bp indicated the presence of *cis*-regulatory upstream element in sequence between 2500 bp and 500 bp upstream the initiation codon. Thus, another response element than $_{-2698}$ARE appears to contribute to CBR3 regulation. The existence of putative NFκB binding sites in the promoter region of the *CBR3* gene indicates that *CBR3* may be a target gene of the NFκB signaling pathway. This hypothesis is further supported by data obtained from microarray analyses that show the up-regulation of CBR3 mRNA in pro-inflammatory environments.

The theoretical part of this thesis summarizes current state of understanding of human CBRs. The aim of the experimental part was to investigate the possible involvement of the NFκB pathway in the transcriptional regulation of CBR3. We have studied the effect of NFκB activation and NFκB inhibition on the mRNA level by means of RT-PCR.

After that, the effect of NFκB activation has been evaluated on the protein level using western blot analysis. The last aim was to examine the functional impact of putative NFκB binding sites in mediating the transcriptional regulation of CBR3.

We showed that CBR3 mRNA expression is inducible in the human cancer cell lines HT-29 and HepG2 by NFκB-dependent mechanism. Treatment with the NFκB activators TNF-α in HT-29 cells and with IL-1β in HepG2 cells regulated the expression of CBR3 mRNA in a time- and concentration-dependent manner. The HT-29 and HepG2 cell lines responded differently with respect to both the degree of CBR3 mRNA inducibility and the kinetics of CBR3 mRNA expression pointing a cell-specific regulation of the CBR3 transcription. In addition, we proved by means of vector-based overexpression of the NFκB subunits p65 and p50 that the inducing effect on CBR3 mRNA level is mediated *via* NFκB pathway.

An interesting finding of this work was that the activation of the NFκB pathway clearly enhanced the CBR3 mRNA level, but this effect did not correlate with protein expression. In HT-29 cells, only marginal changes in the CBR3 protein expression were detected after treatment with TNF-α at various concentrations and for different times. Furthermore, the overexpression of NFκB subunits p65 and p50 enhanced the expression of CBR3 protein only slightly. However, it has frequently been observed that mRNA and protein levels of a certain gene do not correlate. It can be hypothesized that miRNA-based regulation of CBR3 is responsible for lack of CBR3 up-regulation on protein level.

In the last part of the experimental work, we pinpointed areas on the CBR3 promoter that may regulate the transcription of CBR3. It was demonstrated that the $_{-1160}$NFκB binding site may be predominantly responsible for the constitutive activity of the CBR3 promoter construct in HepG2 cells. Furthermore, the $_{-1160}$NFκB and $_{-593}$NFκB binding sites may act as *bona fide* functional elements to activate NFκB-mediated gene transcription in the presence of NFκB activators. In conclusion, we provide for the first time clear evidence that the NFκB signaling pathway is involved in the regulation of the *CBR3* gene.

Abstrakt

Univerzita Karlova v Praze, Farmaceutická fakulta v Hradci Králové
Katedra biochemických věd

Kandidát: **Mgr. Petra Malátková**
Školitel: **Prof. Ing. Vladimír Wsól, Ph.D.**
Název disertační práce: **Regulace exprese lidské karbonylreduktasy 3 (CBR3)**

Regulace exprese lidské karbonylreduktasy 3 (CBR3) byla až donedávna zcela neznámá a stále není plně objasněna. Transkripční regulace genu úzce souvisí s funkcí proteinu, který kóduje. Proto lze očekávat, že objasnění regulace CBR3 pomůže porozumět fyziologické funkci tohoto enzymu, která dodnes zůstává záhadou. Promotor genu *CBR3* byl popsán v roce 2009. CBR3 promotor obsahuje několik potenciálních vazebných míst pro různé transkripční faktory. V roce 2010 jsme prokázali, že exprese CBR3 je regulována signální dráhou Nrf2/ARE. Šlo o první studii popisující transkripční regulaci CBR3. Zapojení Nrf2 do regulace CBR3 bylo nedávno potvrzeno studií jiného výzkumného týmu.

Funkční antioxidační responzivní element (ARE) se nachází 2698 párů bází proti směru iniciace translace CBR3 ($_{-2698}$ARE). Analýza promotoru CBR3 o délce 2500 párů bází nicméně naznačila přítomnost *cis*-regulačního elementu v sekvenci mezi 2500 a 500 párů bází proti směru transkripce. Zdá se tedy, že i jiný responzivní element než $_{-2698}$ARE může regulovat CBR3. Protože promotor genu *CBR3* obsahuje potenciální vazebná místa pro NFκB, může být *CBR3* cílovým genem signální dráhy NFκB. Tuto hypotézu navíc podporují data z microarray analýz, která dokumentují zvýšení CBR3 mRNA v prozánětlivém prostředí.

Teoretická část této disertační práce shrnuje dosavadní poznatky o lidských karbonylreduktasách. Úkolem experimentální práce bylo studium možného zapojení NFκB do regulace transkripce genu *CBR3*. Pomocí RT-PCR jsme zkoumali vliv aktivace a inhibice NFκB na množství CBR3 mRNA. Následně byl studován vliv aktivace NFκB na expresi proteinu použitím western blot analýzy. Posledním úkolem bylo prozkoumat úlohu potenciálních vazebných míst pro NFκB v transkripční regulaci CBR3.

Zjistili jsme, že exprese CBR3 mRNA je indukována v lidských nádorových buněčných liniích HT-29 a HepG2 prostřednictvím NFκB. Inkubace buněk HT-29 s TNF-α a buněk HepG2 s IL-1β ovlivnilo expresi CBR3 mRNA v závislosti na čase i na koncentraci. Odpověď v buněčných liniích HT-29 a HepG2 se značně lišila, a to jak v míře indukce CBR3 mRNA tak v jejím časovém průběhu. Zdá se proto, že regulace transkripce CBR3 je specifická pro buněčný typ. Metodou overexprese vektorů kódujících podjednotky NFκB p65 a p50 bylo ověřeno, že indukční efekt na CBR3 mRNA je zprostředkován dráhou NFκB.

Zajímavým zjištěním bylo, že aktivace NFκB dráhy jednoznačně zvýšila hladinu CBR3 mRNA, ale tento efekt nekoreloval s expresí proteinu. Vystavení buněk HT-29 působení několika rozdílným koncentracím TNF-α způsobilo při různých časech inkubace jen nepatrné změny v expresi CBR3 proteinu. Metodou overexprese NFκB podjednotek p65 a p50 se zvýšila exprese proteinu CBR3 jen mírně. Je často pozorováno, že množství mRNA a proteinu pro určitý gen spolu nekorelují. Příčinou nezvýšení exprese CBR3 proteinu může být regulace prostřednictvím miRNA.

V poslední části experimentální práce jsme určili oblasti promotoru CBR3, které regulují transkripci CBR3. Výsledky naznačují, že vazebné místo $_{-1160}$NFκB je zodpovědné za konstitutivní aktivitu promotorového konstruktu CBR3 v buňkách HepG2. Vazebná místa $_{-1160}$NFκB a $_{-593}$NFκB mohou působit v přítomnosti aktivátorů NFκB jako funkční elementy zvyšující transkripci genu zprostředkovanou NFκB dráhou. Závěrem lze říci, že se podařilo prokázat zapojení signální dráhy NFκB do regulace genu *CBR3*.

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Abbreviations

17β-HSD8	17β-hydroxysteroid dehydrogenase type 8
4-ONE	4-oxonon-2-enal
AhR	aryl hydrocarbon receptor
AKR	aldo-keto reductase
ARE	antioxidant response element
ARNT	aryl hydrocarbon receptor nuclear translocator
B[*k*]F	benzo[*k*]fluoranthene
bp	base pair(s)
CBR	carbonyl reductase
CHF	congestive heart failure
ChIP	chromatin immunoprecipitation
CoQ	coenzyme Q
COX-2	cyclooxygenase-2
Cul3	Cullin-3
DAUN	daunorubicin
DAUNol	daunorubicinol
DEM	diethyl maleate
DMEM	Dulbecco's modified Eagle medium
DOX	doxorubicin
DOXol	doxorubicinol
EMSA	electrophoretic mobility shift assay
FCS	fetal calf serum
IFN-γ	interferon gamma
IKK	IκB kinase
IL-1	interleukin-1
iNOS	inducible nitric oxide synthase

Inr	initiator
IκB	inhibitor NFκB
kb	kilobase pairs
Keap1	Kelch-like ECH-associated protein 1
LPS	lipopolysaccharides
MAPK	mitogen-activated protein kinase
miRNA	micro RNA
NFκB	nuclear factor kappa B
NNK	4-methylnitrosamino-1-(3-pyridyl)-1-butanone
Nrf2	nuclear factor erythroid 2-related factor 2
OSCC	oral squamous cell carcinoma
PBS	phosphate buffered saline
PDTC	pyrrolidine dithiocarbamate
PRT	parthenolide
qPCR	quantitative real-time PCR
ROS	reactive oxygen species
RT-PCR	reverse transcription polymerase chain reaction
SDR	short-chain dehydrogenase/reductase
SDS	sodium dodecyl sulfate
siRNA	small interfering RNA
SNP	single nucleotide polymorphism
sqPCR	semi-quantitative PCR
t-BHQ	*tert*-butylhydroquinone
TNF-α	tumor necrosis factor α
XME	xenobiotic metabolizing enzyme
XRE	xenobiotic response element

Content

1. Introduction

In the post-genomic era, a major effort of biology is the characterization of individual genes and the encoded proteins (Day et al., 2009). The understanding of the genes transcriptional regulation is a related goal from several reasons. For example, the biochemical activity of the protein can be regulated by means of transcriptional regulation. Next, aberrant regulation of the gene may contribute to a particular disease. Therefore, the elucidation of the transcriptional regulatory mechanisms increases our understanding of disease pathogenesis and/or may provide targets for therapeutic intervention. Various transcription factors are the key mediators of drug-induced changes in xenobiotic metabolizing enzymes (XMEs). The increase of XMEs enhances the elimination and clearance of these xenobiotics and, consequently, plays a central role in the protection of the body against stresses from the environment (Xu et al., 2005). In general, the studying of gene regulation greatly contributes to our knowledge of the regulatory circuits that control important cellular processes. Although eukaryotic gene regulation mechanisms have been studied for more than two decades, many fundamental aspects remain to be clarified (Carey and Smale, 2000).

Carbonyl reducing enzymes metabolize a wide variety of endogenous and xenobiotic compounds. Since the chemical carbonyl group is often responsible for the biological activity, these enzymes play significant roles in physiology (Oppermann, 2007). Human carbonyl reductase 3 (CBR3), an enzyme with carbonyl reducing activity, is poorly characterized. Up to the present, just a few substrates have been found for CBR3 and its physiological role remains obscure (Malatkova et al., 2010). The regulation of CBR3 expression has been complete mystery until recently and is still not well understood (Ebert et al., 2010; Cheng et al., 2012). The elucidation of the CBR3 regulation will increase the knowledge about this enzyme. Moreover, it might help to understand the physiological role of CBR3 because the transcriptional regulation of a protein is closely related to its function.

2. Theoretical part

2.1. Biotransformation of xenobiotics and endogenous compounds

Biotransformation means the enzymatic conversion of lipid-soluble substances into polar, water-soluble, and excretable metabolites (Meyer, 1996). Xenobiotic metabolizing enzymes (XMEs) are the enzymes that play central roles in the biotransformation of xenobiotics. Most of the tissues and organs are well equipped with various XMEs. These enzymes are present constitutively at the basal level and/or are induced after exposure to xenobiotics (Xu et al., 2005). In general, XMEs protect the body against the potential harmful effects of xenobiotic compounds. However, some biotransformation products may exhibit enhanced activity or toxic effects (Meyer, 1996). Thus, the knowledge of biotransformation enzymes is crucial for understanding the potential health risks of xenobiotics. Importantly, the process of biotransformation involves not only xenobiotics but endogenous compounds as well. Many endogenous compounds like hormones are inactivated in the same pathways as the xenobiotics (Meyer, 1996), i.e., some XMEs metabolize also endogenous compounds (Honkakoski and Negishi, 2000).

Redox reactions are considered as the most important reactions in xenobiotic metabolism. One of the reasons is that the biotransformation of a xenobiotic often begins with redox reactions (Testa and Kramer, 2007). The well studied enzyme system involved in redox reactions of xenobiotics is the cytochrome P450 system that mediates particularly oxidation reactions. Less attention was initially paid to reducing enzymes such as carbonyl-reducing enzymes (Hoffmann and Maser, 2007). Carbonyl reduction means the formation of a hydroxyl functional group from a ketone or aldehyde moiety. The chemical carbonyl group occurs frequently in xenobiotics as well as endogenous compounds and is often responsible for biological activity of these compounds (Oppermann, 2007).

Enzymes involved in carbonyl reduction are NAD(P)(H) dependent oxidoreductases from two protein superfamilies, namely the aldo-keto reductase (AKR) and the short-chain dehydrogenase/reductase (SDR) superfamilies (Hoffmann and Maser, 2007). While enzymes like aldehyde reductase and aldose reductase are members of the AKR superfamily, the carbonyl reductases *per se* belong to the SDR superfamily.

Several carbonyl reductases from the SDR superfamily are not well characterized and further studies are necessary to elucidate their catalytic activity and roles in xenobiotic as well as endogenous metabolism (Matsunaga et al., 2006; Pilka et al., 2009). The following part focuses on human carbonyl reductases (CBRs) from the SDR superfamily.

2.2. The SDR superfamily

The SDR superfamily is one of the largest enzyme superfamilies. Today, the SDR superfamily contains more than 47000 members (Kallberg et al., 2010). The majority of SDRs have low sequence homologies of only 15-30 % indicating early divergence (Jornvall et al., 1995). The common feature of all SDR enzymes is the occurrence of typical sequence motifs that define the cofactor binding site and the catalytic tetrad (Persson et al., 2009). The cofactor binding motif is composed of βαβ units and called the Rossmann fold. Rossmann fold is highly conserved in the SDR superfamily despite little sequence conservations (Duax et al., 2000). The active site consists of a tetrad of catalytically important Asn, Ser, Tyr, and Lys residues (Filling et al., 2002). Tyr of this catalytic tetrad is the most conserved residue within the whole SDR family (Jornvall et al., 1995).

The SDR enzymes are present in all genomes investigated and in humans, over 80 SDR genes exist. It is now clear that SDRs fulfil essential functions in all forms of life. Members of the SDR superfamily are NAD(P)(H)-dependent oxidoreductases with great functional diversity. Human SDRs have physiological roles in metabolism of steroid hormones, prostaglandins, retinoids, lipids, and xenobiotics. Until now, three SDR enzymes designated as carbonyl reductase (CBR) have been identified in humans. These are: CBR1 (SDR21C1), CBR3 (SDR21C2) and CBR4 (SDR45C1) (Persson et al., 2009).

2.2.1. Human carbonyl reductases

CBR1 has been first isolated in 1973 from the human brain and was named as aldehyde reductase (Ris and von Wartburg, 1973). In 1981, Wermuth et al. reported the wide substrate specificity of this enzyme and suggested the name “carbonyl reductase” (Wermuth, 1981). As lately as in 1998, a novel gene for carbonyl reductase has been identified 62 kb downstream from the original *CBR1* gene. The novel enzyme has been designated as CBR3 (Watanabe et al., 1998). CBR1 and CBR3, in contrast to other members of the SDR superfamily, share remarkably high amino acid sequence identity (72 %). The last carbonyl reductase known so far, CBR4, was revealed during sequencing of the human genome (Strausberg et al., 2002; Ota et al., 2004). CBR4 exhibits low sequence identity with CBR1 and CBR3 (16 % and 18 %, respectively).

2.2.1.1. CBR1

2.2.1.1.1. Introduction

CBR1 is a monomeric cytosolic enzyme with a molecular mass of 30375 Da consisting of 277 amino acids (Wermuth et al., 1988). An unusual property of CBR1 is that it exists *in vivo* in three forms with different charges and slightly different molecular masses (Wermuth, 1981; Sciotti et al., 2000). These multiple isoforms result from covalent modification of a Lys residue at position 239 by an autocatalytic process (Wermuth et al., 1993; Krook et al., 1993; Sciotti et al., 2000).

The presence of CBR1 has been detected by immunohistochemical staining in all organs investigated but the amount of staining varies widely. The high enzyme concentrations are observed in the tissues that are in close contact with exogenous compounds, i.e., the liver, the epithelia of the gastrointestinal tract, and the epidermis (Wirth and Wermuth, 1992). This is in agreement with the suggested role of CBR1 in the detoxification of xenobiotics (Wermuth et al., 1986). On the other hand, CBR1 is not restricted only to tissues exposed to potentially harmful xenobiotic compounds (Wirth and Wermuth, 1992). Thus, the role for CBR1 in the metabolism of endogenous carbonyl substrates is also possible. Indeed, CBR1 catalyzes the NADPH-dependent reduction of a variety of exogenous and endogenous carbonyl compounds (Wermuth, 1981; Kelner et al., 1997). The best substrates are quinones (e.g., menadione, phenanthrenequinone), followed by ketoaldehydes, aromatic aldehydes, and the biogenic aldehydes (Wermuth, 1981). Because the substrates of CBR1 possess biological and pharmacological activities, CBR1 may influence several physiological processes in human body.

Significant interindividual variability in the activity and expression of CBR1 has been repeatedly documented (Rady-Pentek et al., 1997; Lopez de et al., 1999; Gonzalez-Covarrubias et al., 2006; Kassner et al., 2008; Gonzalez-Covarrubias et al., 2009). The molecular basis of these disparities and its potential impact on CBR1-mediated drug metabolism is yet not clear. The interindividual differences in carbonyl reductase activity may in part reflect variable rates of CBR1 transcription, which is regulated by the aryl hydrocarbon receptor (AhR) pathway (see *2.3.3 Transcriptional regulation of CBR1*) (Gonzalez-Covarrubias et al., 2006; Lakhman et al., 2007). Further, CBR1 polymorphism is suspected to play a role in variable CBR1 activity and expression

because is associated with various CBR1 expression levels or isoforms with distinct enzymatic properties (see *2.2.1.1.4 Polymorphisms*) (Lal et al., 2008; Gonzalez-Covarrubias et al., 2009; Bains et al., 2010).

2.2.1.1.2. Xenobiotic substrates

The major role of CBR1 is in the phase I metabolism of xenobiotic compounds. CBR1 reduces a wide variety of xenobiotic aldehydes, ketones and quinones. Quinones are the best substrates of CBR1, namely benzo- and naphtoquinones with short substituents, and the K-region *ortho*-quinones derived from polycyclic aromatic hydrocarbons (Wermuth et al., 1986; Jarabak and Harvey, 1993). Concretely, the best known substrate of CBR1 is 9,10-phenanthrenequinone (Wermuth, 1981; Wermuth et al., 1986; Jarabak and Harvey, 1993).

Quinones can cause a variety of hazardous effects *in vivo*, such as acute cytotoxicity, immunotoxicity, and carcinogenesis (Wermuth et al., 1986; Bolton et al., 2000). Quinones may be enzymatically reduced by both one-electron and two-electron reduction, resulting in the formation of semiquinones or hydroquinones, respectively (Monks et al., 1992). CBR1 catalyzes the two-electron reduction of quinones (Wermuth et al., 1986), which is a detoxification pathway in contrast to one-electron reduction that yields toxic semiquinones (Monks et al., 1992; Gonzalez-Covarrubias et al., 2006). As depicted in Fig. 1, CBR1 may protect the cell against oxidative stress induced by quinones by competing with the single-electron reduction pathway (Wermuth et al., 1986).

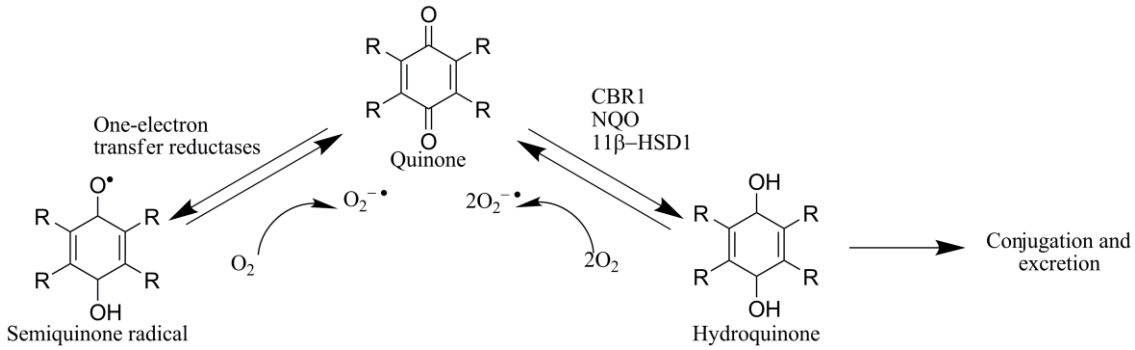

Fig. 1: Quinone reduction and redox cycling. NQO1 = NAD(P)H: quinone oxidoreductase 1, 11β-HSD1 = 11β-hydroxysteroid dehydrogenase type 1.

Another xenobiotic substrate of CBR1 is 4-methylnitrosamino-1-(3-pyridyl)-1-butanone (NNK) that is present in tobacco and tobacco smoke (Atalla et al., 2000; Breyer-Pfaff et al., 2004). After metabolic activation, NNK exerts its carcinogenic potential. The competing pathway for NNK activation is detoxification by means of carbonyl reduction (Hecht, 1998). Based on several lines of evidence, CBR1 is the major cytosolic NNK-reductase in human liver and lung (Atalla et al., 2000; Maser et al., 2000; Breyer-Pfaff et al., 2004). Consequently, CBR1 activity may protect against NNK-induced carcinogenesis.

Several drugs belong to substrates of CBR1, e.g., antipsychotics (haloperidol, bromoperidol, timiperone), diagnostics (metyrapone), non-steroidal anti-inflammatory drugs (loxoprofen), serotonin-antagonists (dolasetron), and anti-cancer anthracyclines (daunorubicin [DAUN] and doxorubicin [DOX]) (Ohara et al., 1995; Breyer-Pfaff and Nill, 2004; Kassner et al., 2008; Lal et al., 2008). Particularly, the role of CBR1 in anthracycline metabolism is suspected to have negative consequences. The results of several studies imply CBR1 as an important contributor to the development of chemoresistance and cardiotoxicity during therapy with DAUN or DOX (reviewed in Malatkova et al., 2010).

2.2.1.1.3. Endogenous substrates

CBR1 catalyzes the carbonyl reduction not only of various xenobiotics but also of endogenous substrates (Wermuth, 1981; Usami et al., 2001; Bains et al., 2010). The best endogenous substrate that has been identified to date is isatin (Usami et al., 2001). Isatin is an endogenous indole that exhibits various physiological functions. It is found in mammalian brain, peripheral tissues, and body fluids. Isatin is increased in a range of states associated with stress (Manabe et al., 1997; Medvedev et al., 2005). CBR1 acts as a major isatin reductase at least in the liver and the kidney (Usami et al., 2001). The apparent K_m value of CBR1 for isatin is within the concentration ranges of isatin in tissues and plasma. Moreover, the turnover number of isatin *via* CBR1 is comparable to that for the excellent xenobiotic substrate 9,10-phenanthrenequinone (Usami et al., 2001; Medvedev et al., 2005). Considerable amounts of isatin are metabolized *in vivo* to the reduction product with diminished biological activities (Usami et al., 2001).

Therefore, CBR1 may be involved in controlling the concentrations of biological active isatin in human tissues (Usami et al., 2001).

CBR1 exhibits reductase activity to prostaglandins, endogenous lipid mediators with broad variety of physiological functions (Russell et al., 1975). CBR1, which is also named prostaglandin 9-ketoreductase, mediates 9-ketoreduction of PGE_1 and PGE_2 (Wermuth, 1981; Schieber et al., 1992; Kelner et al., 1997). By reducing the 9-keto group, CBR1 catalyzes the conversion of PGE_2 to its antagonist $PGF_{2\alpha}$ (Schieber et al., 1992). Although the major route of $PGF_{2\alpha}$ synthesis is the reduction of PGH_2 through prostaglandin-endoperoxide synthase or reductase, CBR1 may at least in part contribute to $PGF_{2\alpha}$ synthesis (Basu, 2007).

In addition to 9-ketoreduction, CBR1 works as 15-hydroxyprostaglandin dehydrogenase (Schieber et al., 1992). Therefore, CBR1 has also been named NADP-linked 15-hydroxyprostaglandin dehydrogenase (Hoffmann and Maser, 2007). CBR1 oxidizes PGE_2, $PGF_{2\alpha}$, and PGD_2 to their corresponding, biologically inactive, 15-keto metabolites (Schieber et al., 1992). Noteworthy, the oxidation of prostaglandins at position 15 is their major inactivation step (Tai et al., 2002). However, the physiological significance of 9-keto reduction activity as well as 15-hydroxyprostaglandin dehydrogenase activity of CBR1 in prostaglandin metabolism is unclear. CBR1 exhibits *in vitro* much lower enzymatic activity toward prostaglandins than to the best substrates such as 9,10-phenanthrenequinone and menadione (Wermuth, 1981).

CBR1 contributes to the biosynthesis of tetrahydrobiopterin in human brain (Park et al., 1991; Iino et al., 2003). Tetrahydrobiopterin is a cofactor for aromatic amino acid hydroxylases that catalyze the initial steps in phenylalanine degradation in the liver and the rate-limiting steps in the biosynthesis of catecholamine and indoleamine neurotransmitters in the brain (Musacchio et al., 1971; Iino et al., 2003). CBR1 reduces 6-pyruvoyl-tetrahydropterin into 1´-hydroxy-2´-oxopropyl-tetrahydropterin that may be directly converted into tetrahydropterin by sepiapterin reductase (Thony et al., 2000; Iino et al., 2003).

As mentioned above, the best substrates of CBR1 are quinones (Wermuth et al., 1986). Environmental quinones may have a variety of hazardous effects since they can form toxic intermediates (Monks et al., 1992; Bolton et al., 2000). On the other hand, quinones constitute an important class of naturally occurring compounds (Monks et al., 1992). Numerous quinones play vital roles in several biological processes, particularly as redox-active cofactors, e.g., vitamin K compounds and ubiquinones (coenzymes Q [CoQs]) (Wermuth et al., 1986; Monks et al., 1992).

Vitamin K is a family of structurally similar fat-soluble 2-methyl-1,4-naphthoquinones, including phylloquinone (vitamin K_1), menaquinones (vitamin K_2), and menadione (vitamin K_3) (Lamson and Plaza, 2003). CBR1 reduces menadione to menadiol but is not able to metabolize phylloquinone that constitutes the major dietary source of vitamin K in most diets (Wermuth et al., 1986; Lamson and Plaza, 2003; Shearer and Newman, 2008). Menadione is not considered a natural vitamin K, but rather a synthetic analogue that acts as a provitamin (Lamson and Plaza, 2003). Nevertheless, several studies provide an evidence that menadione is a catabolic product of phylloquinone formed after administration (Thijssen et al., 2006). Consequently, CBR1 is able to reduce both synthetical menadione and menadione formed in the human body from phylloquinone. Thus, CBR1 may have some impact on vitamin K metabolism since it allows menadione excretion.

CoQ acts in its reduced form (ubiquinol) as an antioxidant (Albano et al., 2002; Lamson and Plaza, 2003). CoQ is composed of a nucleus, i.e., 2,3-dimethoxy-5-methylbenzoquinone, and a side chain at position 6 consisting of isoprene units (Littarru and Tiano, 2007). The reducing activity of CBR1 decreases rapidly with the increasing side chain length of certain quinones (Wermuth et al., 1986). Concretely, CoQ_1 is readily reduced by CBR1, whereas much lower or no enzymatic activity was observed for CoQ_{10} (Wermuth, 1981; Wermuth et al., 1986; Pilka et al., 2009). CoQ_{10} constitutes the predominant form of CoQ in humans (Albano et al., 2002). The low activity of CBR1 toward CoQ_{10} *in vitro* and the fact that this compound is primarily associated with membranes *in vivo* imply that cytosolic CBR1 is not involved in the biological functions of CoQ_{10} (Wermuth et al., 1986).

2.2.1.1.4. Polymorphisms

CBR1 exhibits substantial interindividual variabilities in hepatic expression levels and reductase activity. These variabilities may contribute to the unpredictable pharmacodynamics of CBR1 drug substrates such as DAUN and DOX (Kassner et al., 2008; Gonzalez-Covarrubias et al., 2009). Differences in CBR1 expression may reflect the individual induction status by xenobiotics *via* the XRE in the CBR1 gene promoter (Lakhman et al., 2007; Kassner et al., 2008). Alternatively, variable CBR1 activity may be a consequence of genetic polymorphism (Gonzalez-Covarrubias et al., 2007). Indeed, there are two documented non-synonymous single nucleotide polymorphisms (SNPs) in the CBR1 coding region, CBR1 V88I (262G>A, SNP ID: rs1143663 from The National Center for Biotechnology Information database available at http://www.ncbi.nlm.nih.gov/snp) and CBR1 P131S (820C>T, rs41557318). Studies with recombinant enzymes revealed that both the non-synonymous SNPs lead to CBR1 isoforms with distinctive catalytic properties (Bains et al., 2009; Gonzalez-Covarrubias et al., 2009). Therefore, these non-synonymous SNPs may be responsible for the interindividual variabilities in DAUN and DOX pharmacokinetics (Bains et al., 2009).

A significant association has been found between a SNP in the 3'-untranslated region, i.e., CBR1 1096G>A (SNP ID: rs9024), and hepatic expression of the CBR1 protein (Gonzalez-Covarrubias et al., 2009). Moreover, the polymorphisms CBR1 1096G>A and 627C>T (SNP ID: rs202572) are associated with higher reduction rates for DOX (Lal et al., 2008; Gonzalez-Covarrubias et al., 2009). Furthermore, the results document that the comparatively higher risk of anthracycline-related cardiotoxicity observed among black cancer patients may be in part due to the scarcity of the CBR1 1096G>A polymorphism among individuals with African ancestry (Gonzalez-Covarrubias et al., 2009). In contrast to findings mentioned above, Kassner et al. (2008) failed to detect any association between CBR1 polymorphisms, including CBR1 1096G>A and 627C>T, and CBR1 expression or DOX reductase activities. Therefore, further studies are necessary to confirm a functional impact of the CBR1 1096G>A and 627C>T polymorphism.

2.2.1.1.5. Possible biological functions

As described above (see *2.2.1.1.3. Endogenous substrates*), CBR1 reduces several endogenous compounds and, therefore, may affect various biological processes such as metabolism of vitamin K or isatin. In addition, molecules containing glutathione are endogenous substrates of CBR1 as well (Feldman et al., 1981; Chung et al., 1987; Bateman et al., 2008). For example, CBR1 metabolizes S-nitrosoglutathione (GSNO), the most common low molecular weight S-nitrosothiol (Gaston et al., 1993; Bateman et al., 2008). GSNO is reduced by CBR1 with kinetic constants comparable with the good CBR1 substrate, menadione. Thus, GSNO may be an important CBR1 substrate, comparable to the many xenobiotic substrates of the enzyme. Through the GSNO reductase activity, CBR1 may regulate protein S-nitrosylation. Protein S-nitrosylation has important roles in normal physiology as well as in a broad spectrum of human diseases (Foster et al., 2003; Foster et al., 2009). Next, a novel function for CBR1 was revealed in apoptosis. Concretely, CBR1 is involved in serum-withdrawal-induced apoptosis (Tanaka et al., 2005).

2.2.1.1.6. Significance in pathological states

Carbonyl-containing compounds are present in the human diet and can be formed within the human body as well (Ellis, 2007). Aldehyde and ketone functional groups are inherently highly reactive and may bind to protein, lipid, or nucleic acid cellular components. Consequently, carbonyl agents alter normal biological functions and participate in the progression of various diseases (Shapiro, 1998; Ellis, 2007). Under conditions of oxidative stress, reactive carbonyls are produced by lipid peroxidation or the oxidation of glycation products (Ellis, 2007). Hence, degradation pathways of reactive carbonyls constitute an important part of defense against oxidative stress (Shapiro, 1998). CBR1 possesses activity toward 4-oxonon-2-enal (4-ONE), a major product of lipid peroxidation (Lee and Blair, 2000; Doorn et al., 2004; Maser, 2006). According to the k_{cat}/K_m values for 4-ONE, CBR1 plays an important role in 4-ONE metabolism and detoxification (Doorn et al., 2004). Since the increased lipid peroxidation is associated with neurodegenerative diseases, CBR1 may confer protection against oxidative stress-induced neurodegeneration (Shapiro, 1998; Maser, 2006).

CBR1 protein levels are increased in adult brain from individuals with Down syndrome and it really seems that CBR1 may be implicated in Down syndrome (Balcz et al., 2001). By the fourth decade of life, individuals with Down syndrome display neuropathological features similar to individuals with Alzheimer´s disease (Cheon et al., 2008). It has been proposed that the activity of CBR1 has positive impact in Down syndrome since CBR1, acting as a quinone reductase, could decrease oxygen radical generation (Jarabak and Harvey, 1993). Thus, the proposed neuroprotective role of CBR1 could contribute to protection against brain damages also in Down syndrome (Maser, 2006). Another disease for which an association with CBR1 functions has been suggested is type 1 diabetes mellitus. Type 1 diabetes mellitus is a genetically complex disease characterized by an immune mediated destruction of pancreatic β–cells (Bang-Berthelsen et al., 2006). The exposure of human pancreatic islets to a mixture of cytokines, which simulates the pathogenetic process of type 1 diabetes mellitus, decreased the CBR1 mRNA level (Bergholdt et al., 2007). The observed down-regulation of CBR1 may be an important mechanism that makes β-cells more vulnerable towards oxidative stress (Bang-Berthelsen et al., 2006).

Several studies have been performed on the levels of CBR1 expression or activity in tumor tissues. The results of the studies differ depending upon the method used. Two studies comparing CBR1 reducing activity in paired tumor and normal tissues found an increase in CBR1 activity in colon, lung, and breast cancer (Schlager and Powis, 1990; Lopez de et al., 1999). In contrast, two studies investigating CBR1 expression by immunohistochemical staining revealed reduced or total absence of CBR1 expression in tumor tissues (hepatocellular carcinoma and prostatic adenocarcinoma), compared to normal tissues (Suto et al., 1999; Ismail et al., 2000). Analogous findings have been revealed in studies with ovarian cancer samples employing immunohistochemical staining and studies assessing CBR1 mRNA levels by real-time PCR in non-small-cell lung cancer. In detail, reduced CBR1 expression correlated with poor survival and nodal involvement (Umemoto et al., 2001; Takenaka et al., 2005). It should be considered that immunohistochemical staining and quantification of gene expression by real-time PCR are more accurate than measuring carbonyl reducing activities in the samples, as was performed in elderly studies.

Although the results are not fully consistent, CBR1 may play a role in progression of malignant tumors. Decrease or loss of CBR1 is believed to render the tumor cells more malignant than their intrinsic nature but the mechanism of action remains unclear (Suto et al., 1999; Ismail et al., 2000; Umemoto et al., 2001; Takenaka et al., 2005). Several lines of evidence indicate the prostaglandin inactivation by CBR1 as the possible mechanism. CBR1 inactivates PGE_2 that is involved in inhibition of apoptosis and inducing of angiogenesis (Schieber et al., 1992; Ben-Av et al., 1995; Sheng et al., 1998; Jain et al., 2008). Thus, inactivation of PGE_2 by CBR1 may have a pro-apoptotic effect and inhibit tumor angiogenesis. Indeed, Takenaka et al. (2005) found a significant correlation between reduced CBR1 mRNA expression and active angiogenesis. The pro-apoptotic function of CBR1 would be in agreement with the observed correlation between increased expression of CBR1 and reduced progression of malignant tumors (Umemoto et al., 2001; Takenaka et al., 2005). Undoubtedly, further studies should be conducted to clarify the precise mechanism by which CBR1 alters the behaviour of cancer cells.

2.2.1.2. CBR3

2.2.1.2.1. Introduction

Like CBR1, CBR3 is a cytosolic monomeric NADPH-dependent carbonyl reductase consisting of 277 amino acids (Watanabe et al., 1998; Maser, 2006; Hoffmann and Maser, 2007). CBR3 possesses a molecular weight of 30850 Da which is only slightly different to that of CBR1. The CBR3 gene is located on human chromosome 21q22.2 62 kb downstream from the *CBR1* gene (Watanabe et al., 1998). CBR1 and CBR3 share a 72 % identity and 79 % homology on the amino acid level. This is remarkably high compared to the usually observed low identity levels among other members of the SDR superfamily (Miura et al., 2008). A number of sequence motifs are conserved in most SDRs despite their low sequence homologies. This holds true also for CBR1 and CBR3. The consensus sequence of cofactor binding motif called Rossmann fold is completely conserved in CBR1 and CBR3 (Miura et al., 2008). In addition, an Asn/Ser/Tyr-x-x-x-Lys motif, which constitutes the catalytic tetrad conserved among most SDR enzymes (Filling et al., 2002), is also identical in CBR1 and CBR3 (Miura et al., 2008).

Both CBR1 and CBR3 mRNA are expressed ubiquitously but the expression patterns of the *CBR1* and *CBR3* genes clearly differ. Whereas the highest levels of CBR3 mRNA were detected in ovary and pancreas, CBR1 mRNA occurs at particularly high levels in liver and kidney. Notably, sex-specific CBR3 mRNA expression was observed, that is, lower expression in prostate and testis than that in ovary. In general, the relative expression of CBR3 is much lower than CBR1 in most of the tissues analyzed (Matsunaga et al., 2006; Miura et al., 2008; Kassner et al., 2008). Both genes are expressed on similar levels only in the foetal brain (Mizobuchi et al., 2007).

2.2.1.2.2. Substrates

The first study concerning the catalytic properties of CBR3 have been published as recently as 2008 (Miura et al., 2008). The study reported the considerably less activity of CBR3 compared to CBR1. The K_m values of CBR3 toward NADPH were higher than those of CBR1. Interestingly, the K_m values of CBR3 toward common substrates for measuring carbonyl reductase activity (i.e., menadione, 4-benzoylpyridine, and 4-nitrobenzaldehyde) were similar or lower than the K_m values of CBR1 (Miura et al., 2008). A later study investigating the enzymatic properties of CBR1 and CBR3 showed

similar results. Whereas CBR1 exhibited significant activity for 43 out of 111 substrates, a limited set of substrates were reduced by CBR3. Moreover, the activity of CBR3 towards its substrates was usually significant lower than the activity of CBR1. For CBR3, significant activity was observed for 12 out of the 22 quinones tested. It was found that CBR3 exerts activity toward *ortho*-quinones but not *para*-quinones. 1,2-Naphthoquinone was among the best substrates for CBR3. The non-quinone compounds identified as substrates for CBR3 are, e.g., isatin, oracin, coniferyl aldehyde, and acetohexamide (Pilka et al., 2009).

CBR3 differs from CBR1 in the enzymatic activity not only toward xenobiotics but also to endogenous compounds. Unlike CBR1, CBR3 exhibits no prostaglandin 9-ketoreductase and prostaglandin 15-hydroxydehydrogenase activity. Further, while CBR1 has significant 3-ketosteroid reductase activities, CBR3 exhibits only low 3-ketosteroid reductase activities (Miura et al., 2008; Miura et al., 2009a). Actually, no endogenous substrate for CBR3 except isatin has been detected so far (Pilka et al., 2009). Menadione, a prototypical substrate for measuring carbonyl reductase activity was originally reported as a good substrate of CBR3 (Lakhman et al., 2005). Later studies ruled this possibility out showing very low or even no activity of recombinant CBR3 toward menadione (El-Hawari et al., 2009; Pilka et al., 2009; Miura et al., 2009a; Bains et al., 2010). The chemical structures of some substrates of CBR1 and CBR3 are depicted in Fig. 2.

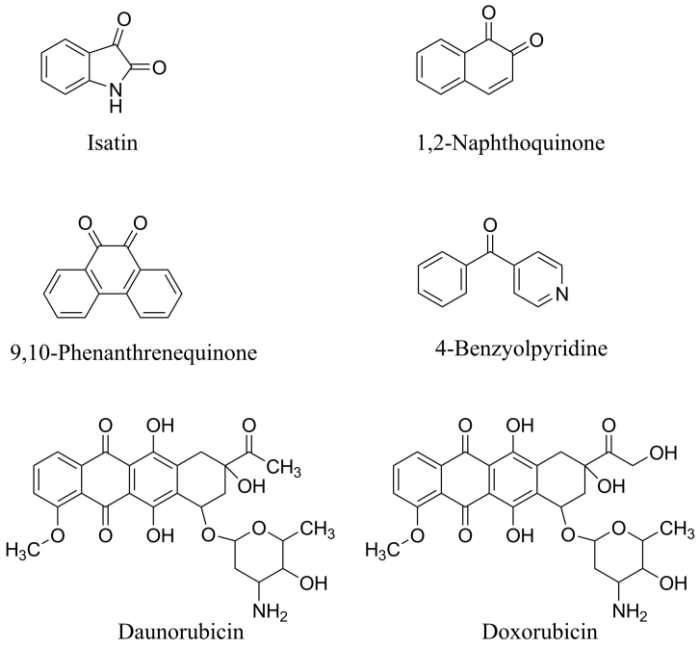

Fig. 2: Substrates of both CBR1 and CBR3.

CBR3 is of particular interest for its role in the metabolism of the anthracycline drugs; DAUN and DOX (see also *2.2.1.2.4. Polymorphisms*). Anthracyclines are the most valuable cytostatic agents in chemotherapy, but their usefulness is limited by the development of cardiotoxicity and the intrinsic or acquired resistance of the tumor cells against these drugs (Minotti et al., 2004). The metabolism of 13-ketone anthracyclines, such as DAUN and DOX, occurs primarily *via* carbonyl reduction to their 13-hydroxy metabolites, daunorubicinol (DAUNol) and doxorubicinol (DOXol), respectively (Soldan et al., 1996). Both DAUNol and DOXol possess significantly lower antineoplastic potency compared to the parent drugs (Ax et al., 2000). Moreover, DAUNol and DOXol are thought to be responsible for the cardiotoxicity observed upon chemotherapy. CBR3 metabolizes both DAUN and DOX *in vitro*, with higher specificity for the latter (Blanco et al., 2008; Bains et al., 2010). According the V_{max} and k_{cat} values, DOX is even a better substrate for CBR3 than it is for CBR1 (Bains et al., 2010). However, the relatively low expression of CBR3 transcripts in all tissues tested argues against any major role of this enzyme in drug disposition (Kassner et al., 2008). CBR1 metabolizes both DOX and DAUN and, moreover, is expressed at higher levels than CBR3, especially in liver (Matsunaga et al., 2006; Miura et al., 2008; Kassner et al., 2008; Bains et al., 2010). Indeed, CBR1 has been suggested as a major hepatic DOX reductase (Kassner et al., 2008).

In conclusion, CBR3 has a much narrower spectrum of substrates and mostly lower catalytic activities against carbonyl substrates compared to CBR1 (Miura et al., 2008; Pilka et al., 2009). The lack of CBR3 activity toward endogenous as well as xenobiotic substrates of CBR1 implies that CBR3 is involved in the metabolism of structurally and chemically different compounds (Pilka et al., 2009). Present findings also suggest a minor role of CBR3 in xenobiotic metabolism. The only exception is the possible participation of CBR3 in the anthracycline metabolism.

2.2.1.2.3. Origin of enzymatic differences between CBR1 and CBR3

As described above, enzymatic activities of CBR1 and CBR3 clearly differ. Several studies has been investigated the origin of this unexpected discrepancy. Miura et al. (2009b) found two low-identity regions in amino acid sequences of CBR1 and CBR3. The one is the N-terminal low identity region at amino acid positions 140-159 near the catalytic Ser-140. The second is the C-terminal low-identity region at amino acid positions 230–244 that forms the substrate-binding loop. The C-terminal low-identity region was identified as the major source of the differences between CBR1 and CBR3 regarding both catalytic and coenzyme-binding properties (Miura et al., 2009b).

El-Hawari et al. (2009) performed the substitution of amino acid residues 230 and 236-244 in the CBR3 by amino acids corresponding to those of the CBR1. In line with previous finding by Miura et al. (2009b), the substitution significantly induced catalytic efficiencies toward both isatin and 9,10-phenanthrenequinone (El-Hawari et al., 2009). Comparison of the amino acid sequences of CBR1 and CBR3 among humans, rats, Chinese hamsters, and mice, shows that the Trp residue at position 230 is highly conserved, while human CBR3 possesses the rigid amino acid Pro at that position. However, single substitution of Trp for Pro in CBR3 had no apparent impact on its carbonyl reductase activities (Miura et al., 2009a).

Recently, the architecture of active sites of CBR1 and CBR3 has been elucidated by comparing their crystal structures. The major difference between the crystal structures of CBR1 and CBR3 is a large hydrophobic wall built by Trp-229 in CBR1, and a more open substrate site in CBR3 (Pilka et al., 2009). Thus, the substrate pockets of CBR1 and CBR3 show fundamental differences in size. Moreover, the enzymes differ in surface properties, since the active site in CBR3 is lined with more polar residues. Especially, the residues at positions 230 (Trp in CBR1, Pro in CBR3) and 236 (Ala in CBR1, Asp in CBR3) were found to strongly influence the substrate and product binding. Other determinants for activity are residues found at position 142, namely Met in CBR1 and Gln in CBR3. These residues form the wall opposite of Trp/Pro at position 230. Despite their similar size, Met-142 in CBR1 and Gln-142 in CBR3 have significantly different effects on the catalytic properties of the active site (Pilka et al., 2009). For sequence alignment of CBR1 and CBR3 see Fig. 3.

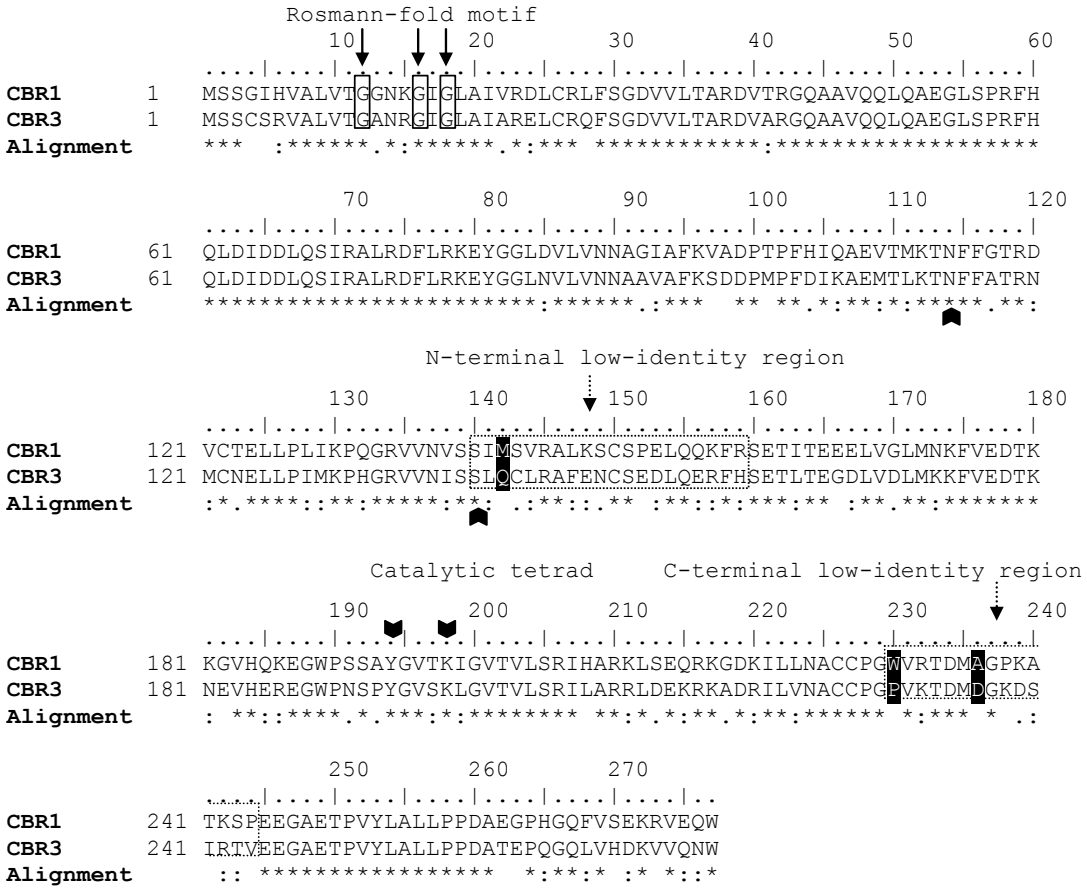

Fig. 3: Amino acid sequence alignment between human CBR1 and human CBR3 with highlighted active sites. Identical, strongly conservative, and weakly conservative residues are indicated by asterisks, colons, and dots, respectively. Critical residues for substrate binding and catalysis are marked black. Residues of the catalytic tetrad are labelled by thick arrows. N-terminal and C-terminal low identity regions are highlighted by dashed boxing. The sequence alignment was conducted using BioEdit version 7.1.3 for Windows, Ibis Biosciences, Carlsbad California USA.

2.2.1.2.4. Polymorphisms

The wide interindividual variabilities in carbonyl reductase activity in humans raised a question about the significance of genetic polymorphisms of CBR1 and CBR3 (Lakhman et al., 2005). The research especially focuses on the impact of the CBR1 and CBR3 polymorphisms on the anthracycline pharmacokinetics and hematologic toxicities (Blanco et al., 2008; Fan et al., 2008; Lal et al., 2008; Bains et al., 2009; Bains et al., 2010). Several studies describe the genetic polymorphisms of CBR1 and/or CBR3 and their association with anthracycline metabolism (i.e., DOX and DAUN metabolism) but with partially inconsistent results. The first study about CBR3 polymorphism was published by Lakhman et al. (2005). They identified a common CBR3 V244M (730G>A, rs1056892) polymorphism occurring at higher frequency among Africans compared to Caucasians. This CBR3 V244M polymorphism encodes for isoforms with distinctive enzymatic properties. Concretely, the CBR3 M244 isoform exhibited higher catalytic efficiency for menadione (Lakhman et al., 2005).

Fan et al. (2008) assessed polymorphisms in CBR3 in 101 Southwest Asian breast cancer patients receiving DOX. Two non-synonymous SNPs of CBR3, C4Y (11G>A, rs8133052) and V244M (730G>A, rs1056892), were common in the population studied. Both polymorphisms correlated with DOX pharmacokinetics. Patients with the CBR3 C4 or CBR3 M244 isoform had a significant higher DOXol AUC and metabolite ratios suggesting a higher catalytic activity of CBR3 C4 and CBR3 M244 isoform. Concerning the CBR3 V244M (730G>A) polymorphism, the patients with the A allele had a higher intratumoral CBR3 expression than those with the G allele. In addition, the CBR3 C4Y (11G>A) polymorphism correlated with hematologic toxicities and tumor reduction. Moreover, significant interethnic differences were shown for the distribution of the CBR3 C4Y (11G>A) polymorphism. Chinese had a significantly higher frequency of the CBR3 11A variant allele, which is associated with lower conversion efficiency of DOX to DOXol. Consequently, this results in a higher exposure to the more active and toxic parent drug, DOX. Indeed, DOX-induced myelosuppression is greater in Chinese compared with Caucasians. Thus, the results indicate that CBR3 genotypes may contribute to the interindividual as well as interethnic variability of DOX pharmacology (Fan et al., 2008).

In their study, Fan et al. (2008) also assessed the genetic variants of CBR1 in breast cancer patients. They have failed to find any associations between CBR1 polymorphisms and DOX pharmacokinetics and hematologic toxicities. Contrary results were obtained by Lal et al. (2008), who observed an association of CBR1 polymorphisms with DOX pharmacokinetics but detected no influence of the CBR3 polymorphism, including CBR3 V244M isoform (Lal et al., 2008). In another study, no significant associations were observed between the genotypes for CBR3 V244M and V93I and breast cancer outcomes. Nevertheless, there was a suggestion of an association between breast cancer survival and CBR3 V244M polymorphism, although not statistically significant. Patients receiving the anthracycline-containing therapy (DOX, cyclophosphamide, and 5-fluorouracil) and being homozygous for the A allele (CBR3 M244 isoform) had an increased risk of cancer recurrence and mortality compared to G allele carriers (CBR3 V244 isoform) (Choi et al., 2009).

Blanco et al. (2008) revealed that the CBR3 V244M polymorphism has an impact on the risk of anthracycline-related congestive heart failure (CHF). Anthracycline-related CHF is an important long-term complication among childhood cancer survivors but the role of genetic variabilities in the pathogenesis of CHF remains to be elucidated. Individuals homozygous for the G allele (CBR3 V244 isoform) exhibited an 8-fold increased risk of anthracycline-related CHF compared to individuals who were homozygous for the A allele (CBR3 M244 isoform). In concordance, the CBR3 V244 isoform exhibited a 2.6-fold higher rate of DOXol synthesis compared to the CBR3 M244 isoform. Thus, the individuals with CBR3 V244 genotype seemed to have an increased rate of myocardial synthesis of cardiotoxic C-13 alcohol metabolites compared to the individuals with the CBR3 M244 isoform (Blanco et al., 2008). Interestingly, these results are contrary to previous findings that CBR3 M244 isoform possesses higher catalytic activity toward DOX than CBR3 V244 isoform (Fan et al., 2008; Choi et al., 2009). However, later study from Blanco et al. (2011) confirmed the association between CBR3 V244M genotype (730G>A) and cardiomyopathy. The risk of cardiomyopathy was higher for individuals with homozygous CBR3 GG genotype (CBR3 V244 isoform) compared with those carrying at least one copy of the variant A allele (Blanco et al., 2011).

Bains et al. (2010) reported consistent results with those of Blanco et al. (2008 and 2011). Wild-type CBR3 was compared with seven known variants of CBR3 (P131S,

rs16993929; V244M, rs1056892; C4Y, rs8133052; M235L, rs4987121; L84V, rs9282628; V93I, rs2835285; D239V, rs11701643) regarding *in vitro* metabolism of DAUN and DOX. In comparison to the wild-type enzyme, the CBR3 isoforms M244, Y4 and I93 had a significantly lower reductase activity toward both anthracyclines. Further, the CBR3 L235 isoform showed a significantly decreased reductase activity for DOX only (Bains et al., 2010).

The epidemiological studies showed that black ethnicity constitutes a risk factor for the development of anthracycline-related cardiotoxicity. Noteworthy, the hepatic expression of CBR3 mRNA was found 1.9-fold higher in samples from black donors than from white donors. Thus, the interethnic differences in the expression of CBR3 may influence the risk of anthracycline related cardiotoxicity. However, no associations were detected between two common SNPs in the CBR3 promoter (CBR3 −326T>A and CBR3 −725T>C) and relative hepatic CBR3 mRNA levels (Zhang and Blanco, 2009). Still, the impact on the CBR3 expression of the number of common SNPs both in the coding and non-coding region of CBR3 remains to be investigated.

Polymorphic CBR3 is suspected to influence the pharmacology of anthracyclines, but the findings are partially controversial. Although some studies demonstrated the correlation between genetic variants of CBR3 and pharmacokinetics of DOX (Fan et al., 2008; Blanco et al., 2008), the exact role of CBR3 in DOX metabolism *in vivo* is not clear. Both CBR1 and CBR3 metabolize DOX and DAUN but CBR3 is expressed at much lower level than CBR1 (Kassner et al., 2008). Therefore, the variability seen in patients undergoing treatment with anthracyclines might be associated with non-synonymous SNPs in the gene encoding CBR1. Indeed, the CBR1 isoforms show distinct kinetic properties for DOX and DAUN (Gonzalez-Covarrubias et al., 2007; Bains et al., 2009; Gonzalez-Covarrubias et al., 2009). Moreover, an association of CBR1 polymorphism and DOX pharmacokinetics has been observed (Lal et al., 2008). On the other hand, few studies have revealed an association of CBR3 genotype status with anthracycline metabolism and cardiotoxicity but failed to find any association for CBR1 polymorphism (Fan et al., 2008; Blanco et al., 2011). Larger studies with a higher sample size must be carried out to draw definitive conclusions and to further evaluate the role of carbonyl reductase polymorphisms in influencing anthracycline-treatment efficacy and anthracycline-related cardiotoxicity.

2.2.1.2.5. Association with cancer

Similar to CBR1, CBR3 is also suspected to have pro-apoptotic functions. Based on a study conducted by Espana et al. (2005), CBR3 functions have been related to the behaviour of metastatic cells. CBR3 was underexpressed in metastasis cells, particularly in bone metastasis, with regard to the tumor. In general, redox system, involving CBR3, exhibited a connection to the mechanism implicated in the selective growth of cells at the metastatic foci (Espana et al., 2005).

The link between CBR3 and apoptosis is further supported by study of Ohkura-Hada et al. (2008). They have demonstrated that CBR3 mediates, at least in part, the cytostatic effects of 9-*cis*-retinoic acid in oral squamous cell carcinoma (OSCC) cells. The anticancer activities of retinoic acids are attributable to growth suppression, the induction of apoptosis, and the suppression of cell migration (Garattini et al., 2007). 9-*cis*-Retinoic acid induced CBR3 expression in the OSCC cell line, Ca9-22. Next, the transfection of Ca9-22 cells with the CBR3 expression vector reduced cell growth and migration potential of the cells. Moreover, the CBR3 mRNA expression was markedly reduced in OSCCs compared to the expression in premalignant dysplasias and hyperplasias. Among OSCCs, the expression of CBR3 was significantly down-regulated in highly invasive tumors compared to less invasive ones (Ohkura-Hada et al., 2008).

2.2.1.3. CBR4

2.2.1.3.1. Introduction

The gene *CBR4* is located on chromosome 4 and encodes a 237-amino acid protein with a molecular mass of 25301 Da (UniProt Knowledgebase; The Universal Protein Resource available at http://www.uniprot.org/). CBR4 shows low sequence identity, i.e., <30 %, with six known CBRs and enzymes with CBR activity (Endo et al., 2008). Further, CBR4 differs from the other known human CBRs by its subcellular localization and subunit structure. Concretely, CBR4 is a tetrameric protein found in the mitochondrial matrix. The expression of CBR4 was observed in human liver and kidney. In these tissues, CBR4 is expressed at lower levels compared to CBR1 (Endo et al., 2008).

2.2.1.3.2. Substrates

CBR4 exhibits NADPH-dependent reductase activity for *ortho*- and *para*-quinones. Among the best substrates are 9,10-phenanthrenequinone and 1,4-benzoquinone (Endo et al., 2008). Unlike CBR1, CBR4 exhibits low catalytic efficiency for menadione and does not reduce DAUN (Wermuth, 1981; Ohara et al., 1995). The substrate specificity of CBR4 is also distinct from that of CBR3 since CBR3 exhibits no activity toward *para*-quinones (Pilka et al., 2009). The most apparent difference between CBR4 and further enzymes with carbonyl reducing activity was the inability of CBR4 to reduce carbonyl compounds other than quinones. CBR4 did not reduce aromatic and aliphatic ketones, aldehydes, sugars, and ketosteroids. Thus, CBR4 has been considered as a quinone reductase (Endo et al., 2008).

2.2.1.3.3. Biological functions

Recently, CBR4 has been identified as a part of the human mitochondrial fatty acid synthesis pathway. The β-ketoacyl thioester reductase, which has not been characterized before, was identified as heterotetrameric $\alpha_2\beta_2$ assembly composed of 17β-hydroxysteroid dehydrogenase type 8 (17β-HSD8) and CBR4. The activity measurements showed that 17β-HSD8 and CBR4 are fully functional only when they form a complex (Chen et al., 2009). It should be noted that β-ketoacyl thioester reductase catalyzes the reduction of fatty acyl CoA substrates with more higher catalytic efficiencies (k_{cat}/K_m) than 9,10-phenanthrenequinone (Chen et al., 2009). This argues against the major role of CBR4 in of exogenous quinones as it has been proposed initially (Endo et al., 2008).

2.3. Transcriptional regulation

2.3.1. Concept of transcriptional regulation in eukaryotes

In the eukaryotic organism, expression of its tens of thousands of genes requires the proper regulation. Transcription is a key step at which gene expression is controlled. The DNA sequence of a gene contains the information that specifies the transcriptional program of the gene (Kadonaga, 2002). Proteins that are necessary for the initiation of transcription, but which are not themselves part of RNA polymerase, are defined as transcription factors (Lewin, 2007). Transcription factors binds to the sequences called *cis*-acting elements. The *cis*-acting elements include enhancers, silencers, proximal promoter regions, core promoters, and insulator elements (Kadonaga, 2002). The core promoter binds RNA polymerase II and its accessory factors (so called "basal" factors) creating the basal apparatus. The basal apparatus directs the RNA polymerase II to begin transcribing at the correct start site. The proximal promoter is located immediately upstream of the core promoter while enhancer sequences lie farther away either upstream or downstream (Carey and Smale, 2000).

A common control sequence in core promoters is the TATA box. In higher eukaryotes, the TATA box is located 25–30 bp upstream of the transcription start site and directs accurate transcription initiation. However, many promoters do not contain TATA boxes and are called TATA-less promoters. Some TATA-less promoters retain the ability to direct transcription initiation from a specific nucleotide, whereas others direct transcription initiation from multiple start sites (Smale, 1997). Other common sequence motif in core promoters is the initiator (Inr). The Inr element encompasses the transcription start site and may be present in both TATA-containing and TATA-less core promoters (Butler and Kadonaga, 2002). An important element for basal transcription activity is a downstream core promoter element. The downstream core promoter element is commonly found in TATA-less promoters (Kadonaga, 2002). In the promoter region of housekeeping genes, there are regions of DNA rich in CpG dinucleotides termed as CpG islands (Caiafa and Zampieri, 2005). The typical structure of a eukaryotic mRNA gene is depicted in Fig. 4.

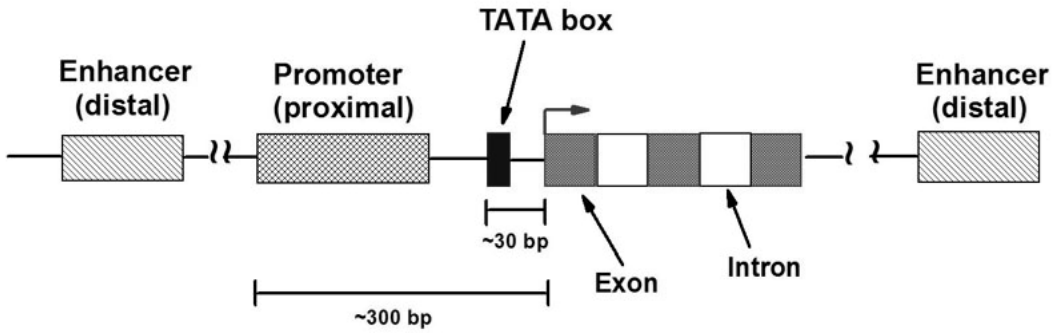

Fig. 4: Typical structure of a eukaryotic gene coding mRNA (from http://themedicalbiochemistrypage.org/gene-regulation.html#initiation).

Proximal promoters and enhancers bind transcription factors called activators that activate transcription of the gene (Carey and Smale, 2000). The activators bind to specific sequences referred to as response elements. The response elements for the same transcription factor found in different genes are closely related, but not necessarily identical (Lewin, 2007). Another group of factors essential for efficient transcription are coactivators. Coactivators provide a connection between activators and basal apparatus but do not themselves bind to DNA (Lewin, 2007). Repressors are transcription factors that bind to silencers and thereby inhibit activators and reduce transcription (Griffiths et al., 2000). Insulators prevent a gene from being influenced by the enhancers and silencers of adjacent genes (Kadonaga, 2002).

Eukaryotic transcription is most often under positive regulation. That means a transcription factor activates a promoter or a set of promoters that contain a common target sequence (Lewin, 2007). Some activators are expressed ubiquitously, whereas others have a regulatory role and are provided only under certain conditions (Lewin, 2007). Thus, the action of transcriptional activators is modulated. Many activators are regulated at the transcriptional level or by posttranslational mechanisms. The posttranslational mechanisms include, e.g., phosphorylation, acetylation, or dissociation of an inhibitory protein (Kadonaga, 2002). Combinatorial control represents another important mechanism employed in transcriptional regulation. Combinatorial control means different combining of ubiquitous and cell-type specific regulatory proteins that are used to turn genes on and off in different contexts (Carey and Smale, 2000).

Activators have distinct domains for the recognition of target genes (DNA-binding domains) and for stimulating the transcriptional machinery (activating domains).

Transcription factors are classified according to the structure of their DNA-binding domain (Triezenberg, 1995). Some common motifs of the DNA-binding domains include basic helix-loop-helix, helix-turn-helix, leucine zipper, and zinc finger motifs. A transcription-activating domain interacts with components of the general transcription machinery. When the activator does not itself have a transcription-activating domain, it binds a coactivator that has the transcription-activating domain (Lewin, 2007).

Various transcription factors have been shown to be the key mediators of drug-induced changes in xenobiotic metabolizing enzymes (XMEs), including phase I, phase II metabolizing enzymes as well as phase III transporters. It appears that, in general, xenobiotics exposure may trigger cellular stress response leading to the up-regulation of XMEs expression. The increase of XMEs enhances the elimination and clearance of these xenobiotics and/or other "cellular stresses" including harmful reactive intermediates such as reactive oxygen species (ROS). This mechanism plays a central role in the protection of the body against stresses from the environment (Xu et al., 2005). Therefore, the understanding of the transcriptional mechanisms that regulate both basal and inducible expression of drug metabolizing enzymes is crucial.

2.3.2. Analysis of the transcriptional regulatory mechanism

After completion of the human genome, a major effort of biology is the characterization of individual genes (Day et al., 2009). When a new gene is isolated, the most important objectives are to elucidate the structure, biochemical activities, and biological functions of the encoded protein. A related goal is to understand the mechanisms governing the gene expression. A gene's regulatory mechanisms are of interest because the biochemical activity of the protein can be regulated at the level of gene expression. Importantly, aberrant regulation of the gene may contribute to a particular disease, or the gene may be specifically expressed in a cell type associated with a disease. In such cases, the elucidation of the regulatory mechanism increases our understanding of disease pathogenesis or may provide targets for therapeutic intervention. In general, the studying of gene regulation greatly contributes to our knowledge of the regulatory circuits that control cellular processes (Carey and Smale, 2000).

An essential step before gene regulation analysis is to determine whether the protein's biochemical activity is truly regulated at the level of gene expression. The activity could be modulated at least in part at the gene expression level if the protein's activity and

abundance, as well as its mRNA abundance, differ in the two cell types. Steady-state protein levels can be measured by a variety of methods, including western blot or immunoprecipitation assays. Steady-state mRNA levels can be determined, e.g., by Northern blot, primer extension, or quantitative PCR analyses (Carey and Smale, 2000). The analysis of transcriptional regulation usually begins with the identification of the promoter sequence and/or distant control elements, such as enhancers and silencers. The potential regulatory elements are searched using a computer algorithm that reveals homologies with known control elements (Carey and Smale, 2000).

The identification of a control region can be followed by various experiments. These experimental strategies lead to the identification of relevant DNA elements and/or proteins binding to those elements. The transfection assay is most commonly used for identification of relevant *cis*-acting sequence elements. In this assay, plasmids containing the control region of interest are introduced into cells. Typically, the control region regulates transcription of a so-called "reporter gene". The reporter gene is a known gene whose mRNA or protein level can be measured easily and accurately. Mutations introduced into the control region allow to identify important regulatory elements and, ultimately, important transcription factors (Carey and Smale, 2000). DNA-binding proteins that interact with the DNA elements can be identified by the electrophoretic mobility shift assay (EMSA). Another technique for studying protein-DNA interactions is chromatin immunoprecipitation (ChIP). ChIP investigates protein-DNA interactions in the living cell (*in vivo*) in contrast to EMSA that is an *in vitro* assay (Jiang et al., 2009).

A transcription factor can be linked to a putative target gene also by abolishing expression of the factor in a cell line or animal using RNA interference such as small interfering RNA (siRNA). The absence of the transcription factor might result in a reduction of target gene expression, implicating the protein as a positive regulator of the gene. The overproduction of a transcription factor may be also employed in studying the transcriptional regulation (Carey and Smale, 2000). Similarly, activation of a putative transcription factor or its signaling pathway will affect the amount of mRNA and/or protein. An opposite approach, i.e., inhibition of the transcription factor may be used as well.

2.3.3. Transcriptional regulation of CBR1

Lakhman et al. (2007) performed a functional characterization of the CBR1 promoter. It lacks a CAAT and TATA box and contains a CpG island extending into the first exon (Lakhman et al., 2007). This structure is characteristic of many housekeeping genes (Butler and Kadonaga, 2002). By several lines of evidence, the aryl hydrocarbon receptor (AhR) signaling pathway has been identified as the key regulator of CBR1 expression (Lakhman et al., 2007). AhR is a ligand-dependent transcription factor that upon ligand binding translocates into the nucleus and heterodimerizes with aryl hydrocarbon receptor nuclear translocator (ARNT). The heterodimer AhR:ARNT interacts with xenobiotic response element (XRE) in the regulatory region of target genes (Denison and Nagy, 2003). Multiple xenobiotic enzymes, such as CYP1A1, are target genes of AhR. The AhR/XRE signaling system mediates most of the toxic and carcinogenic effects of a wide variety of environmental contaminants (Barouki et al., 2007). The ligands of AhR with the highest affinities are halogenated aromatic hydrocarbons and nonhalogenated polycyclic aromatic hydrocarbons (Denison and Nagy, 2003).

The prototypical AhR ligand *β*-naphthoflavone is able to induce the CBR1 mRNA expression (Forrest et al., 1990; Lakhman et al., 2007). Moreover, DNA sequence analysis revealed two XREs ($_{-122}$XRE and $_{-5783}$XRE) with potential regulatory functions in 5'-flanking region of CBR1. Experiments with engineered reporter constructs confirmed that $_{-122}$XRE and $_{-5783}$XRE may act as functional elements to activate AhR-mediated gene transcription in the presence of AhR ligands. Furthermore, experiments with heterozygous Ahr $^{+/-}$ and homozygous Ahr $^{-/-}$ mice clearly showed that AhR plays a pivotal role in mediating CBR1 induction *in vivo*. The potent AhR ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin induced hepatic CBR1 mRNA and CBR1 protein levels in Ahr $^{+/-}$ mice compared to vehicle-injected controls. In contrast, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin treatment failed to induce CBR1 expression in homozygous null (Ahr $^{-/-}$) animals (Lakhman et al., 2007).

Interindividual differences in CBR1 activity were found in several tissues. This variability may be a result of CBR1 regulation *via* the AhR signaling pathway. The increased CBR1 activity after AhR activation may influence the state of human health. For example, it has been proposed that benzo[*a*]pyrene may modulate CBR1 expression in the lungs of smokers *via* the AhR pathway (Lakhman et al., 2007). Benzo[*a*]pyrene is one of the best-characterized carcinogens in cigarette smoke and is the powerful AhR ligand (Denison and Nagy, 2003). Thus, through the AhR pathway, benzo[*a*]pyrene may affect CBR1 mediated detoxification of other smoke carcinogens relevant to the pathogenesis of lung cancer such as NNK (Lakhman et al., 2007).

2.3.4. Transcriptional regulation of CBR3

2.3.4.1. Promoter analysis of CBR3

In 2009, Zhang and Blanco have described the promoter of CBR3. The promoter analysis has been performed with a region encompassing up to 1.3 kb upstream from the ATG translation initiation codon of CBR3. The promoter of CBR3 represents a typical CpG island promoter. The 361 bp DNA sequence of the 5'-flanking region and 839 bp downstream of the start codon ATG contains a high frequency of CpG dinucleotides. The proximal promoter of CBR3 was further confirmed by the presence of the Inr element TTAGTC, several conserved motifs for the Sp1 transcription factor, and a proximal GC-box (Zhang and Blanco, 2009). Zhang et al. (2009) have found no TATA box and no downstream core promoter element in the promoter of CBR3. Nevertheless, the presence of downstream core promoter element has been identified in a later study (Warnatz et al., 2010).

The region encompassing up to 1.3 kb upstream from the translation start site of CBR3 exerted significant gene promoter activity in two different cell lines. These were liver hepatocellular carcinoma cell line HepG2 and breast adenocarcinoma cell line MCF-7. The promoter activities of CBR3 reporter constructs were distinct in HepG2 and MCF-7 cells indicating a cell-dependent regulation of CBR3 expression. However, reporter gene experiments pinpointed areas that may regulate transcription of CBR3 in both cell lines. The sequence located 172-313 bp upstream of the transcription initiation site was essential for CBR3 promoter activity in both cell lines. The significance of this segment is in agreement with previous results of the promoter analysis. The region contains two transcription start sites and a GC box with an embedded consensus sequence for the binding of Sp1 transcription factor. The Sp1 transcription factor may be responsible for constitutive activity of CBR3 because the Sp1 transcription factor induces the transcription of several TATA-less genes. Detailed screening of the CBR3 promoter revealed potential consensus motifs for several transcription factors (Zhang and Blanco, 2009). Recently, other conserved consensus sequences have been identified in more distal parts of the CBR3 promoter (Cheng et al., 2012). These are the antioxidant response element (ARE) and two conserved XREs. ARE identified in this study ($_{-2698}$ARE) has been proved as the functional element mediating *CBR3* gene transcription (see *2.3.4.2. Regulation of CBR3 expression by Nrf2*).

2.3.4.2. Regulation of CBR3 expression by Nrf2

In 2010, we have published the first study about regulation of CBR3 expression (Ebert et al., 2010). The results clearly show that CBR3 is regulated *via* the Nrf2-dependent signaling pathway. The Nrf2 ("nuclear factor erythroid 2-related factor 2")/Keap1 ("Kelch-like ECH-associated protein 1") signaling system plays a key role in the transcriptional activation of genes in the response to oxidative stress (Motohashi and Yamamoto, 2004). Under normal conditions, a cytosolic repressor protein Keap1 binds to leucine zipper transcription factor Nrf2 and retains it in the cytoplasm (Kensler et al., 2007). Keap1 in complex with Cullin-3 (Cul3), Roc1 and E2 proteins provides ubiquitination of Nrf2 followed by proteosomal degradation (Lushchak, 2011). Oxidative stress or chemopreventive agents disrupt the interaction between Nrf2 and Keap1. Dissociation of Nrf2 from Keap1 requires phosphorylation of Nrf2. Liberated Nrf2 enters the nucleus and forms a heterodimer with other transcription factors such as Maf proteins. As depicted in Fig. 5, the heterodimer binds to the ARE and activates the expression of antioxidant enzymes and phase II detoxifying enzymes (Zhang, 2006).

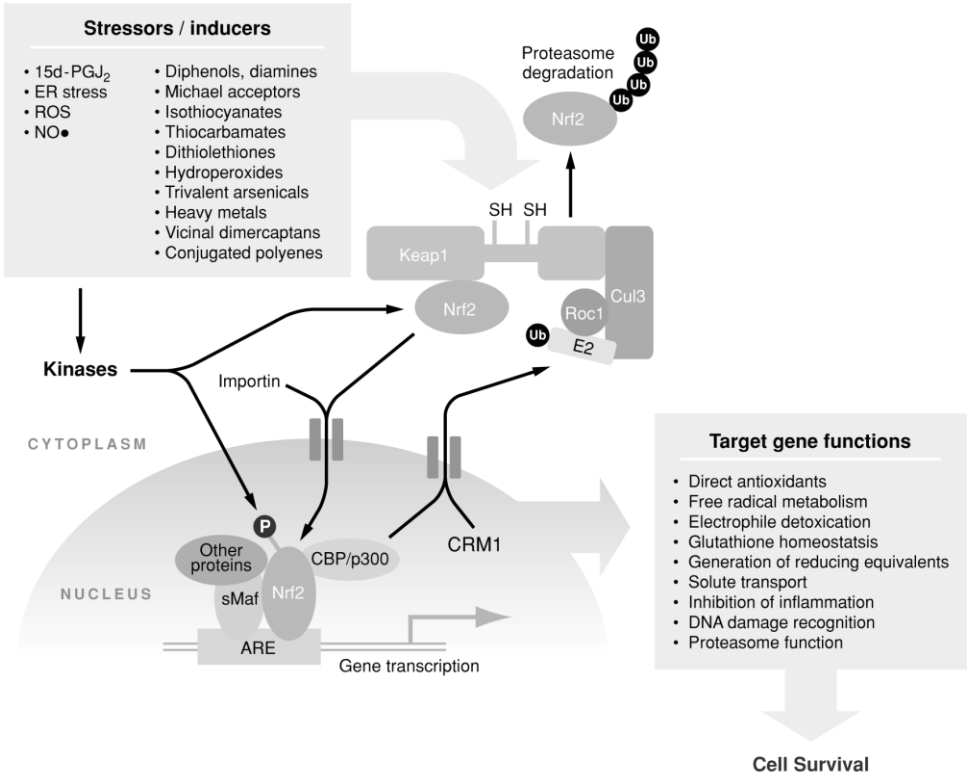

Fig. 5: General scheme of the induction of gene expression through the Nrf2/ARE signaling pathway. Various agents are able to disrupt the association of Nrf2 with Keap1. Nrf2 together with other transcription factors such as small Maf proteins (sMaf) and CBP/p300 binds to ARE within the regulatory regions of target genes. Nuclear export of Nrf2 is mediated by chromosomal region maintenance 1 (CRM1) (adapted from Kensler et al., 2007).

The involvement of Nrf2 in the regulation of CBR3 has been confirmed at different levels in experiments with cultured cancer cells. First, the Nrf2 inducer *tert*-butylhydroquinone (*t*-BHQ) increased the expression of CBR3 mRNA in HCT116 cells after 8 h. The so-called bifunctional inducer benzo[*k*]fluoranthene (B[*k*]F) failed to induce CBR3 mRNA after 8 h but elevated the CBR3 mRNA after longer treatment (24 h) in HCT116 and HT-29 cells (Ebert et al., 2010). Fig. 6 shows that the bifunctional inducer B[*k*]F can directly activate AhR/XRE driven genes but for the activation of the Nrf2/ARE signaling pathway, the metabolism of B[*k*]F is required (Burczynski et al., 1999). The ability of B[*k*]F to affect CBR3 expression only after long-term treatment shows that Nrf2 but not the AhR signaling pathway may regulate CBR3 expression. The strong inducing effects of diethyl maleate (DEM) and sulforaphane on CBR3 expression in HT-29 cells further support the involvement of Nrf2. DEM and sulforaphane are established Nrf2 activators without any AhR agonistic activity (Nguyen et al., 2003). Sulforaphane induced the CBR3 mRNA expression in a time- and concentration-dependent manner and, moreover, elevated the level of CBR3 protein (Ebert et al., 2010).

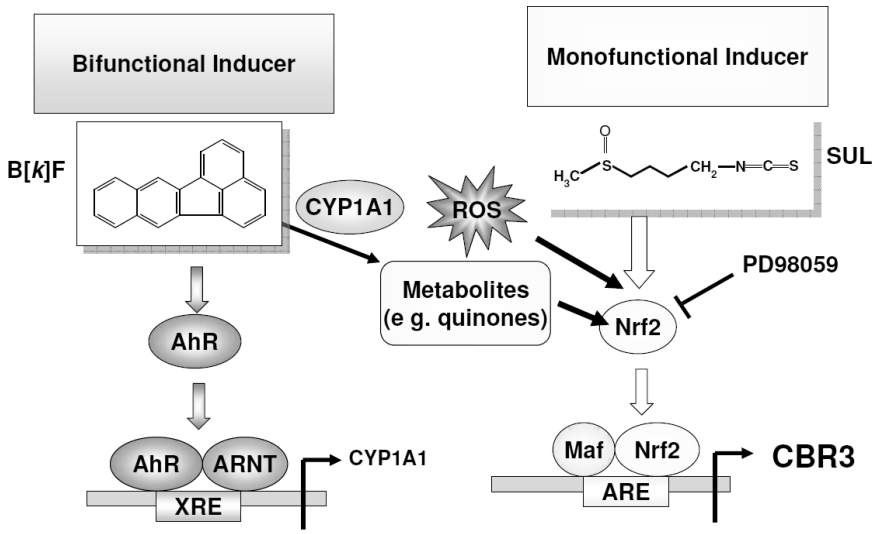

Fig. 6: Regulation of AhR- and Nrf2-mediated gene transcription by bifunctional and monofunctional inducers of xenobiotic metabolizing enzymes (adapted from Ebert et al., 2010).

Proteasome inhibitors induce the Nrf2 activity through blockade of its degradation. The treatment with the proteasome inhibitors MG-132 or bortezomib strongly elevated the levels of CBR3 mRNA. The co-incubation of cells with MG-132 and B[*k*]F or DEM strengthened the inducing effect of B[*k*]F and DEM on CBR3 mRNA expression. Inhibition of Nrf2 activity was another approach that confirmed the involvement of Nrf2 in CBR3 expression. Blocking of Nrf2 phosphorylation by MAPK kinase inhibitor PD98059 prevented DEM-mediated CBR3 induction. Finally, knockdown studies targeting either Nrf2 or Keap1 verified that the CBR3 expression is mediated *via* the Nrf2/Keap1 signaling pathway. The transfection with Nrf2 siRNA clearly down-regulated the expression of Nrf2 as well as CBR3. Next, the Nrf2 siRNA diminished the sulforaphane-mediated inducing effect on CBR3 mRNA. On the other hand, knockdown targeting Keap1, the repressor of Nrf2, strongly induced CBR3 mRNA in HT-29 and HepG2 cells.

A recent study by Cheng et al. (2012) confirmed that the expression of CBR3 is regulated by Nrf2. In this study, the prototypical Nrf2 activator *t*-BHQ significantly induced CBR3 mRNA and protein levels. The functional ARE was identified in the CBR3 promoter located at 2698 bp upstream ($_{-2698}$ARE) of the start codon of CBR3. Deletion of $_{-2698}$ARE from a CBR3 promoter construct impacted basal promoter activity as well as promoter activity induced by *t*-BHQ treatment or Nrf2 overexpression. Moreover, EMSA demonstrated increased binding of specific nuclear protein complexes to $_{-2698}$ARE in nuclear extracts from *t*-BHQ treated cells. These specific nuclear protein-$_{-2698}$ARE complexes contained Nrf2. Therefore, $_{-2698}$ARE is suspected to mediate the transcriptional regulation of CBR3 (Cheng et al., 2012).

2.3.4.3. Hypothesis about involvement of NFκB in CBR3 regulation

Recent findings have demonstrated the regulation of CBR3 expression *via* Nrf2/ARE signaling pathway (Ebert et al., 2010; Cheng et al., 2012). However, some additional regulation elements may modulate CBR3 expression as well. The basal CBR3 mRNA and protein expression vary widely in different cell lines. Noteworthy, there are differences in the up-regulation of the CBR3 expression in response to Nrf2 activators between various cell lines (Ebert et al., 2010). In addition, reporter gene experiments have shown differences in basal and inducible CBR3 promoter activity in the two cell lines, namely HepG2 and MCF-7 cells (Zhang and Blanco, 2009; Cheng et al., 2012).

Quite recently, $_{-2698}$ARE has been identified as the functional response element in the CBR3 promoter (Cheng et al., 2012). Functional analysis of the CBR3 promoter encompassing 2500 bp indicated the presence of putative *cis*-regulatory upstream elements in the sequence between 2500 and 500 bp upstream the initiation codon (Warnatz et al., 2010). Thus, a response element other than $_{-2698}$ARE may contribute to CBR3 regulation. The promoter of CBR3 contains several putative binding sites for various transcription factors. By analysing a 1300 bp portion of the 5′-UTR of the *CBR3* gene, Zhang and Blanco (2009) found two potential nuclear-factor kappa-B (NFκB) consensus motifs in the CBR3 promoter. These are located at 1160 bp upstream ($_{-1160}$NFκB: 5′-GGGTTTCAC-3′) and 593 bp upstream ($_{-593}$NFκB: 5′-GGGAATCCC-3′) of the start codon. Our own promoter analysis revealed the existence of a third putative NFκB binding site ($_{-364}$NFκB: 5′-GGGGTTTTCCC-3′) (unpublished results). Some of these NFκB consensus motifs, especially $_{-1160}$NFκB or $_{-593}$NFκB, may be involved in CBR3 expression.

The hypothesis that CBR3 may be regulated *via* NFκB-dependent pathway is further supported by data obtained from microarray analyses. The data records from microarray analyses are available in the NCBI database GEO Profiles ("Gene Expression Omnibus", available at http://www.ncbi.nlm.nih.gov/geoprofiles). The data found in GEO document the up-regulation of CBR3 mRNA in pro-inflammatory environments in different cellular contexts (see *5.2.5. Summary and discussion of the effect of NFκB activation on CBR3 mRNA expression*). It will be interesting to prove if the NFκB signaling pathway is involved in the up-regulation of CBR3 mRNA in such conditions.

2.3.4.3.1. NFκB signaling pathway

NFκB transcription factors

NFκB ("nuclear factor kappa B") comprises a family of transcription factors controlling a wide range of critical physiological processes in the organism such as inflammation, immune response and cell survival (Pahl, 1999; Hayden and Ghosh, 2008). In mammals, the NFκB family consists of five different members grouped in two classes. The first class includes Rel proteins, i.e., c-Rel, RelB, and RelA (p65). Rel proteins are synthesized as mature products and contain a transactivating domain required to promote transcription. A second group consists of p105 and p100 that are processed to active DNA-binding proteins p50 and p52, respectively, by either limited proteolysis or arrested translation (Gilmore, 1999).

NFκB subunits interact with each other creating homodimers and heterodimers (Hayden and Ghosh, 2004). The term NFκB commonly refers specifically to a p50:p65 heterodimer that is the major NFκB dimer in most cells (Gilmore, 1999). The dimers of NFκB transcription factors bind to promoter and enhancer regions containing NFκB binding sites (named also as κB sites). The κB sites display a varying degree of consensus (Chen and Ghosh, 1999). The general consensus sequence of NFκB binding sites is GGGRNNYYCC (N = any base, R = purine, and Y = pyrimidine) (Hayden and Ghosh, 2004).

Activation of the NFκB signaling pathway

In their inactive state, NFκB dimers are associated with the IκB ("inhibitor of kappa B") proteins. The prototypical and most extensively studied member from the IκB proteins is IκBα. IκBα is primarily bound to p65:p50 heterodimer (Hayden and Ghosh, 2008). The binding of IκB masks the nuclear localization sequence and retains the NFκB dimers in the cytoplasm in an inactive form. The regulation of the IκB-NFκB interaction is a key step for the control of NFκB activity (Gilmore, 1999). Degradation of IκB is initiated upon specific phosphorylation of these molecules by activated IκB kinase (IKK). A great variety of biological factors (e.g., pathogens, cytokines) and environmental conditions (e.g., UV irradiation, oxidative stress) can activate IKK. The activation of IKK results in phosphorylation and proteasomal degradation of IκBα and release of the NFκB dimers. The liberated dimers translocate into the nucleus and exert their transcriptional activity by binding to κB sites (Chen and Ghosh, 1999).

There are two main signaling pathways activating NFκB, called as "classical" (canonical) and "alternative" (non-canonical) pathway (Gilmore, 2006). The classical pathway is essential for the innate immunity (Bonizzi and Karin, 2004). This pathway is activated by endogenous inflammatory stimuli (e.g., pro-inflammatory cytokines such as tumor necrosis factor-α [TNF-α] and interleukin-1 [IL-1]), and in response to viruses and invading microorganisms or their products (e.g. lipopolysaccharides [LPS]). In many cases, the activators of the classical pathway bind to a cell surface receptor, e.g., tumor necrosis factor-receptor or a Toll-like receptor. The major NFκB dimer released in the classical pathway is the p50:p65 heterodimer (Bonizzi and Karin, 2004). The alternative pathway is crucial in lymphoid organ development and the adaptive immunity (Senftleben et al., 2001; Bonizzi and Karin, 2004). In contrast to the classical pathway, the alternative pathway is activated only by certain receptor signals in a limited set of cells (e.g., B cells, fibroblasts, and macrophages). Activation of the alternative pathway results in the nuclear translocation of the p52:RelB dimer (Razani et al., 2010).

The biochemical characteristics of the two major NFκB pathways reflect their functional differences. While the classical pathway is fast acting (it responds within minutes), the alternative pathway responds more slowly (over hours and days). Moreover, the classical pathway is reversible due to the presence of negative feedback mechanisms (Hoffmann and Baltimore, 2006). The negative feedback mechanism is mediated by IκBα whose expression is induced after the NFκB activation. The newly synthesized IκBα enters the nucleus and removes the NFκB dimer from DNA and re-sequestrates them in the cytoplasm (Pahl, 1999). The negative feedback mechanism is responsible for post-induction repression of NFκB activity upon stimulus removal. On the other hand, the alternative pathway provides long-lasting nuclear NFκB activity without an increase of IκBα expression (Hoffmann and Baltimore, 2006).

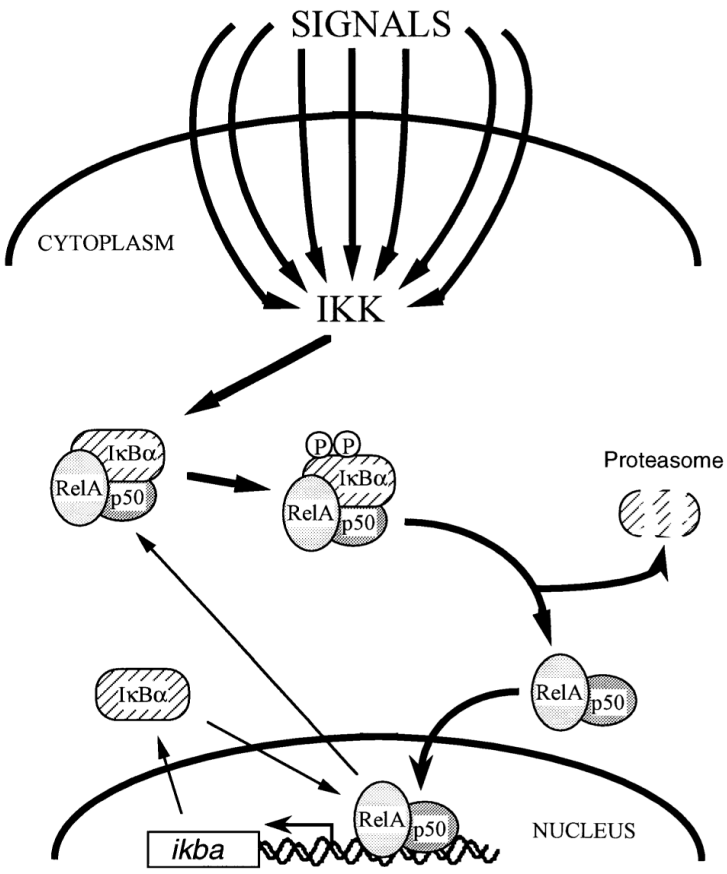

Fig. 7: NFκB classical pathway (adapted from Gilmore, 1999).

Roles of NFκB

Because the activation of NFκB results in the release of different NFκB dimers, NFκB selectively controls the transcription of diverse sets of genes (Bonizzi and Karin, 2004). Active NFκB transcription factors enhance the expression of over 150 target genes. NFκB represents a major transcription factor that controls genes mediating the immune response. These genes include, e.g., cytokines, chemokines, immunoreceptors, cell adhesion molecules, and growth factors. In addition, NFκB regulates the transcription of genes with other cellular functions, such as acute phase proteins, stress response genes and regulators of apoptosis (Pahl, 1999).

Numerous studies show that the NFκB signaling pathway is essential for immune system development and normal cellular proliferation. The dysregulation of the NFκB pathway has been associated with the pathogenesis of many diseases such as cancer, rheumatoid arthritis, diabetes mellitus, atherosclerosis, and neurodegenerative diseases.

Therefore, appropriate regulation and control of NFκB activity can be a powerful therapeutic strategy for the treatment of NFκB-related human diseases. Modulation of NFκB activity may be achieved by gene modification or pharmacological strategies (Kumar et al., 2004).

In recent years, several studies have been focused on the role of ROS and the NFκB signaling. It is now widely accepted that NFκB is engaged in a mutual cross-talk with ROS (reviewed in Bubici et al., 2006; Morgan and Liu, 2011). ROS can mediate both the activation and inhibition of NFκB signaling, depending on the cellular context. In turn, the transcription of NFκB target genes influences the level of ROS in the cells. Thus, NFκB attenuates the production of ROS by increased expression of antioxidant proteins. On the other hand, some transcriptional targets of NFκB promote the production of ROS, especially enzymes important for the inflammatory response, such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) (Morgan and Liu, 2011).

3. Specific aims of the thesis

The main topic of this thesis is the transcriptional regulation of human CBR3. The understanding of CBRs is continuously growing but is still not complete. CBR3, a member of the SDR superfamily, is poorly characterized. Until recently, the transcriptional regulation of CBR3 was fully unknown. The aim of the thesis was not only the experimental investigation of CBR3 regulation but also the review of literature about human CBRs, especially CBR3.

The first part of this thesis summarizes the current knowledge about human CBRs. The topics of the theoretical part are:

1. characterization of human CBRs (i.e., CBR1, CBR3, and CBR4) including their substrates and possible biological roles
2. transcriptional regulation in eukaryotes and the methods of its analysis
3. transcriptional regulation of CBR1 and CBR3.

Several lines of evidence indicate the involvement of NFκB in transcriptional regulation of CBR3. Therefore, in the experimental part, the role of the NFκB signaling pathway in CBR3 regulation was investigated. The goals were:

1. to investigate the expression of CBR3 in various cultured cell lines and to find an appropriate model cell line for studying the regulation of CBR3
2. to study the effect of NFκB activation on CBR3 mRNA level
3. to study the effect of NFκB inhibition on CBR3 mRNA level
4. to investigate the CBR3 protein expression after activation of the NFκB pathway
5. to identify a functional NFκB binding site in the promoter of CBR3.

4. Experimental part

4.1. Materials

4.1.1. Chemicals and related products

6 × Loading dye solution (Fermentas, St. Leon-Rot, Germany)

Agarose NEEO ultra-quality (Carl Roth, Karlsruhe, Germany)

Ampicillin natrium (AGS GmbH, Heidelberg, Germany)

Anti-CBR3 antibody *(sc-70218)* (Santa Cruz Biotechnology, Santa Cruz, USA)

Anti-rabbit HRP-conjugated antibody (GE healthcare, Heidelberg, Germany)

Anti-β-actin antibody *(RB-9421-R1)* (NeoMarkers, Fremont, USA)

BCA Protein assay kit (Novagen, Darmstadt, Germany)

Bovine serum albumin (Behringwerke, Marburg, Germany)

Cell line HCT116 was generously provided by J. Abel (IUF, University of Duesseldorf, Duesseldorf, Germany)

Cell lines A549, HT-29, Caco-2, and SW-480 (DSMZ, Braunschweig, Germany)

Cell lines HepG2, PANC-1, and A431 (Cell lines service, Eppelheim, Germany)

Complete protease inhibitor cocktail tablets (Roche, Mannheim, Germany)

Dimethyl sulfoxide (Sigma-Aldrich, Steinheim, Germany)

DNAse I (Promega, Heidelberg, Germany)

dNTPs (Fermentas, St. Leon-Rot, Germany)

Dual Glo® Luciferase Assay System (Promega, Heidelberg, Germany)

Dulbecco's modified Eagle medium (DMEM) high glucose, DMEM/Ham's F-12 (PAA Laboratories, Cölbe, Germany)

ECL advance Western Blotting detection kit, ECL Western Blotting detection kit (GE healthcare, Heidelberg, Germany)

EDTA (Sigma-Aldrich, Steinheim, Germany)

Fetal calf serum (PAA Laboratories, Cölbe, Germany)

G-418 (Biochrom AG, Berlin, Germany)

GelRed™ (Biotium Inc., Hayward, CA)

GeneRuler™ 100 bp Plus DNA ladder (Fermentas, St. Leon-Rot, Germany)

Human CBR3 cDNA (Deutsches Ressourcenzentrum für Genomforschung, Berlin, Germany)

IL-1β (Cell Systems, Troisdorf, Germany)

Insulin-Transferrin-Selenium-X Supplement (Invitrogen, Darmstadt, Germany)

Kalium chloride (Merck, Darmstadt, Germany)

Kalium dihydrogenphosphate (Sigma-Aldrich, Steinheim, Germany)

L-Glutamine solution (PAA Laboratories, Cölbe, Germany)

Lipofectamine 2000™ (Invitrogen, Darmstadt, Germany)

Lipopolysacharides (Sigma-Aldrich, Steinheim, Germany)

MasterPure™ RNA Purification Kit (Epicentre Biotechnologies, Madison, Wisconsin, USA)

Non-essential amino acids solution (PAA Laboratories, Cölbe, Germany)

Nonidet P40 (Carl Roth, Karlsruhe, Germany)

NuPAGE® Novex® Bis-Tris Gels (Invitrogen, Darmstadt, Germany)

Oligo(dT)$_{18}$ primer (Fermentas, St. Leon-Rot, Germany)

OptiMEM I (Invitrogen, Darmstadt, Germany)

PageRuler™ Prestained Protein Ladder (Fermentas, St. Leon-Rot, Germany)

Parthenolide (Sigma-Aldrich, Steinheim, Germany)

pCI-neo mammalian expression vector (Promega, Mannheim, Germany)

pCMV4 p65 and pCMV4 p50 plasmids (Addgene Inc., Cambridge, MA, USA)

PCR purification kit (Qiagen, Hilden, Germany)

Pefabloc®SC (Sigma-Aldrich, Steinheim, Germany)

pGL4.17[*luc2*/Neo] vector and pGL4.73[*hRluc*/SV40] vector (Promega, Heidelberg, Germany)

Phire Hot-Start DNA polymerase (Biozym Scientific, Hessisch Oldendorf, Germany)

Phusion® High-Fidelity DNA Polymerase (New England Biolabs, Frankfurt am Main, Germany)

Powdered milk blotting grade (Carl Roth, Karlsruhe, Germany)

Primers (Eurofins MWG Operon, Ebersberg, Germany

PureLink™ Genomic DNA Mini Kit (Invitrogen, Darmstadt, Germany)

Pyrrolidine dithiocarbamate (Sigma-Aldrich, Steinheim, Germany)

QIAGEN plasmid Midi kit (Qiagen, Hilden, Germany)

Rapid DNA Dephos & Ligation Kit (Roche, Mannheim, Germany)

Restriction enzymes: EcoRI, HindIII, KpnI and NotI (New England Biolabs, Frankfurt am Main, Germany)

RevertAid™ H minus M-MuLV Reverse Transcriptase (Fermentas, St. Leon-Rot, Germany)

siRNAs targeting NFκB p65 *(sc-29410)* and Nrf2 *(sc-37030)*, non-targeting control siRNA-A *(sc-37007)* (Santa Cruz Biotechnology, Santa Cruz, USA)

Sodium chloride (Mallinckrodt Baker, Griesheim, Germany)

Sodium desoxycholate (Sigma-Aldrich, Steinheim, Germany)

Sodium dodecyl sulfate (Sigma-Aldrich, Steinheim, Germany)

TaqMan Fast Universal PCR Mastermix (Applied Biosystems, Darmstadt, Germany)

Taq-man probes for Real-Time PCR Hs01025918_m1 (CBR3) and Hs99999903_m1 (ATCB, β-Actin) (Applied Biosystems, Darmstadt, Germany)

TNF-α (Cell Systems, Troisdorf, Germany)

Tris base (Sigma-Aldrich, Steinheim, Germany)

Trypsin/EDTA solution (PAA Laboratories, Cölbe, Germany)

Tween 20 (Sigma-Aldrich, Steinheim, Germany)

4.1.2. Apparatus

AB 7500 Fast Real-time PCR system (Life Technologies, Darmstadt, Germany)

Analytical balance (Sartorius AG, Göttingen, Germany)

Centrifuges: Biofuge Fresco and Sorvall Legend RT Plus (Heraeus, Hanau, Germany)

GENios Pro (Tecan, Männedorf, Germany)

Incubator BB6220 (Heraeus, Hanau, Germany)

Intas Gel-imaging system (Intas, Göttingen, Germany)

PCR-Thermocyclers: TGradient and TProfessional (Biometra, Göttingen, Germany)

Power Supply: Power Pack 35/60 (Phase, Lübeck, Germany), Power Pack 300 (BioRad, München, Germany)

Spectrophotometer GeneQuant II (Pharmacia Biotech, Freiburg, Germany)

XCell SureLock® Mini-Cell and XCell II™ Blot Module Kit (Invitrogen, Darmstadt, Germany)

4.2. Cell culture

The human colon cancer cell lines Caco-2, HT-29, HCT116, and SW-480, and human lung cancer cell line A549 were maintained in DMEM (high glucose) supplemented with 2 mM L-glutamine, 1 % of non-essential amino acids and 10 % heat-inactivated fetal calf serum (FCS). PANC-1 (pancreas) and A431 (skin) cells were cultured in DMEM supplemented with 4 mM L-glutamine and 10 % FCS. HepG2 (liver) cells were maintained in a DMEM/Ham's F12 (1:1) mixture supplemented with 2 mM L-glutamine and 10 % FCS. All cells were routinely cultured without antibiotics in a humidified atmosphere of 5 % CO_2 in air at 37 °C.

4.3. Gene expression experiments

4.3.1. Incubation with test compounds

Cells were seeded in 60 mm Petri dishes or six-well plates (9.3 cm²) and grown until they reached 60-90 % of confluence. Stock solutions of IL-1β and LPS were prepared in sterile H_2O, stock solution of TNF-α in sterile H_2O containing 0.1 % BSA, and stock solution of parthenolide (PRT) and pyrrolidine dithiocarbamate (PDTC) in dimethyl sulfoxide. The aliquots were stored at -20 °C until used. After serum-starvation for 18 h to 24 h, the cells were incubated with test compounds freshly dissolved in serum-free medium for times as indicated in the text. In case of PRT and PDTC treatment, the final concentration of dimethyl sulfoxide in test medium was 0.1 %. The medium was changed every 24 h.

4.3.2. Transient transfections

Cells were seeded in 6-well plates and 35 mm Petri dishes and grown to 40-60 % confluence. Expression plasmids for NFκB subunits p65 (pCMV4 p65) and p50 (pCMV4 p50) were constructed by Warner Greene (Ballard et al., 1992) and obtained from Addgene. The plasmids pCMV4 p65 and pCMV4 p50 were purified from bacterial cultures with QIAGEN plasmid Midi kit. Expression plasmids pCMV4 p65 and pCMV4 p50 or siRNA were diluted in 250 µl of OptiMEM I, combined with 250 µl of OptiMEM I containing 10 µl of Lipofectamine 2000^{TM}. After incubation for 20 min at room temperature, the mixture was added to the cells. The medium was replaced after 7 h with fresh culture medium. Transfected cells were harvested for RNA isolation after 24 h to 72 h.

4.3.3. RNA isolation and cDNA synthesis

RNA was isolated using the MasterPure™ RNA Purification Kit. The cell-monolayer was washed with phosphate buffered saline (PBS), and the cells were harvested with 600 µl of Tissue and cell lysis solution. RNA isolation was performed following the manufacturer's protocol for cell samples including removal of contaminating DNA and with an additional ethanol wash step. RNA concentration and purity was assessed by measuring absorbance at 260 nm and the A260/A280 ratio. Isolated RNA was stored at -80 °C until cDNA synthesis.

To avoid the co-amplification of genomic DNA, prior to reverse transcription, two micrograms of RNA were subjected to DNAse I digestion following manufacturer´s instruction. Then, the treated RNA was reverse transcribed using RevertAid^{TM} H minus M-MuLV reverse transcriptase with 100 pmol of oligo $(dT)_{18}$ as primer for 60 min at 42 °C. The cDNA was used for PCR analysis or stored at -20 °C.

4.3.4. Semi-quantitative RT-PCR (sqPCR)

All primers were designed to span at least one exon-exon-boundary to avoid co-amplification of genomic DNA. Semi-quantitative RT-PCR (sqPCR) was performed using Phire Hot-start DNA polymerase in a total volume of 20 µl containing 2 µl of cDNA, 500 nM RT-Primer, 200 µM dNTPs and 1.5 mM $MgCl_2$. The thermal cycling comprised an initial denaturation step at 98 °C for 30 min, followed by 19-34 cycles of denaturation at 98 °C for 10 s, annealing at 59 °C for 15 s, and extension at 72 °C for 15 s and a final extension step at 72 °C for 2 min. β-Actin served as the housekeeping gene. Amplified PCR products were separated electrophoretically on 1.5 % agarose, visualized by staining with GelRed^{TM} and documented digitally with Intas Gel-imaging system. Densitometric analyses were carried out with GIMP 2.6 software. Primer sequences and amplicon sizes are shown in Table 1.

Table 1: RT-PCR primers and amplicon sizes

Gene	**GenBank Acc. No**	**Forward primer/ Reverse primer (sequence from 5' to 3')**	**Amplicon size (bp)**
β-Actin	NM_001101	ACTCTTCCAGCCTTCCTTCCT AGGTTTTGTCAAGAAAGGGTGT	394
CBR3	NM_001236	GCTCAACGTACTGGTCAACAAC ATCCTCGATAAGACCGTGACC	372
IκBα	NM_020529	CTACACCTTGCCTGTGAGCA GCTCGTCCTCTGTGAACTCC	466
COX-2	NM_000963.2	GAATGGGGTGATGAGCAGTT GGTCAATGGAAGCCTGTCAT	397
p50 (NFκB1)	NM_003998.3	TGCCAACAGCAGATGGCCCAT AAACATGAGCCGCACCACGCT	544
p65 (RelA)	NM_021975.3	AAGTTCCTATAGAAGAGCAGCG TGCTCTTGAAGGTCTCATATG	509
Nrf2	NM_006164	GAGAGCCCAGTCTTCATTGC ACTGGTTGGGGTCTTCTGTG	343

4.3.5. Quantitative real-time RT-PCR (qPCR)

Two micrograms of RNA were reverse transcribed into cDNA in a total volume of 35 µl as described above. Each cDNA sample was diluted 2-fold and 2 µl of this dilution were used for real-time RT-PCR (qPCR) analysis. One 20 µl reaction contained 1 µl of TaqMan Gene expression Assay Hs01025918_m1 (CBR3) or Hs99999903_m1 (ATCB, β-Actin), 10 µl of TaqMan Fast Universal PCR Mastermix, 2 µl cDNA and 7 µl sterile H_2O. The thermal cycling conditions included an initial activation-step at 95 °C for 20 s, followed by 40 cycles of denaturation (95 °C , 3 s) and annealing/extension (60 °C, 30 s). Samples and no-template controls were run in triplicates. The fold-changes in the mRNA expression levels were calculated by the comparative quantitation ($2^{-\Delta\Delta Ct}$) method by using the instrument's software.

4.4. Protein expression experiments

4.4.1. Construction of CBR3 expression plasmid pCI-neoCBR3

I have not participated in the construction of expression plasmid pCI-neoCBR3, therefore, it follows just brief description of the procedure. Human CBR3 cDNA was amplified by PCR and cloned into the EcoRI/NotI restriction sites of pCI-neo mammalian expression vector using „Rapid Ligation Kit“. This vector contains the neomycin phosphotransferase gene which allows for selection of cells stably expressing CBR3 by treatment with G-418.

4.4.2. Incubation with test compounds

Cells were seeded in 100 mm Petri dishes and grown until they reached 90-100 % confluence. Stock solution of TNF-α was prepared in sterile H_2O containing 0.1 % BSA. The aliquots were stored at -20 °C until used. After serum-starvation for about 20 h, the cells were incubated with TNF-α freshly dissolved in serum-free medium for times indicated in the text below. The medium was changed every 24 h. The serum-reduced medium consisting of DMEM, Insulin-Transferrin-Selenium-X and 2 % FCS was used in the experiments with HCT116 cells instead of serum-free medium.

4.4.3. Transient transfections

HT-29 cells were seeded in 60 mm Petri dishes and grown to 90 % confluence. Expression plasmids pCMV4 p65 and pCMV4 p50 were diluted in 500 µl of OptiMEM I, combined with 500 µl of OptiMEM I containing 20 µl of Lipofectamine 2000™. After incubation for 20 min at room temperature, the mixture was added to the cells. The medium was replaced after 7 h with fresh culture medium. Transfected cells were harvested for western blotting after 24 h to 72 h.

4.4.4. Western blotting

To obtain whole cell lysates, cells were harvested by scraping into a small volume of ice-cold PBS containing 50 µM Pefabloc® SC protease inhibitor, pelleted by centrifugation and passively lyzed for 30 min on ice in RIPA buffer (50 mM Tris, 150 mM NaCl, 1 % Nonidet P40, 0.5% sodium desoxycholate, 0.1 % SDS, pH 7.5) supplemented with protease inhibitor cocktail. Soluble fractions were obtained by centrifugation and protein contents of samples were determined by the bicinchoninic

acid method with bovine serum albumin as a standard. Proteins (70-100 µg) were separated on NuPAGE® Novex® Bis-Tris Gels (4-12 % acrylamide, Invitrogen) and electrotransferred onto PVDF membranes. After blocking overnight in blocking buffer (5 % dry milk in 0.1 % PBS-T) at 4 °C, probing was performed for 1 h with anti-CBR3 antibody (dilution 1:500 in 0.1 % PBS-T containing 2.5 % dry milk). Blots were then incubated with secondary anti-rabbit horse-radish peroxidase (HRP)-conjugated antibody (1:4000) for 1.5 h at room temperature. Bands were visualised by enhanced chemiluminescence detection kit (ECL™) following the manufacturer's instructions. Blots were stripped using glycine buffer (100 mM glycine, pH 2.5) and reprobed for β-actin (anti-β-actin primary antibody, dilution 1:100; anti-rabbit HRP conjugated secondary antibody, dilution 1:10000).

4.5. Reporter gene assay

Genomic DNA was isolated from SW480 cells with PureLink™ Genomic DNA Mini Kit. A 1297 bp portion of the CBR3 promoter was amplified by PCR using Phusion® High-Fidelity DNA Polymerase. Three 5'-progressive deletion constructs were amplified using the product from the first PCR as a template. Primer sequences and amplicon sizes are shown in Table 2. The 1297 bp portion of the CBR3 promoter and its deletion constructs were digested with KpnI and HindIII for 3 h at 37 °C, column-purified with PCR purification kit and cloned into the KpnI and HindIII restriction site of the basic firefly luciferase vector pGL4.17 using the Rapid DNA Dephos & Ligation Kit. HB101 cells were transformed with vectors and plated on ampicillin-containing plates. Colony PCR was used to screen the ampicillin resistant colonies using appropriate primers. Positive clones were verified by sequence analysis.

HepG2 cells were seeded in 96 well plate. For each well, 50000 cells in 100 µl of growth medium containing FCS were used. Medium was changed 24 h after seeding and the transfection was performed. Cells were co-transfected with 200 ng of one of the CBR3 firefly luciferase plasmid constructs or the pGL4.17 empty vector and 10 ng of the normalizer plasmid pGL4.73 containing the Renilla luciferase gene. In detail, for each well, plasmid DNA was diluted in 25 µl OptiMEM I and combined with 25 µl OptiMEM I containing 0.5 µl of Lipofectamine 2000™. After incubation for 20 min at room temperature, the mixture was added to the cells. Twenty-four hours after

transfection, the medium was replaced with 150 µl of serum-free medium. After serum starvation for 24 h, the cells were incubated for 4 h with 75 µl of IL-1β (10 ng/ml) freshly dissolved in serum-free medium or with 75 µl of serum-free medium only. Then, luciferase reporter gene activities were determined with the Dual Glo® Luciferase Assay System according to the manufacturer´s instructions. Light release was quantified for 10 s using a luminometer. Firefly luciferase activities from each reporter construct and from the pGL4.17 empty vector were first normalized to their corresponding renilla (reniformis) luciferase activities. Normalized luciferase activities from each reporter construct were corrected by subtracting the mean luciferase activity from the pGL4.17 empty vector.

Table 2: Primers for construction of 5'-progressive deletion constructs of the CBR3 promoter. Primers with suffix _KpnI or _HindIII contain restriction sites for KpnI or HindIII, respectively.

Designation	**Sequence from 5' to 3'**
Fwd1	CAGTGGTGCAATCTCGGTTTACTGCAACCTC
Fwd2	GAACTCCTGACCGCGTAATCCACCCG
Fwd3	GCGCACACCCAAGAAAATTACTCAGCACTTG
Rev1	GGCTGAGCGGGGAGCGCG
Fwd1_KpnI	AGCTTTGGTACCCAGTGGTGCAATCTC
Fwd2_KpnI	AGCTTTGGTACCGAACTCCTGACCGCG
Fwd3_KpnI	AGCTTTGGTACCGCGCACACCCAAGAA
Rev1_HindIII	AAGTCGTTCGAAGGCTGAGCGGGGAGC

4.6. Statistical analysis

Statistical analyses were performed with GraphPad Prism version 5.04 for Windows, GraphPad Software, San Diego California USA. Differences between mean values were determined by a two-tailed paired Student`s t-test or a one-way ANOVA followed by a Tukey's post-test. Statistically significant differences were set at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$ indicated highly significant data.

5. Results and discussion

5.1. CBR3 expression in cultured cell lines

5.1.1. CBR3 mRNA and protein levels in various cell lines

In order to find an appropriate model cell line for studying the regulation of CBR3, several human cell lines were screened for basal level of CBR3 expression. These cell lines were originated from different tissues, namely, colon (Caco-2, HT-29, HCT116, and SW-480 cells), lung (A549 cells), skin (A431 cells), pancreas (PANC-1 cells), and liver (HepG2 cells).

The expression of CBR3 mRNA as well as protein varied widely. As shown in Fig. 8A, the cell lines A549 and SW-480 expressed the largest amounts of CBR3 mRNA. In contrast, small amounts of CBR3 mRNA were detected in HT-29 and HepG2 cells. Next, the expression of CBR3 protein was analyzed by western blotting using the rabbit anti-CBR3 primary antibody (Santa Cruz, sc-70218; 1:500 dilution) (Fig. 8B). CBR3 protein levels correlated well with that of CBR3 mRNA expression levels for almost all cell lines. The only exception was the HCT116 cell line with high level of CBR3 mRNA but no detectable CBR3 protein. No CBR3 protein was also detected in HepG2 cells that expressed a very low level of CBR3 mRNA.

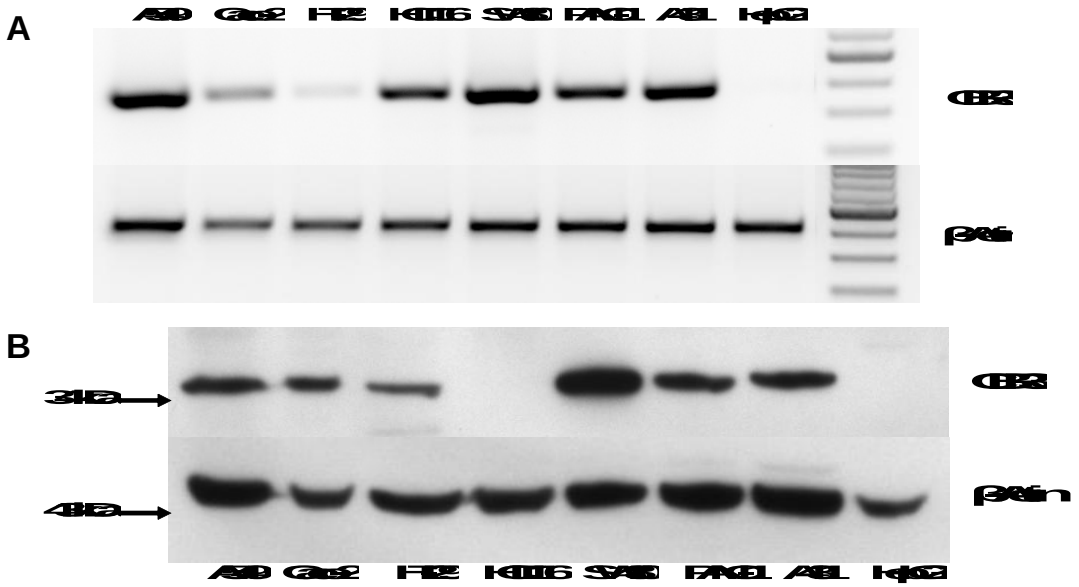

Fig. 8: (A) Constitutive expression of CBR3 mRNA in different human cancer cell lines. Total RNA (2 µg) was reverse transcribed in cDNA and gene specific DNA fragment were amplified by 34 (CBR3) and 19 (β-actin) PCR cycles. (B) Basal expression levels of CBR3 protein in different human cancer cell lines. Whole cell lysates were prepared from different cell lines and 70 µg of protein were analyzed by western blotting. β-Actin served as the loading control.

5.1.2. Changes of CBR3 expression during Caco-2 differentiation

Caco-2 cells, in contrast to many other established colon cancer cell lines, such as the HT-29 cell line, undergo spontaneous differentiation after reaching confluence. The differentiation of Caco-2 cells is completed within 20 days and is characterized by the morphological and functional changes. The same differentiation features are typical for the absorptive cells of the colonic epithelium (Chantret et al., 1988; Meunier et al., 1995). To assess if CBR3 protein expression is changing during differentiation of Caco-2 cells, the level of CBR3 protein was analysed by means of western blotting.

Caco-2 cells were harvested in subconfluent state, confluent state (day 0) and 4, 8, 14 and 20 days after reaching confluence. CBR3 protein expression increased rapidly during differentiation from subconfluent state through day 14, when the maximum level of CBR3 protein was detected (9.5-fold increase compared to subconfluent cells). After longer cultivation, by day 20, CBR3 protein level decreased slightly to 7.4-fold increase compared to subconfluent cells.

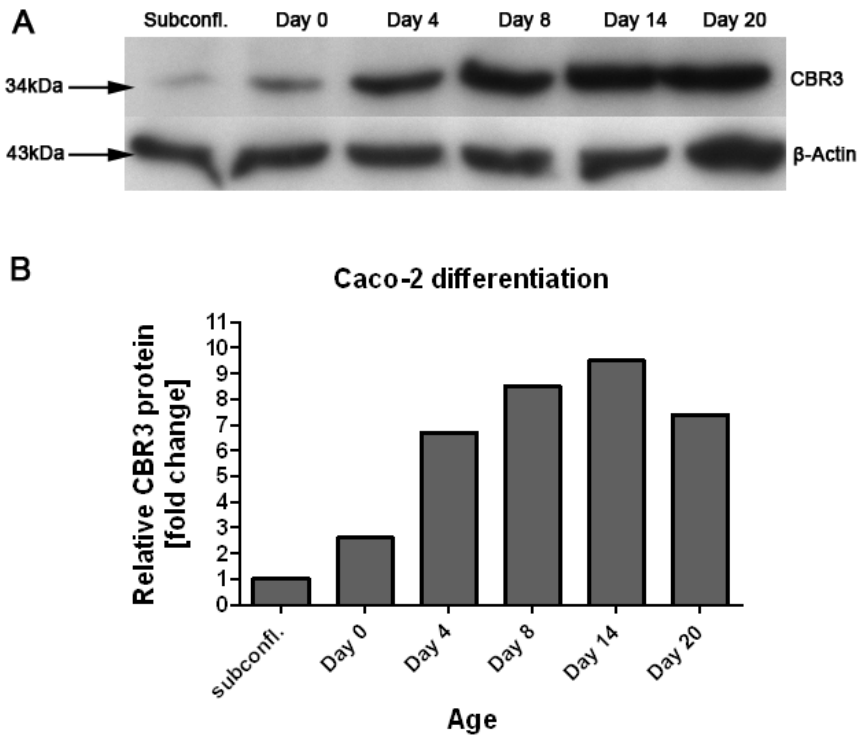

Fig. 9: (A) Changes of CBR3 protein level during differentiation of Caco-2 cells. Each lane contained 100 µg of protein (whole cell lysate). (B) The bar graph represents the densitometric analysis of the gel shown in panel A. The subconfluent cells ("subconfl.") were set to 1.

5.1.3. Summary of CBR3 expression in cultured cell lines

Human CBR3 mRNA was found to be expressed ubiquitously but the expression pattern is different among various tissues (Miura et al., 2008). By analyzing several human cancer cell lines of various origin, we found that CBR3 mRNA is expressed differentially in these cell lines. Very low levels of CBR3 mRNA were found in HT-29 and HepG2 cells (Fig. 8A). Therefore, the cell lines HT-29 and HepG2 were chosen as suitable models to investigate the regulation of CBR3. Their low basal levels of CBR3 mRNA allow the clear detection of any increase of CBR3 mRNA expression. Levels of CBR3 protein exhibited a similar expression pattern as CBR3 mRNA.

The changes of CBR3 protein expression has been studied in detail in Caco-2 cells because they undergo spontaneous enterocytic differentiation. Longer culture times were accompanied by increases in CBR3 protein levels (Fig. 9). This correlation between CBR3 expression and cell differentiation suggests that the enzyme may play some important role in the differentiated cells.

5.2. The effect of NFκB activation on CBR3 mRNA expression

5.2.1. NFκB activation in HT-29 cells

5.2.1.1. TNF-α

HT-29 cells were incubated with TNF-α (50 ng/ml) for 2, 4, 8, 12, 24 and 48 h. As presented in Fig. 10A-C, TNF-α increased the expression levels of CBR3 mRNA in HT-29 cells throughout whole time course of the experiment. Four independent cell culture experiments were subjected to semi-quantitative RT-PCR (sqPCR). β-Actin served as the housekeeping gene. Densitometric analysis (Fig. 10B) showed that CBR3 mRNA levels continuously increased from the 2 h treatment on (2-fold increase vs. control, $P<0.05$) until it reached a peak after 48 h (9.2-fold vs. control, $P<0.05$). The mRNA levels of two established NFκB-target genes, COX-2 and IκBα, which served as controls for the activation of the NFκB signaling pathway, were also assessed (Fig. 10A). Both COX-2 and IκBα were induced by TNF-α treatment throughout the experiment, proving that TNF-α successfully activated NFκB in HT-29 cells.

Two representative experiments were analysed by means of quantitative real-time RT-PCR (qPCR). The results correlated well with these from the sqPCR analysis except for the 48 h treatment. As presented in Fig. 10C, the amounts of CBR3 mRNA increased substantially as early as 4 h post-treatment (3.1-fold vs. control), peaked at 24 h (10.3-fold vs. control) and then, after 48 h, decreased but still remained elevated 5.3-fold compared to untreated control cells.

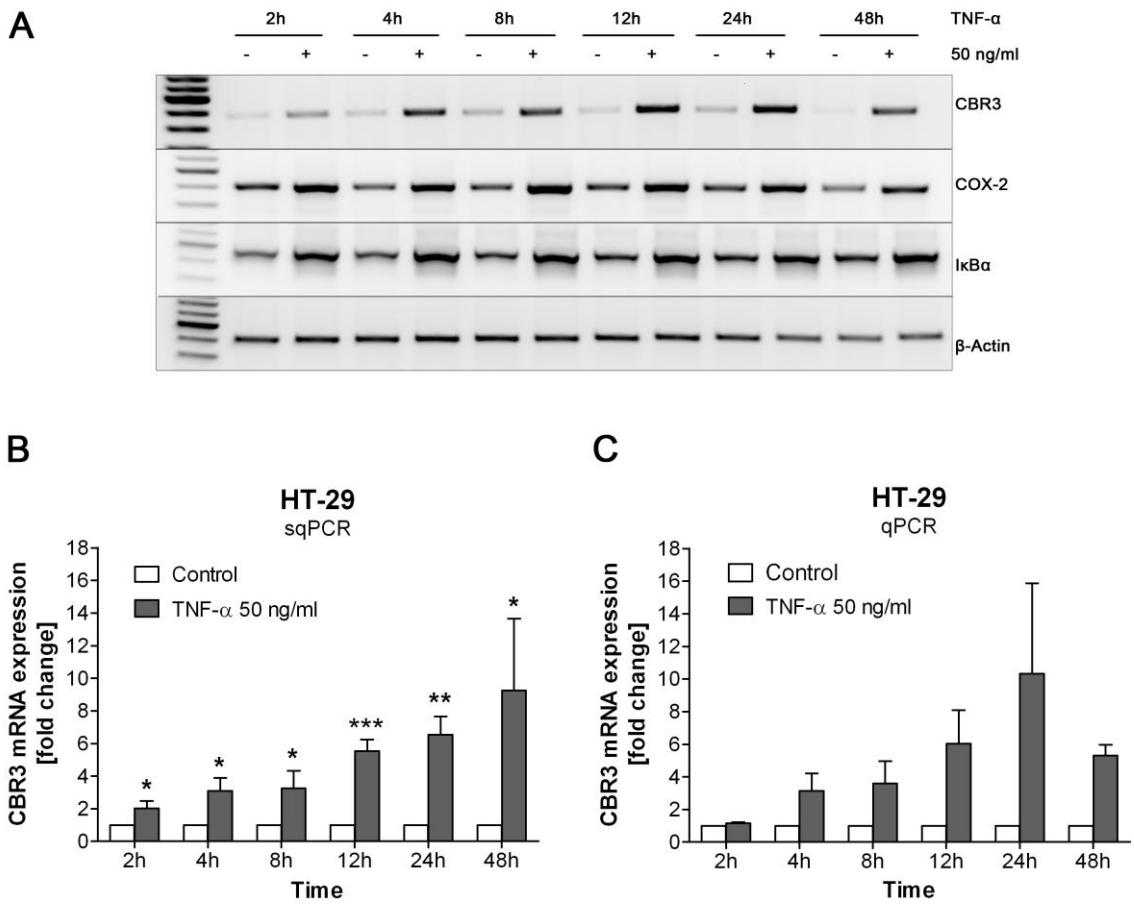

Fig. 10: HT-29 cells were incubated with (+) or without (−) TNF-α (50 ng/ml). Four independent experiments were subjected to sqPCR. (A) One representative gel is shown. (B) The densitometric analysis of the changes in CBR3 mRNA expression relative to untreated cells (control). Bars represent means of n=4 experiments ± the standard deviation (three asterisks denote $P<0.001$; two asterisks denote $P<0.01$; one asterisk denotes $P<0.05$) *(two-tailed paired Student`s t-test)*. (C) Two representative experiments were analysed by real-time PCR. Bars represent means of n=2 experiments ± the standard deviation.

Next, we investigated the concentration-dependent effect of TNF-α on the expression of CBR3 mRNA. HT-29 cells were exposed to 10, 25, 50 and 100 ng/ml of TNF-α for 24 h (Fig. 11A-C). sqPCR analysis was performed for five independent experiments and qPCR for two representative experiments. Both methods clearly showed that TNF-α induced CBR3 mRNA expression in a concentration-dependent manner. As expected, the values for the fold induction differed slightly between the PCR approaches due to the application of different methods.

The sqPCR (Fig. 11B) showed that CBR3 mRNA was inducible even by the lowest concentration of 10 ng/ml of TNF-α (2.3-fold vs. control, $P<0.05$) and this effect was continuously growing with increasing concentrations of TNF-α up to 6.6-fold compared to control ($P<0.001$) when 100 ng/ml of TNF-α was applied. The exposure to TNF-α elevated also the levels of the NFκB-driven control genes COX-2 and IκBα (Fig. 11A). The qPCR analysis (Fig. 11C) confirmed the concentration-dependent induction of CBR3 mRNA by TNF-α. The strongest induction of the *CBR3* gene was observed by qPCR analysis after incubation with the highest concentration of 100 ng/ml of TNF-α (15.3-fold vs. control).

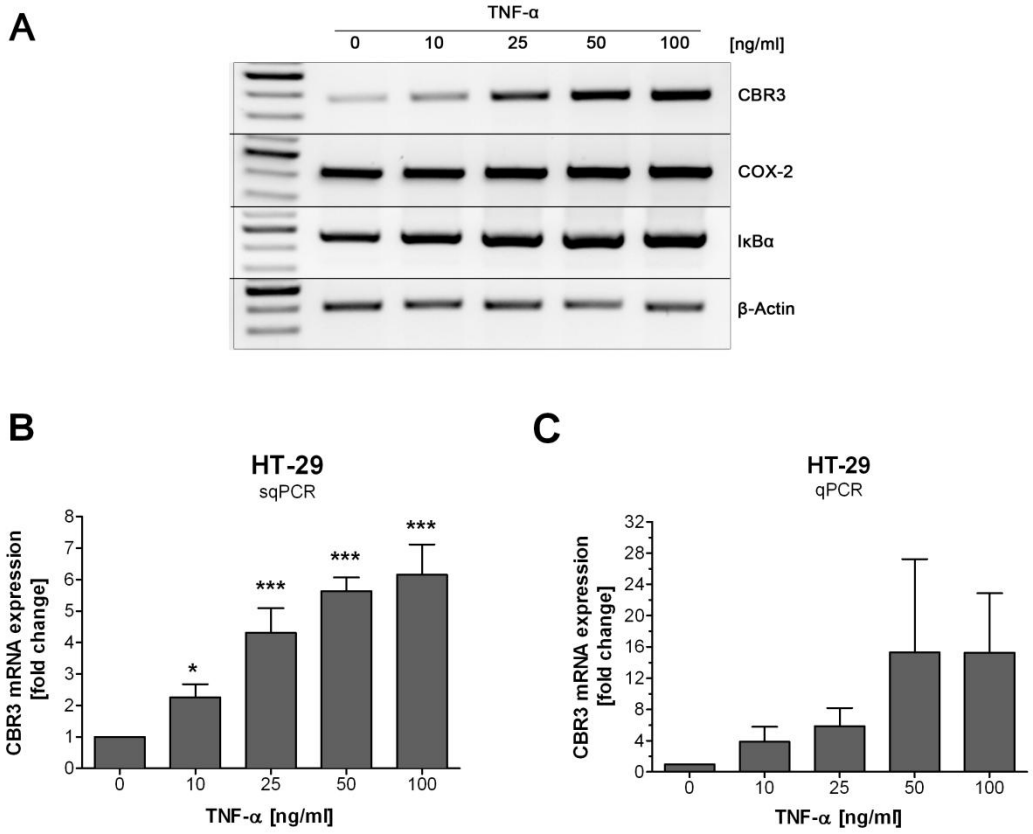

Fig. 11: Concentration-dependent effect of TNF-α on the expression of CBR3 mRNA in HT-29 cells. Different concentrations of TNF-α were applied for 24 h. (A) A representative gel shows the effect on CBR3 and NFκB-regulated control genes COX-2 and IκBα. (B) Densitometric analysis of five independent experiments that were analysed by sqPCR. Bars represent means of n=5 experiments ± the standard deviation (three asterisks denote $P<0.001$; one asterisk denotes $P<0.05$) *(ANOVA)*. (C) Real-time PCR analysis of two representative experiments. Bars represent means of n=2 experiments ± the standard deviation.

5.2.1.2. IL-1β

Next, the effect of another well-established NFκB activator, IL-1β, on CBR3 mRNA expression was studied in HT-29 cells. IL-1β (10 and 20 ng/ml) was added to cells for 4, 8, and 24 h (Fig. 12A). Densitometric analysis of two independent cell culture experiments that were subjected to sqPCR revealed that CBR3 mRNA level increased only as late as 24 h after treatment (Fig. 12B). IL-1β at a concentration of 20 ng/ml had stronger effect (2.2-fold increase vs. control) than lower concentration of 10 ng/ml (1.6-fold increase vs. control). When the incubation period was extended to 48 h, CBR3 mRNA levels decreased to the basal level (data not shown).

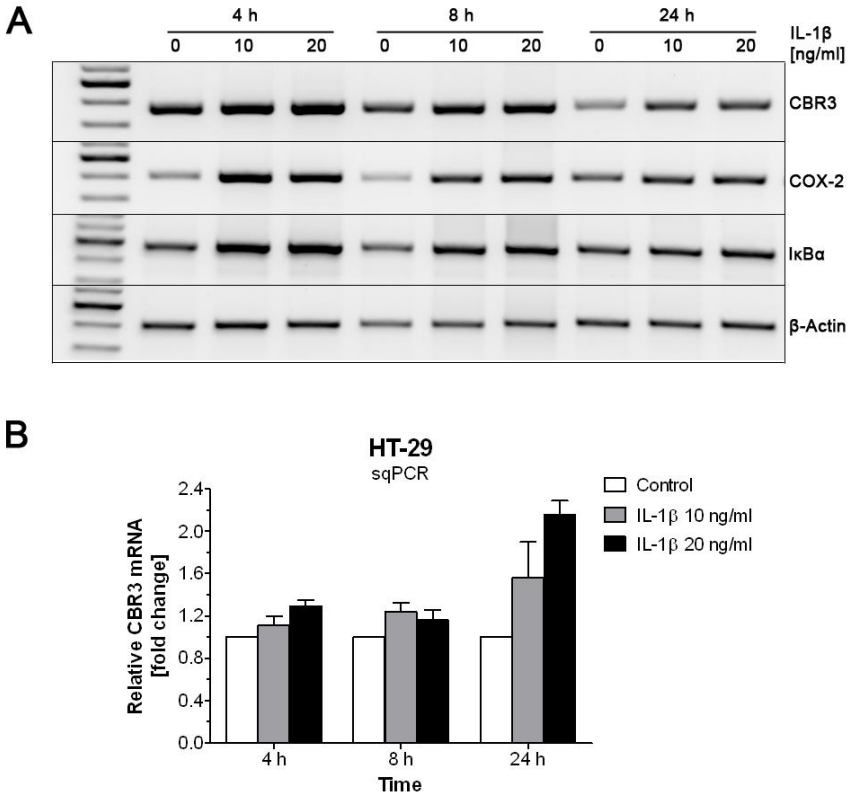

Fig. 12: HT-29 cells were treated with IL-1β (10 and 20 ng/ml) or serum-free medium only (0 ng/ml, control) for 4, 8 and 24 h and prepared for sqPCR analysis. (A) One representative gel is shown. (B) The densitometric analysis of two independent experiments. Bars represent means of n=2 experiments ± the standard deviation.

5.2.1.3. LPS

LPS was the last from the three NFκB activators used for studying the CBR3 expression. HT-29 cells were exposed to LPS (100 and 1000 ng/ml) and CBR3 mRNA levels were assessed by sqPCR after 4, 8, and 24 h. Fig. 13 shows that LPS slightly increased CBR3 mRNA levels after 8 h while both shorter and longer incubation times did not affect the expression of CBR3 mRNA. Eight hours of treatment with LPS (1000 ng/ml) up-regulated the expression of CBR3 mRNA 1.5-fold compared to control ($P<0.05$) (Fig. 13B).

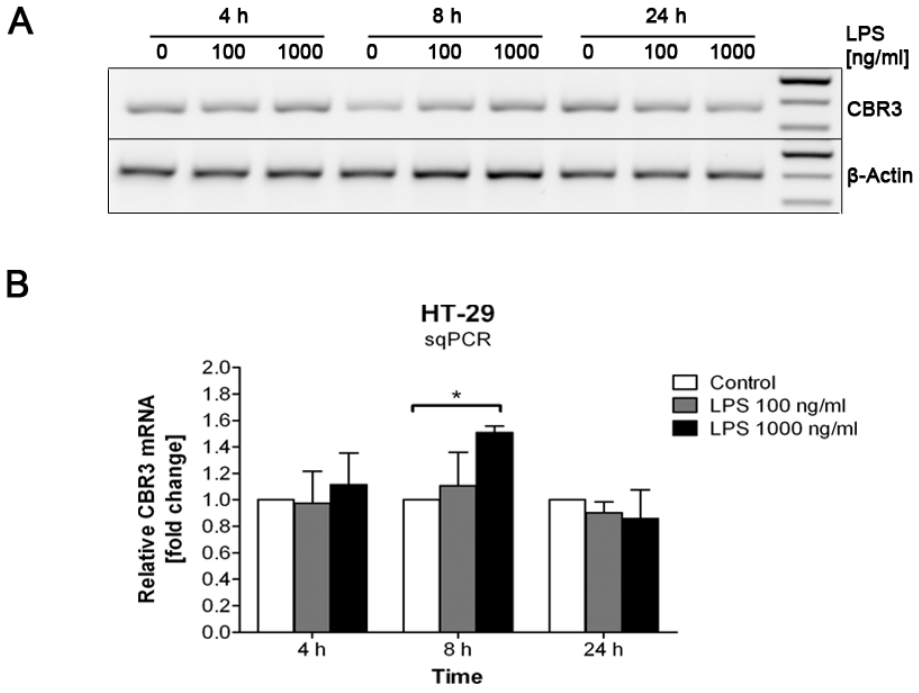

Fig. 13: HT-29 cells were treated with LPS (100 and 1000 ng/ml) or without LPS (0 ng/ml, control) for 4, 8 and 24 h and prepared for sqPCR analysis. (A) One representative gel of the treatment with LPS. (B) The densitometric analysis of two independent experiments. Bars represent means of n=2 experiments ± the standard deviation (one asterisk denotes $P<0.05$) *(ANOVA).*

5.2.2. NFκB activation in Caco-2 cells

The same NFκB activators that were tested in HT-29 cells were applied to another colon carcinoma cell line, namely Caco-2 cell line. Caco-2 cells were treated with two concentrations of each TNF-α (10 and 50 ng/ml), IL-1β (10 and 20 ng/ml) or LPS (100 and 1000 ng/ml). The changes in CBR3 mRNA levels were determined after 4, 8 and 24 h by sqPCR. All activators caused only slight changes of the CBR3 mRNA levels. Treatment with TNF-α (10 ng/ml, 8 h) and IL-1β (20 ng/ml, 4 h) elevated the CBR3 mRNA 1.7- and 1.6-fold, respectively (Fig. 14, A and B). In contrast, the 4 h treatment with LPS (100 ng/ml) significantly decreased the level of CBR3 mRNA expression by 1.7-fold compared to control ($P<0.01$). However, the higher concentration of LPS and/or longer treatment did not affect CBR3 mRNA levels (Fig. 14C).

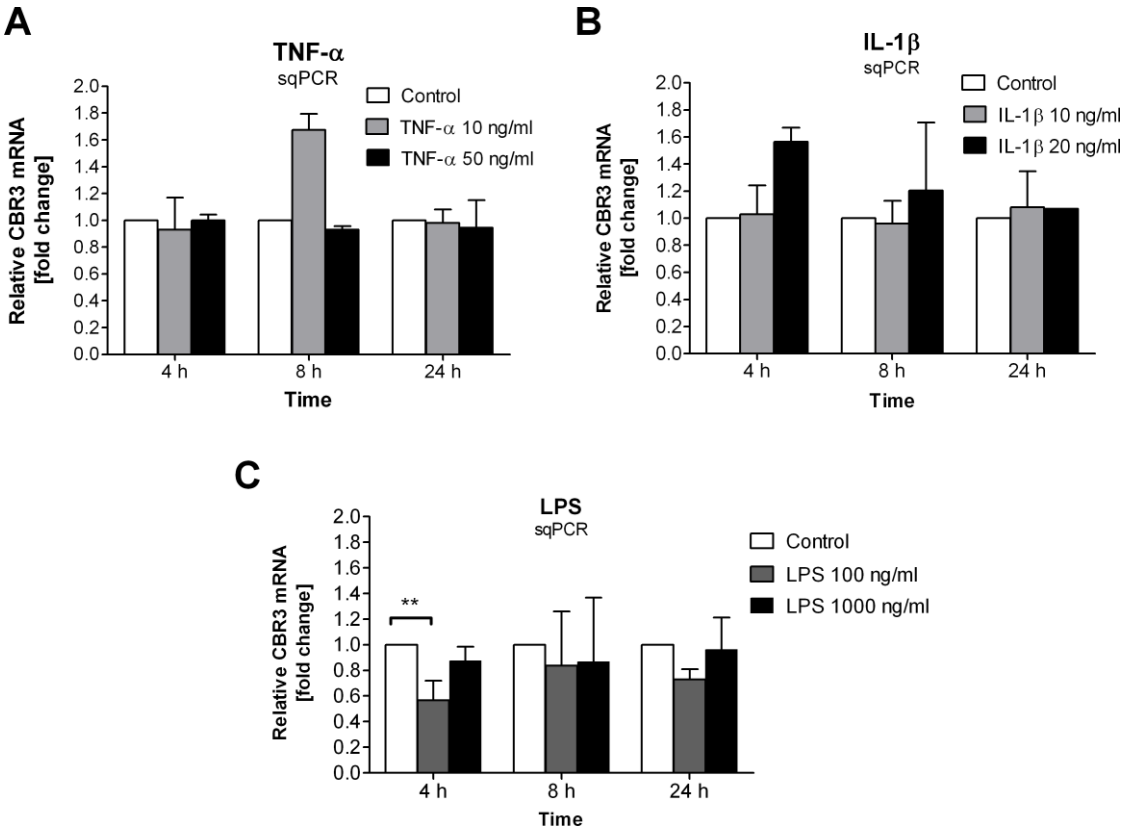

Fig. 14: Caco-2 cells were treated with TNF-α (10 and 50 ng/ml), IL-1β (10 and 20 ng/ml), or LPS (100 and 1000 ng/ml) or serum-free medium only (control) for 4, 8 and 24 h and prepared for sqPCR analysis. The densitometric analysis of two independent experiments with TNF-α (A) and IL-1β (B) is shown. Bars represent means of n=2 experiments ± the standard deviation. (C) The densitometric analysis of three independent experiments with LPS. Bars represent means of n=3 experiments ± the standard deviation (two asterisks denote $P<0.01$) *(ANOVA)*.

5.2.3. NFκB activation in HepG2 cells

5.2.3.1. Preliminary experiment

In order to investigate whether CBR3 mRNA expression is induced upon NFκB activation in a cell line derived from a different tissue, HepG2 cells were treated with TNF-α, IL-1β, and LPS for 4, 8 and 24 h. All NFκB activators tested greatly induced CBR3 mRNA expression in HepG2 cells with the strongest inducing effect after 4 h (Fig. 15) that decreased continuously after 8 and 24 h (data not shown). The most effective inducer of CBR3 mRNA was IL-1β (5.8- and 5.3-fold increase vs. control; 10 and 20 ng/ml, respectively) followed by TNF-α (2.2-fold increase vs. control; 50 ng/ml) and LPS (2.0-fold increase vs. control; 1000 ng/ml). Therefore, IL-1β was chosen for further experiments to study its effect on CBR3 mRNA expression in HepG2 cells in more detail.

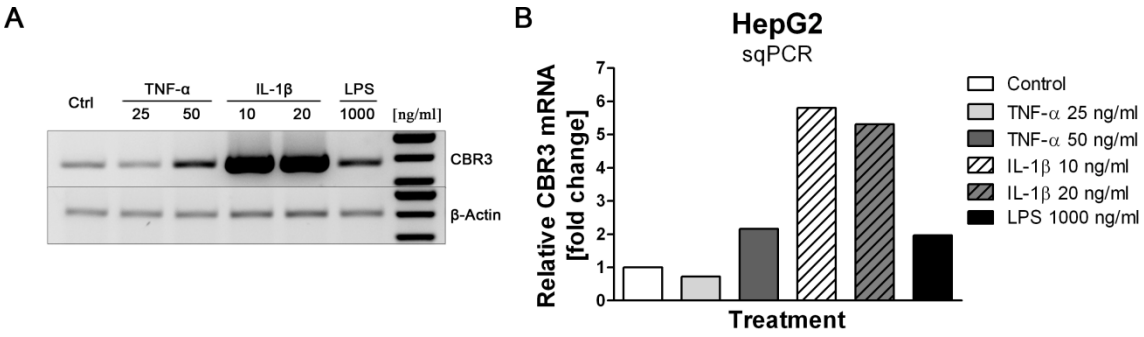

Fig. 15: HepG2 cells were treated with activators of the NFκB signaling pathway TNF-α (25 and 50 ng/ml), IL-1β (10 and 20 ng/ml), and LPS (1000 ng/ml) or serum-free medium only (control, "Ctrl") for 4, 8 and 24 h. Expression levels of CBR3 mRNA were examined by sqPCR. One representative gel (A) and its densitometric analysis (B) for the 4 h incubation are shown.

5.2.3.2. IL-1β

The experiment with HepG2 cells corresponding to that with HT-29 cells (see above *5.2.1.1. TNF-α*) was performed, however, with another test compound, namely IL-1β. This cytokine was chosen because it had the strongest effect on CBR3 expression in HepG2 cells in the preliminary experiment. According to the results from the preliminary experiment, shorter incubation times of 2 h to 8 h were used. IL-1β was added to HepG2 cells at concentrations of 1 ng/ml and 10 ng/ml. The changes in the expression of CBR3 mRNA were assessed after 2, 4, and 8 h by means of sqPCR. From four independent cell culture experiments, two were analyzed by qPCR. IκBα served as a control gene for monitoring the NFκB activation because the second NFκB-regulated control gene, COX-2, was undetectable in this cell line. When HepG2 cells were subjected to IL-1β treatment, IκBα exhibited an expression pattern similar to that of CBR3 (Fig. 16A).

The densitometric analysis of the sqPCR experiment (Fig. 16B) showed that IL-1β (1 ng/ml and 10 ng/ml) significantly induced CBR3 mRNA expression (to 4.2-fold, $P<0.05$ and 10.5-fold, $P<0.001$, respectively) even after 2 h treatment of HepG2 cell line. Longer incubation with 10 ng/ml of IL-1β did not further increase the amount of CBR3 mRNA, but rather decreased (to 7.8-fold, $P<0.001$, at 4 h and to 2.6-fold at 8 h). As presented in Fig. 16C, the qPCR analysis revealed the comparable pattern of the CBR3 mRNA induction to that of sqPCR analysis. Namely, IL-1β treatment (1 ng/ml and 10 ng/ml) after 2 h clearly induced the *CBR3* gene on mRNA level to 3.7- and 20.7-fold, respectively. After 4 h, a slight further increase was observed only in cells exposed to lower concentration of 1 ng/ml of IL-1β (5.5-fold vs. control). The effect of 10 ng/ml of IL-1β remained unchanged after 4 h treatment (20.9-fold induction vs. control) but decreased rapidly after 8 h to 4.1-fold change relative to untreated control. Similarly, CBR3 mRNA expression declined to 1.5-fold after 8 h treatment with 1 ng/ml of IL-1β.

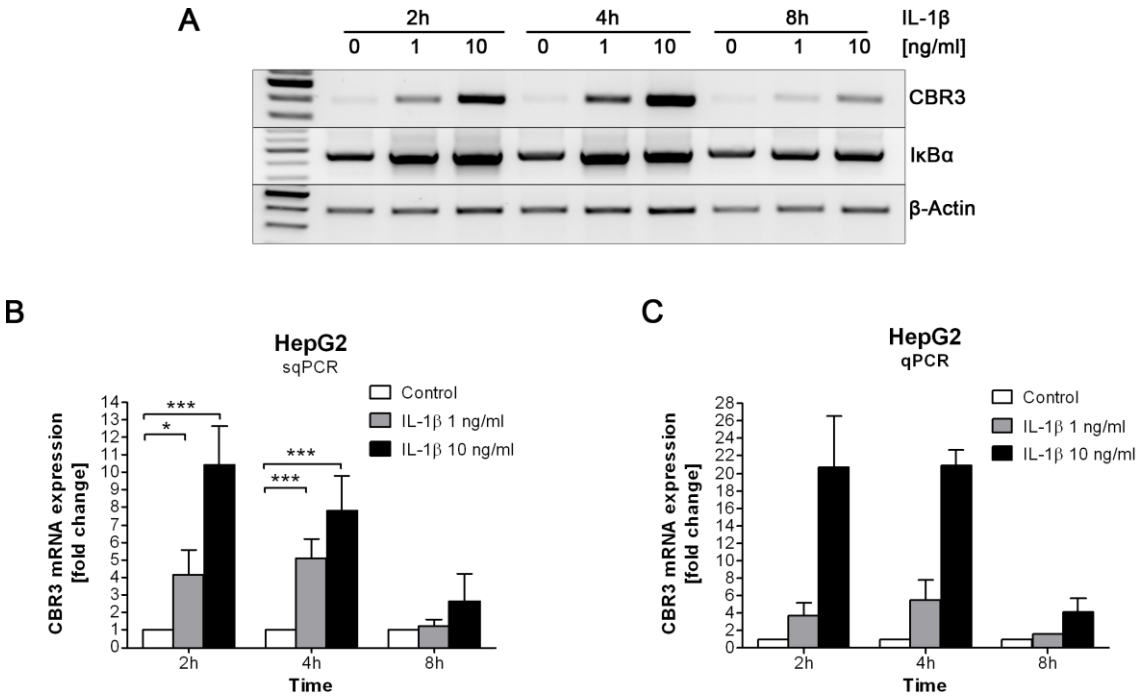

Fig. 16: HepG2 cells were incubated for 2, 4 and 8 h with 1 ng/ml and 10 ng/ml of IL-1 β or serum-free medium only (0 ng/ml, control). This experiment was performed four times. Panel A shows one representative gel. (B) Densitometric analysis of the changes in CBR3 mRNA expression relative to untreated cells is shown. Bars represent means of n=4 experiments analyzed by sqPCR ± the standard deviation (three asterisks denote $P<0.001$; one asterisk denotes $P<0.05$) *(ANOVA)*. (C) Two representative experiments were analysed by qPCR. Bars represent means of n=2 experiments ± the standard deviation.

5.2.4. Overexpression of p65 and p50

To directly activate the NFκB pathway, HT-29 cells were transfected with the expression vectors pCMV4 p65 and pCMV4 p50 encoding the NFκB subunits p65 and p50, respectively. The successful transfection of the cells was demonstrated with increased amounts of p65 mRNA (lanes 2, 3, and 6 of Fig. 17A) or p50 mRNA (lanes 4-6). Moreover, the activation of the NFκB signaling pathway after transfection with the NFκB subunits was monitored by the induction of the NFκB-target genes COX-2 and IκBα (Fig. 17A).

Each plasmid pCMV4 p65 and pCMV4 p50 was either transfected alone in two different concentrations (2 µg and 6 µg) or co-transfected as a combination (2 µg each). As shown in Fig. 17B, after 24 h, the transfection of 2 µg of only one of the both expression vectors significantly induced the *CBR3* gene by 3.4-fold (pCMV4 p65) and 3.1-fold (pCMV4 p50) compared to control cells (P<0.0001). A larger amount of plasmid pCMV4 p50 (6 µg) increased the expression level of CBR3 mRNA slightly to 3.6-fold (P<0.0001). The transfection with a larger amount of plasmid pCMV4 p65 (6 µg) did not further increase CBR3 mRNA level. The co-transfection of both pCMV4 p65 and pCMV4 50 further slightly elevated the expression of CBR3 mRNA to 3.9-fold vs. control (P<0.0001).

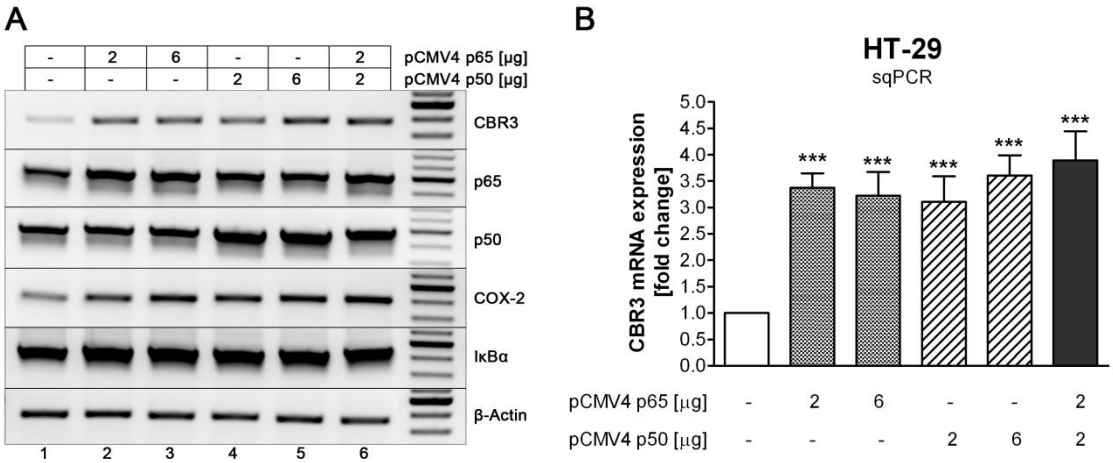

Fig. 17: HT-29 cells were transfected with different amounts of expression plasmids pCMV4 p50 and/or pCMV4 p65 and the changes in the expression of CBR3, p65, p50, as well as of the control genes COX-2 and IκBα were determined by sqPCR 24 h post-transfection. The amounts of transfected plasmid DNA are indicated in the table above or below the figures, respectively. (A) One representative gel is shown. (B) Densitometric analysis of n=3 independent experiments ± the standard deviation (three asterisks denote P<0.001) *(ANOVA)*. The non-transfected control was set to 1.

5.2.5. Summary and discussion of the effect of NFκB activation on CBR3 mRNA expression

The existence of putative NFκB binding sites in the CBR3 promoter region (Zhang and Blanco, 2009) was the basis for our working hypothesis that *CBR3* may be a target gene of the NFκB signaling pathway. The microarray data available through public databases such as GEO ("Gene Expression Omnibus", available at http://www.ncbi.nlm.nih.gov/geo/) supported our hypothesis. When searching this database, we found several studies showing CBR3 mRNA expression being up-regulated in response to pro-inflammatory stimuli. For example, increased CBR3 mRNA expression was observed after treatment of macrovascular umbilical vein endothelial cells with TNF-α **(**GenBank accession no. GDS1542 in GEO database). Elevated levels of CBR3 mRNA were detected in colon biopsy samples from patients suffering from ulcerative colitis, a type of inflammatory bowel disease (GenBank accession no. GDS3119), and in bronchial epithelial cells treated with interferon gamma (IFN-γ), a prototypical inflammatory cytokine (GenBank accession no. GDS1256). In the latter study, incubation with IFN-γ for 8 h led to a 5.6-fold increase in the expression of CBR3 mRNA (Pawliczak et al., 2005).

In our study, incubation with well-established NFκB activators was used to activate the NFκB signaling pathway. We found that the CBR3 mRNA was clearly inducible upon treatment with the NFκB activators TNF-α, IL-1β and LPS in HT-29 and HepG2 cells. By contrast, in Caco-2 cells, the NFκB activators caused only marginal changes of CBR3 mRNA expression. Interestingly, even a decrease of the CBR3 mRNA expression was detected in this cell line (Fig. 14). However, a high inter-assay variability between experiments performed with Caco-2 cells and weak effects on CBR3 mRNA levels in Caco-2 cells indicated that the CBR3 mRNA was not as inducible in Caco-2 cells as it was in HT-29 cells. This confirmed that both the cell lines HT-29 and HepG2, that express low basal levels of CBR3 mRNA, are suitable models for the investigation of the CBR3 transcriptional regulation.

The experiments with HT-29 and HepG2 cells provided different results pointing to a possible cell-specific regulation of CBR3. The cell lines responded differently with respect to both the degree of CBR3 mRNA inducibility and the kinetics of CBR3 mRNA expression. In HT-29 cells, TNF-α significantly induced the expression of CBR3 mRNA, whereas IL-1β and LPS had only slight effects (cf. Fig. 10 and Fig. 12 and 13). By contrast, in HepG2 cells, CBR3 mRNA expression was highly inducible after exposure to IL-1β. Treatment of HepG2 cells with TNF-α or LPS caused a weaker induction of CBR3 mRNA levels than IL-1β did (cf. Fig. 15).

Because the NFκB activators used for our experiments mediate their effects through binding to specific receptors (e.g., TNF receptors [TNF-α], IL-1 receptors [IL-1β], and Toll-like receptors [LPS]), our findings might result from a cell-specific expression of these receptors. In addition, mature colon cells show a relatively low inflammatory response upon exposure to LPS. The low response results from a micro RNA (miRNA-146)-mediated repression of the NFκB signaling pathway. The NFκB repression prevents an unwanted chronic inflammation in response to the normal gut flora (Sonkoly and Pivarcsi, 2009). This phenomenon may explain the almost undetectable increase of CBR3 mRNA after exposure of the colon cell line HT-29 to LPS (Fig. 13) in contrast to HepG2 cells (cf. Fig. 15).

Treatment with either TNF-α (HT-29 cells) or IL-1β (HepG2 cells) regulated the expression of CBR3 mRNA in a time- and concentration-dependent manner. However, in TNF-α-treated HT-29 cells (50 ng/ml), CBR3 mRNA levels did continuously increase over the whole time course from 2 h through 48 h (Fig. 10). By contrast, in HepG2 cells that were exposed to IL-1β (10 ng/ml), the maximum level of CBR3 mRNA was reached after the relatively short incubation time of 2 h and then constantly declined (cf. Fig. 16). Furthermore, HepG2 cells were more sensitive to stimulation with IL-1β than HT-29 cells. The relatively low concentration of 1 ng/ml of IL-1β substantially elevated the level of CBR3 mRNA after 2 h of incubation in HepG2 cells, while this treatment had no effect on the amount of CBR3 mRNA in HT-29 cells (data not shown). In HT-29 cells, only long-term treatment for 24 h with IL-1β (10 and 20 ng/ml) increased the expression of CBR3 mRNA (cf. Fig. 12).

Taking together, the most striking difference between HT-29 and HepG2 cells towards pro-inflammatory cytokines was the very prompt response of HepG2 cells followed by a rapid decline in the mRNA levels of both CBR3 and the control gene IκBα (Fig. 16A). The corresponding effect in HT-29 cells (cf. Fig. 10A) was comparatively slow but more persistent. In addition, the CBR3 mRNA expression was inducible more pronounced in HepG2 cells than in HT-29 cells. Different IκBα activities in colonic cells and hepatocytes may explain the high and rapid inducibility of CBR3 mRNA in HepG2 cells in contrast to the low but prolonged effects in HT-29 cells. For HT-29 cells as well as for some other human colonic epithelial cells it is known that IκBα-degradation is incomplete after NFκB stimulation, however, without preventing the NFκB binding activity (Jobin et al., 1997). In contrast, NFκB activation in HepG2 cells cause a strong and rapid proteolysis of IκBα followed by a prompt re-synthesis of IκBα, resulting in an inhibition of NFκB-mediated gene transcription (Han and Brasier, 1997; Han et al., 1999). In addition, different mRNA-stabilities may also contribute to the observed effects.

To prove that the inducing effect on CBR3 mRNA level is mediated *via* the NFκB pathway, we employed a vector-based overexpression of the NFκB subunits p65 and p50. Activation of the classical NFκB pathway leads predominantly to creation of p65:p50 heterodimers (Ghosh and Karin, 2002). However, p65:p65 or p50:p50 homodimers are able to activate gene transcription as well (Rahman et al., 1999; Vancurova et al., 2002; Agrawal et al., 2003; Rajaiya et al., 2009). In HT-29 cells, overexpression of either p65 or p50 had equivalent effects on the induction of CBR3 mRNA expression. The co-transfection with both expression plasmids (pCMV4 p65 and pCMV4 p50) further enhanced the CBR3 mRNA level only slightly (Fig. 17). If both homodimers (p65:p65 and p50:50) and heterodimers (p65:p50) were involved in the transcription of CBR3 mRNA, one would expect to observe a much larger amount of transcribed CBR3 mRNA in p50 and p65 co-transfected cells. Thus, the same effectiveness of overexpressed subunits alone and in combination indicates the involvement of p65:p50 heterodimers rather than homodimers. In our case, it seems that recombinantly expressed p50 or p65 subunits form dimers with the p65 and p50 subunits, respectively, that were endogenously expressed in HT-29 cells.

To confirm the results of the most important experiments, qPCR analyses were performed additionally to the sqPCR. The qPCR provides an exact quantification of the changes in the mRNA levels over an extraordinarily wide dynamic range. Therefore, qPCR allows reproducible analysis of subtle gene expression changes even at low levels of expression (Valasek and Repa, 2005). The results of experiments analysed by qPCR correlated well with that from sqPCR analysis. However, the values for the fold induction differed slightly between both approaches. Especially when the mRNA levels reach the point of saturation in the sqPCR, the qPCR method delivers higher values for the fold induction.

In summary, we show for the first time that CBR3 mRNA expression is inducible in the human cancer cell lines HT-29 and HepG2 by NFκB-dependent mechanism. The results imply a cell-specific regulation of CBR3 transcription. This is supported by previous reports on the differential expression of the CBR3 mRNA in various tissues and cell lines (Miura et al., 2008; Ebert et al., 2010). Furthermore, different basal luciferase activities were observed in HepG2 and MCF-7 cells after transfection of the CBR3 luciferase reporter constructs (Zhang and Blanco, 2009).

5.3. The effect of NFκB inhibition on CBR3 mRNA expression

5.3.1. NFκB inhibitors

The NFκB inhibition was performed in order to confirm the involvement of the NFκB pathway in the transcriptional regulation of CBR3. HT-29 cells were treated with two well-established NFκB inhibitors, parthenolide (PRT) and pyrrolidine dithiocarbamate (PDTC). In contrast to the expected inhibition of CBR3 mRNA, the 24 h incubation with PRT (10 μM) elevated the basal CBR3 mRNA level by 2.0-fold and further enhanced the inducing effect of TNF-α (50 ng/ml) from 4.1- to 6.1-fold (Fig. 18A). Co-incubation with PDTC (50 and 100 μM) and TNF-α (50 ng/ml) did not prevent the TNF-α-mediated induction but rather marginally increased the CBR3 mRNA level (Fig.18B). The 8 h treatment with PDTC (50 μM) alone caused no change in the CBR3 mRNA expression.

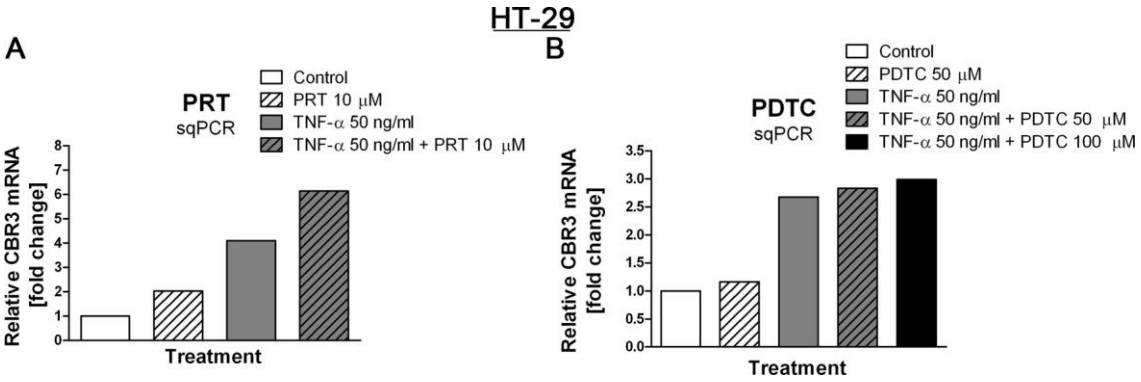

Fig. 18: HT-29 cells were treated with TNF-α (50 ng/ml) and/or PRT (10 μM) for 24 h (A) or PDTC (50 and 100 μM) for 8 h (B). The experiments were analysed by sqPCR. The densitometric analysis of the changes in CBR3 mRNA expression relative to untreated cells (control) is shown.

To prove the effects of NFκB inhibitors PRT and PDTC in cell line expressing higher basal level of CBR3 mRNA than HT-29 cells, experiments were performed with Caco-2 cells. Fig. 19 shows that after 4 h, both PDTC (100 μM) and PRT (20 μM) clearly induced CBR3 mRNA (2.3- and 1.7-fold, respectively). Noteworthy, PDTC (100 μM) and PRT (20 μM) did not change the expression of NFκB-driven control gene IκBα (Fig. 19A). In concordance with previous findings, treatment with TNF-α (50 ng/ml) did not affect CBR3 mRNA level in Caco-2 cells after 4 h. However, TNF-α increased the expression of NFκB-regulated control gene IκBα, proving the successful activation of the NFκB pathway (Fig. 19A). The co-incubation of PDTC (50 μM and 100 μM) or PRT (10 μM and 20 μM) with TNF-α (50 ng/ml) elevated the CBR3 mRNA to a similar extent as the incubation with PDTC (100 μM) or PRT (20 μM) alone.

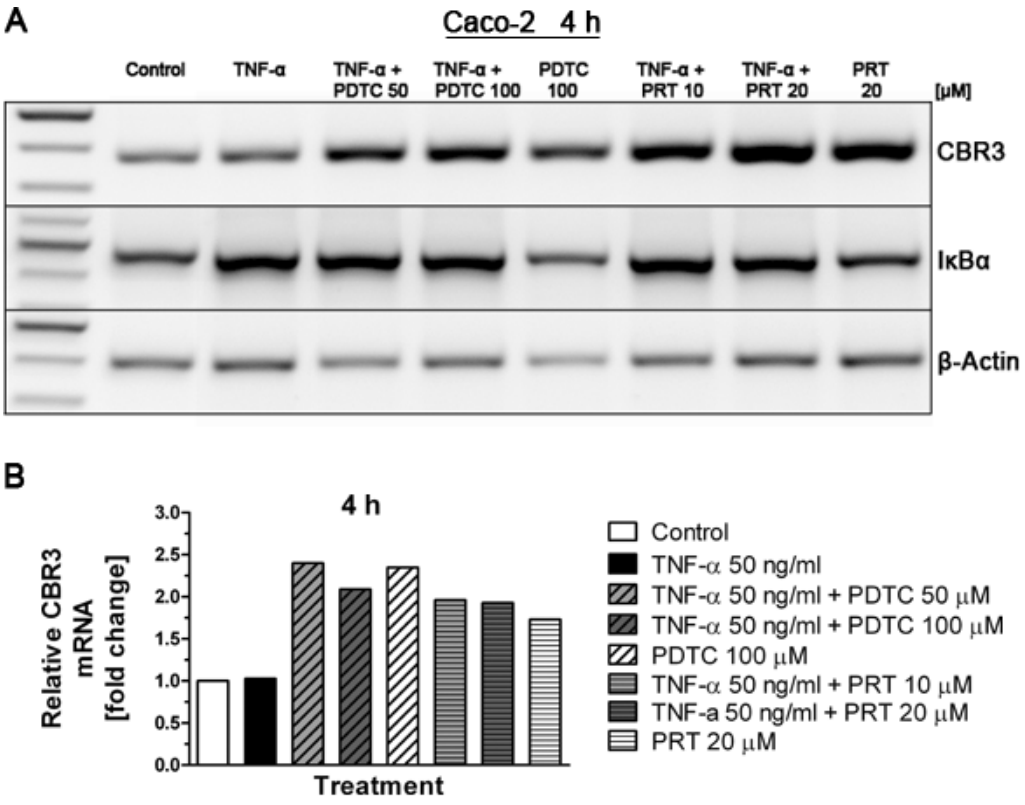

Fig. 19: HT-29 cells were treated with TNF-α (50 ng/ml), PDTC (100 μM), PRT (20 μM) or co-incubated with TNF-α (50 ng/ml) and PDTC (50 μM and 100 μM) or PRT (10 and 20 μM) for 4 h. (B) The bar graph represents the densitometric analysis of the gel shown in panel A.

Next, we performed a similar experiment with Caco-2 cells for 24 h (Fig. 20). Interestingly, the treatment with PDTC (50 μM) reduced the CBR3 mRNA by 1.8-fold while PRT (10 μM) caused slight decrease by 1.2-fold. Similarly to the 4 h incubation, the co-treatment with PDTC (50 μM) or PRT (10 μM) and TNF-α (50 ng/ml) influenced the effect of PDTC and PRT on CBR3 mRNA level only marginally (decrease by 2.4-fold and 1.1-fold, respectively, compared to control).

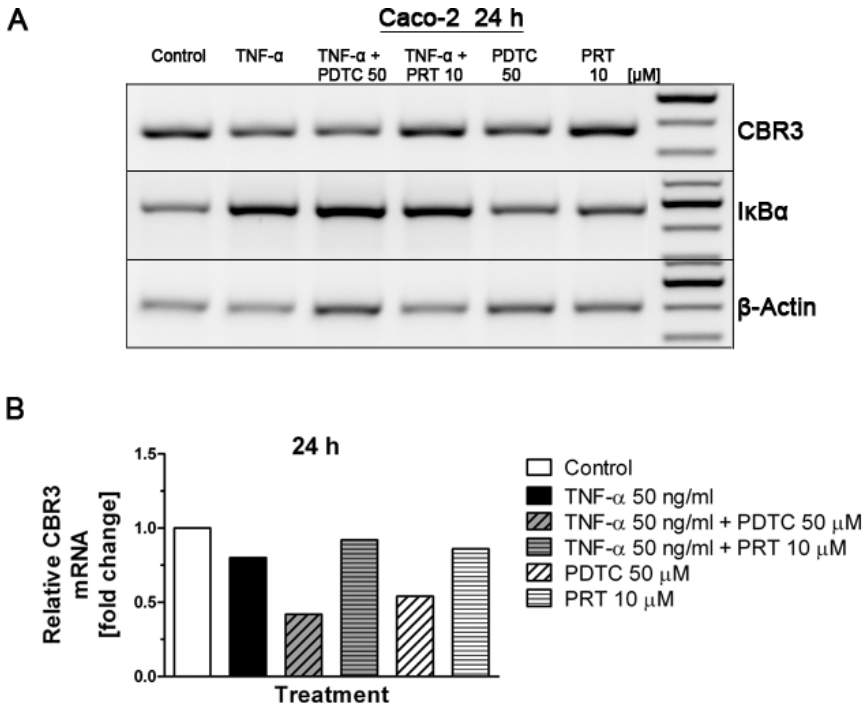

Fig. 20: Caco-2 cells were treated with TNF-α (50 ng/ml) and/or PDTC (50 μM) and/or PRT (10 μM) for 24 h. (B) The bar graph represents the densitometric analysis of the gel shown in panel A.

5.3.2. siRNA

Another approach for the inhibition of the NFκB pathway, the gene silencing with p65 siRNA, was performed. Both the cell lines with low basal levels of CBR3 mRNA (HT-29 and Caco-2 cells), and the cell lines with high basal levels of CBR3 mRNA (A549 and HCT116 cells) were used in the experiments. However, the siRNA-mediated knockdown effect of p65 on mRNA level was not greater than 50 %. Consequently, no significant changes of mRNA level of CBR3 as well as of known NFκB target genes COX-2 or IκBα were observed.

To prove the functionality of the transfection, the Nrf2 siRNA that has been previously shown be effective for CBR3 knockdown (Ebert et al., 2010) was used as a positive control. A549 cells were transfected with either Nrf2 or p65 siRNA or without adding siRNA (Mock control). After 48 h, cells were harvested for the RNA isolation followed by sqPCR analysis (Fig. 21A). The Fig. 21B shows that Nrf2 siRNA caused a knockdown of Nrf2 mRNA level by 73 % while p65 siRNA decreased the p65 mRNA only by 23 %. Furthermore, Nrf2 siRNA decreased CBR3 mRNA by 33 % but the p65 siRNA did not affect CBR3 mRNA level (Fig. 21C).

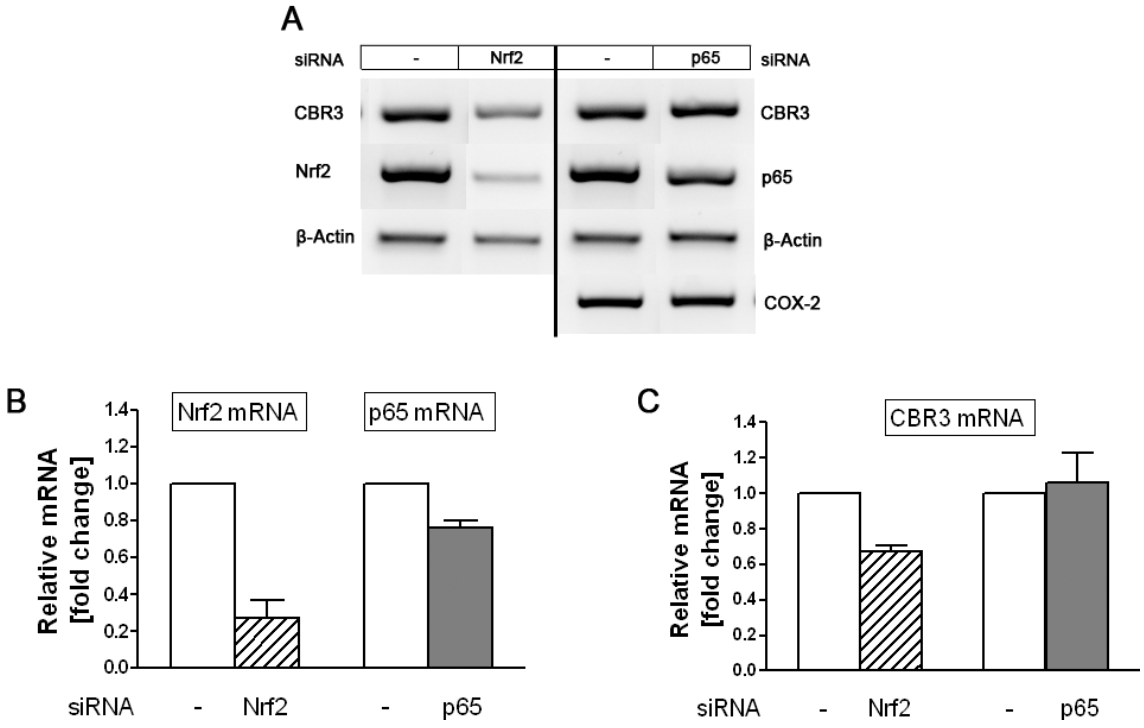

Fig. 21: A549 cells were transfected with Nrf2 or p65 siRNA or mock transfected without siRNA addition ("−") as indicated in the table above or below the figures. (A) One representative gel of sqPCR analysis. (B) Densitometric analysis of the changes in Nrf2 and p65 mRNA expression. (C) Densitometric analysis of the changes in CBR3 mRNA expression. Bars represent the means of n=2 experiments ± the standard deviation.

Apparently, p65 siRNA had no effect on the basal level of CBR3. Next, we tried to block the up-regulation of CBR3 by means of p65 siRNA. Forty-eight hours after transfection with p65 siRNA, HT-29 cells were treated with TNF-α (50 ng/ml, 4 h) and HepG2 cells with IL-1β (10 ng/ml, 2 h), respectively (Fig. 22, A and C). Fig. 22B shows that in HT-29 cells, the p65 siRNA decreased the effect of TNF-α by 27 % (from 3.0-fold to 2.3-fold induction). In HepG2 cells (Fig. 22D), p65 mediated knockdown reduced the induction by 7 % (from 12.0-fold to 11.2-fold induction). When the samples from HepG2 cells were subjected to more sensitive analysis by qPCR (Fig. 22E), a reduction by 25 % was detected (from 28.0-fold to 20.9-fold induction).

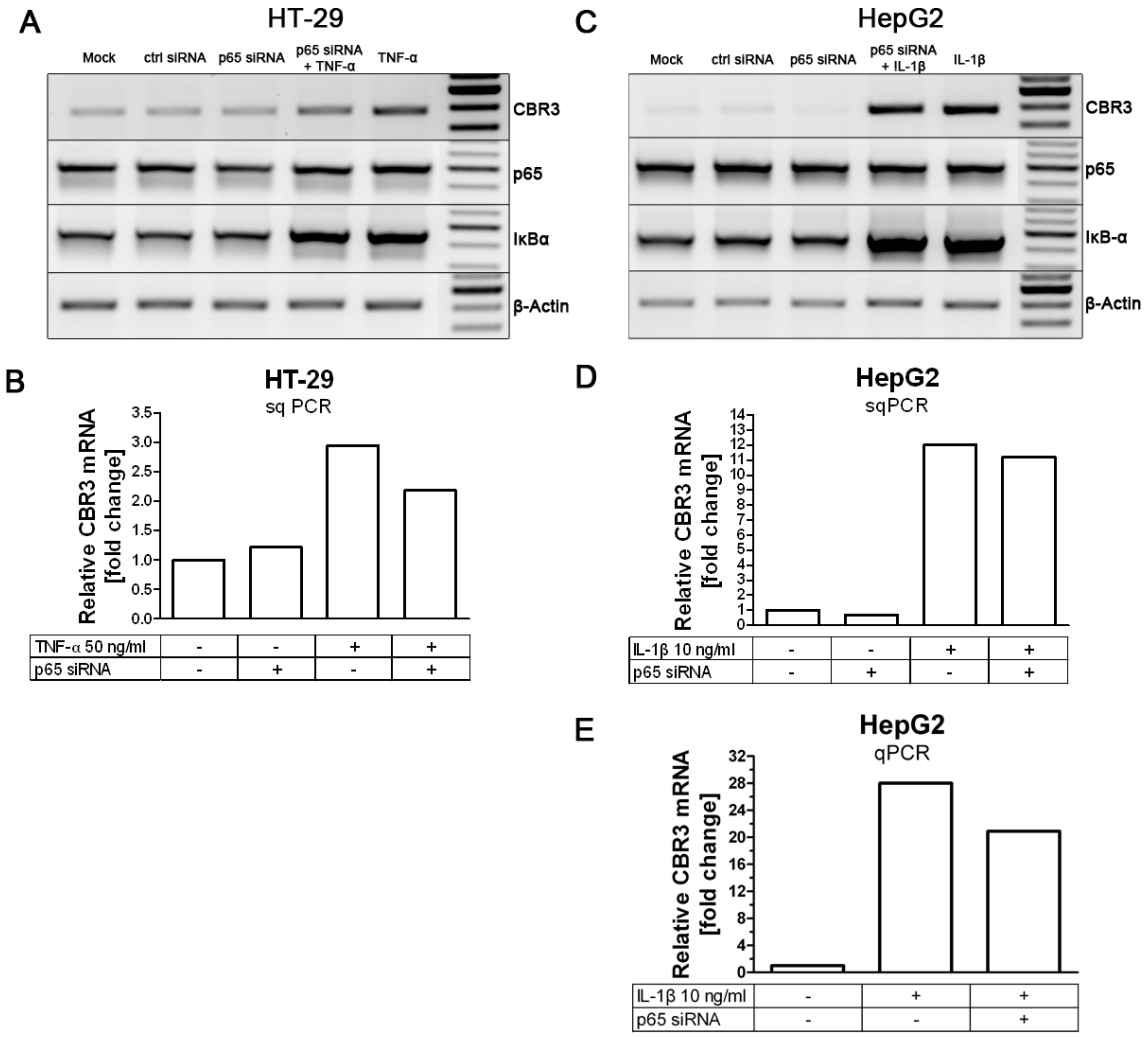

Fig. 22: HT-29 (A) and HepG2 cells (C) were transfected with 100 pmol control siRNA ("ctrl") or p65 siRNA or cells were left untransfected and treated with reagents only ("Mock"). After 48 h, HT-29 cells were treated with TNF-α (50 ng/ml, 4 h) and HepG2 cells with IL-1β (10 ng/ml, 2 h), respectively. The sqPCR analyses were performed. The densitometric analysis of the changes in CBR3 mRNA expression in HT-29 cells (B) and HepG2 cells (D) is shown. (E) The samples of HepG2 cells were analysed also by qPCR.

5.3.3. Summary and discussion of the effect of NFκB inhibition on CBR3 mRNA expression

In previous experiments, CBR3 mRNA expression was induced in a dose- and concentration-dependent manner in HT-29 and HepG2 cells after NFκB activation. Therefore, we attempted to prove the involvement of the NFκB pathway in the regulation of the CBR3 mRNA expression also by means of the NFκB inhibition. For these experiments, HT-29 cells were used because they express higher levels of CBR3 mRNA than HepG2 cells. However, two NFκB inhibitors, PRT and PDTC, had inconsistent effects on the basal level of CBR3 mRNA in HT-29 cells. Furthermore, both inhibitors failed to block the inducing effect of pro-inflammatory stimuli and, on the contrary, rather increased the CBR3 mRNA expression (Fig. 18, A and B).

Because Caco-2 cells express higher basal level of CBR3 mRNA than HT-29 cells, they should be a more suitable model cell line for the experiments with NFκB inhibitors. Therefore, Caco-2 cells were incubated with PDTC or PRT for 4 h and 24 h. The 4 h treatment with PDTC (100 μM) and PRT (20 μM) increased CBR3 mRNA level by 2.3- and 1.7-fold, respectively (Fig. 19). However, after 24 h, PDTC (50 μM) substantially decreased the CBR3 mRNA (cf. Fig. 20). This is in contrast to the experiment with HT-29 cells, where only a marginal effect of PDTC on CBR3 mRNA expression was observed (cf. Fig. 18). In summary, we detected no definite inhibitory effects of NFκB inhibitors PRT and PDTC neither in HT-29 nor in Caco-2 cells.

Although PRT and PDTC are commonly used as monofunctional inhibitors of the NFκB pathway, they have been reported to influence other pathways as well. Since PRT generates oxidative stress (Wen et al., 2002) and PDTC activates AP-1 (Kim et al., 2003), they both can trigger Nrf2-mediated gene expression and consequently might increase the CBR3 mRNA level through this pathway. The simultaneous activation of the Nrf2 signaling pathway upon NFκB inhibition with PRT or PDTC may explain the lack of the inhibition of CBR3 expression in our experiments. The activation of another pathway such as Nrf2 is supported by the observation that PDTC and PRT increased the CBR3 mRNA expression in Caco-2 cells (Fig. 19). However, these compounds did not change the expression of NFκB-driven gene IκBα indicating involvement of another pathway. Furthermore, PDTC exhibited different effects depending on the incubation time (2.3-fold increase after 4 h but 1.8-fold decrease after 24 h). This implies that after

a shorter incubation time, PDTC activates a signalling pathway that enhances the expression of CBR3 mRNA.

Next, the siRNA-mediated gene silencing of p65 was used as a direct method for the inhibition of the NFκB pathway. Cell lines with either low (HT-29 and Caco-2 cells) or high (A549 and HCT116 cells) basal levels of CBR3 mRNA expression were transfected with siRNA targeting NFκB subunit p65. However, the siRNA-mediated knockdown of p65 never exceeded 50 %, which was not sufficient to observe a significant effect on the constitutive expression of CBR3 mRNA (Fig. 21). Consequently, the expression of the NFκB-driven control gene COX-2 also remained unaffected. Further, we investigated the effect of p65 siRNA on the inducible expression of CBR3 mRNA. The pre-treatment with p65 siRNA blocked the up-regulation of CBR3 mRNA induced by NFκB activators just partially (Fig. 22).

In conclusion, we did not observe any definite effect of NFκB inhibition on CBR3 mRNA expression due to the limitations of applied methods. Namely, the effect of inhibitors was probably influenced by the activation of other pathways. Further, the poor effectivity of the p65 siRNA may have been responsible for lack of significant CBR3 knock-down.

5.4. The effect of NFκB activation on CBR3 protein level

5.4.1. NFκB activators

The results on mRNA level clearly demonstrated, that the activation of the NFκB pathway elevates CBR3 mRNA expression. Therefore, we investigated the effect on CBR3 protein level. In HT-29 cells, TNF-α (50 ng/ml) had no inducing effect on CBR3 protein expression in any time tested (4-48 h). On the contrary, we detected a marginal decrease of CBR3 protein levels after 48 h incubation (Fig. 23, A and B). When the concentrations 10, 50 and 100 ng/ml of TNF-α were applied to cells for 24 h, the expression of CBR3 also changed only slightly. TNF-α at concentrations of 10 and 50 ng/ml caused 1.1-fold increase of the CBR3 protein level while treatment with a concentration of 100 ng/ml resulted in a decrease of CBR3 protein by 1.4-fold compared to untreated cells (Fig. 23C). The same treatment was performed with Caco-2 cells. As depicted in Fig. 24 A-C, similar to the experiments with HT-29 cells, just marginal changes of CBR3 protein expression were detected. However, this observation corresponds to the results on mRNA level (see *5.2.2 NFκB activation in Caco-2 cells*).

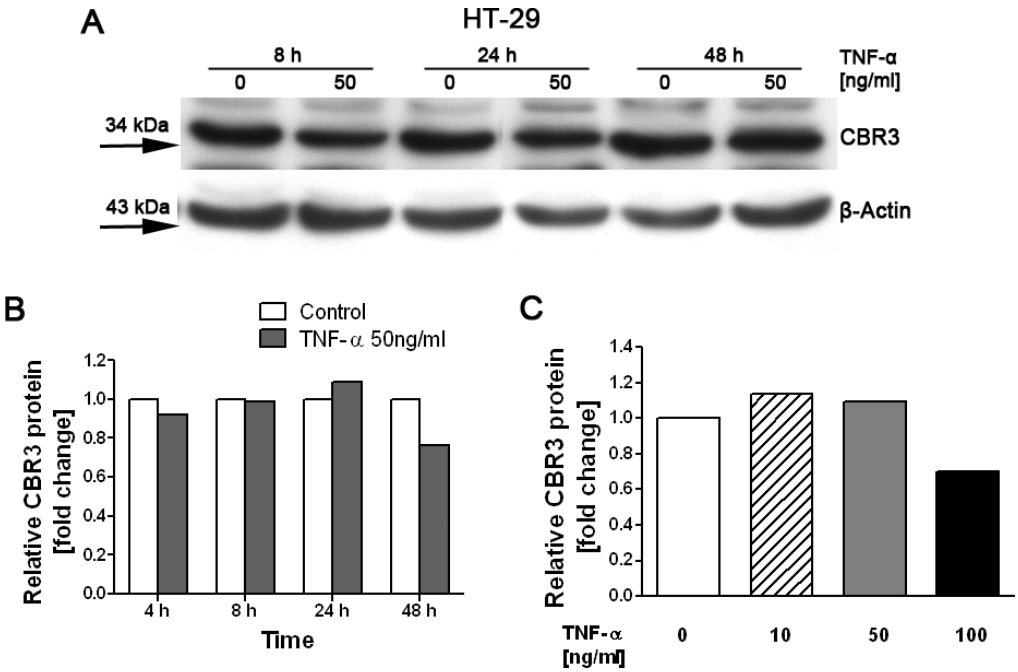

Fig. 23: (A) Western blot of HT-29 cells treated with TNF-α (50 ng/ml) or serum-free medium (0 ng/ml) for the times indicated above. Each lane contained 100 µg of protein (whole cell lysate). Proteins were analyzed using the rabbit anti-CBR3 primary antibody (Santa Cruz, sc-70218; 1:500 dilution). (B) The densitometric analysis of the changes in CBR3 protein expression in HT-29 cells after treatment with serum-free medium (control) or TNF-α (50 ng/ml) for 4-48 h. (C) The densitometric analysis of the samples treated with TNF-α (10, 50 and 100 ng/ml) for 24 h.

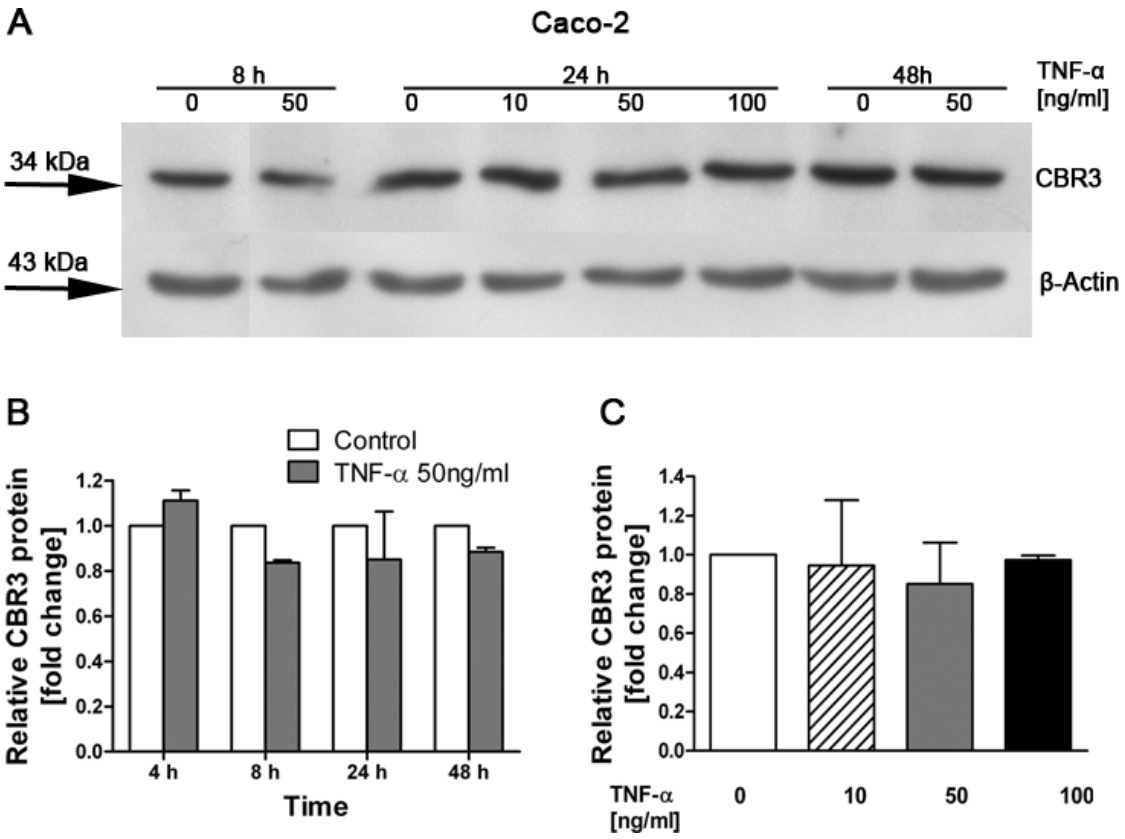

Fig. 24: (A) One representative western blot of Caco-2 cells treated with TNF-α (0-100 ng/ml) as indicated in the figure above. Each lane contained 80 µg of protein (whole cell lysate). (B) The densitometric analysis of the changes in CBR3 protein expression after treatment of Caco-2 cells with serum-free medium (control) or TNF-α (50 ng/ml) for 4-48 h. (C) The densitometric analysis of the samples treated with TNF-α (10, 50 and 100 ng/ml) for 24 h. Bars represent means of n=2 experiments ± the standard deviation.

In HepG2 cells, although IL-1β strongly induced the level of CBR3 mRNA, no effect could be assessed on protein level. The CBR3 protein was not detectable in IL-1β-treated or untreated cells even when as much as 100 µg of protein were loaded onto the western blot.

5.4.2. Overexpression of p65 and p50

The transfection with expression vectors encoding NFκB subunits p65 and p50 clearly induced CBR3 mRNA levels in HT-29 cells. Therefore, an analogous experiment to that on mRNA level was performed on protein level. HT-29 cells were transfected with 12 µg of either pCMV4 p65 or pCMV4 p50 or co-transfected with combination of 4 µg of both plasmids. The amounts of plasmids for transfection were increased twice compared to the gene expression experiment because larger culture vessels were used. The transfection with pCMV4 p65 changed the CBR3 protein content marginally (0.9-fold decrease compared to control cells). On the other hand, the transfection with pCMV4 p50 and the co-transfection with both expression plasmids pCMV4 p65 and pCMV4 p50 increased the expression level by 1.3-fold relative to control cells (Fig. 25).

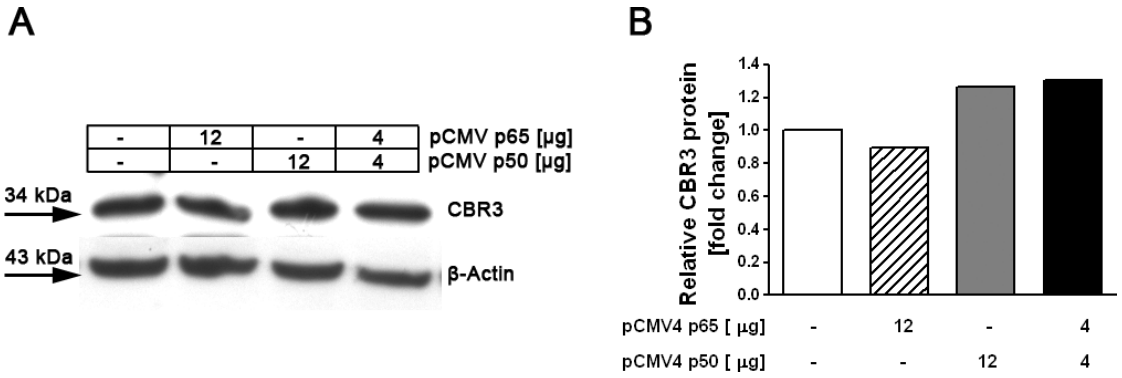

Fig. 25: HT-29 cells were transfected with expression plasmids pCMV4 p50 and/or pCMV4 p65 and the changes in the expression of CBR3 protein were determined by western blotting. The amounts of transfected plasmid DNA are indicated in the table above or below the figures, respectively. (A) Western blot is shown. Each lane contained 100 µg of protein (whole cell lysate). (B) The densitometric analysis of the blot. The non-transfected control was set to 1.

5.4.3. The experiment with HCT116 cells

The treatment with TNF-α greatly increased CBR3 mRNA levels but caused no induction on protein level. Similarly, the overexpression of NFκB subunits p65 and p50 elevated the CBR3 protein marginally in contrast to significant induction of the CBR3 mRNA. The lacking increase in CBR3 protein expression upon NFκB activation may be caused by rapid degradation of newly synthesized CBR3 protein. To test this hypothesis, we performed an experiment with HCT116 cells in which endogenous CBR3 protein is absent.

The parental HCT116 cell line ("HCT116-WT") was stably transfected with CBR3 expression plasmid pCIneoCBR3 ("HCT116-CBR3") or empty vector pCIneo ("HCT116-pCIneo"). If the NFκB activation *via* TNF-α treatment, will target the CBR3 protein for degradation, we should have observed the decrease of recombinantly expressed CBR3 protein. However, after incubation with TNF-α (50 ng/ml), only slight changes of CBR3 protein expression were detected in HCT116-CBR3 cells. The Fig. 26B shows that 8 h treatment elevated the CBR3 protein level by 1.2-fold whereas the treatment for 24 h and 48 h decreased the CBR3 protein expression by 1.1- and 1.2-fold, respectively. As expected, control cells HCT116-WT and HCT116-pCneoI that were treated with TNF-α (50 ng/ml) or serum-free medium for 24 h did not express any CBR3 protein (Fig. 26A).

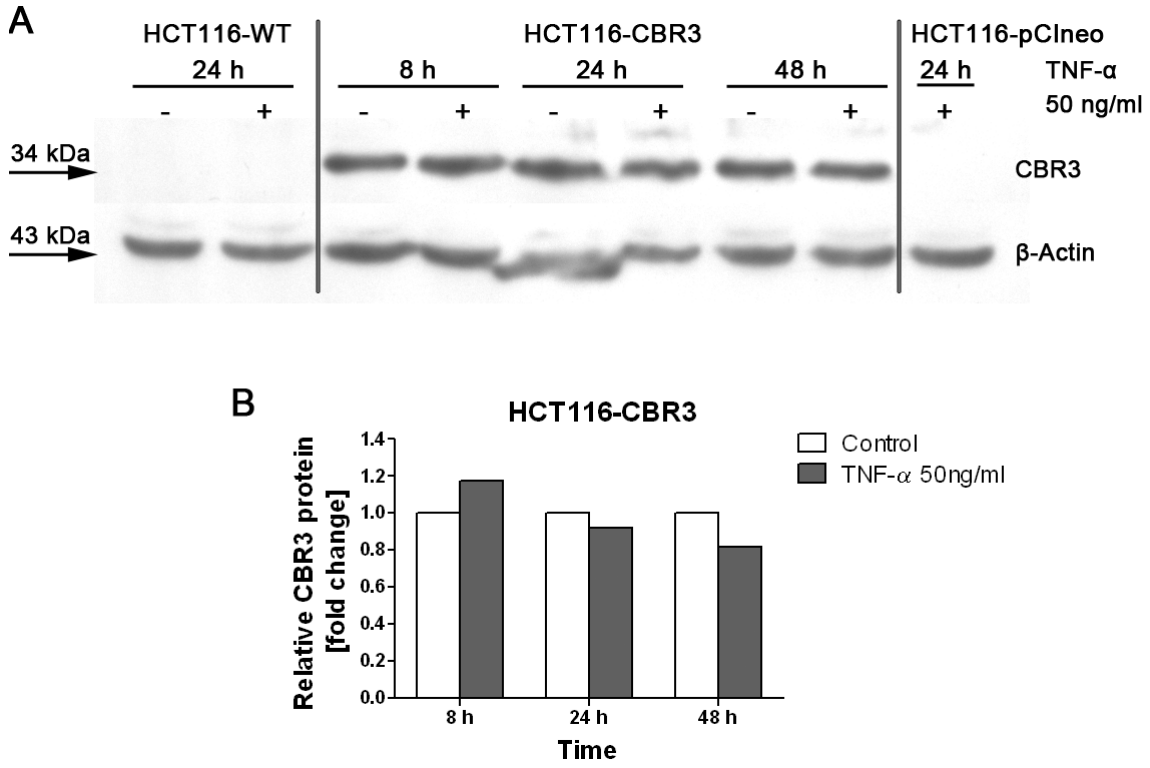

Fig. 26: The parental HCT116 cell line ("HCT116-WT"), and HCT116 cells stably transfected with CBR3 expression plasmid ("HCT116-CBR3") or empty vector pCIneo ("HCT116-pCIneo") were incubated with serum-free medium ("−") or TNF-α (50 ng/ml) for the indicated times. (A) Aliquots of whole cell lysates corresponding to 80 µg proteins were subjected to western blot analysis. (B) The densitometric analysis of the changes in CBR3 protein level in HCT116-CBR3 cells.

5.4.4. Summary and discussion of the effect of NFκB activation on CBR3 protein level

The activation of the NFκB pathway clearly enhanced the CBR3 expression on mRNA level but this effect did not correlate with the protein expression. In HT-29 cells, the CBR3 protein level remained unchanged or slightly decreased after treatment with TNF-α at various concentrations and for different time (Fig. 23). Similar results were obtained in analogous experiments with Caco-2 cells (Fig. 24). Further, the overexpression of NFκB subunits p65 and p50 in HT-29 cells enhanced the expression of CBR3 protein only slightly (Fig. 25). For the analysis of the protein expression after NFκB activation, HT-29 and Caco-2 cells were used because in HepG2 cells, CBR3 protein was undetectable in both untreated and cytokine-treated cells.

When HT-29 cells were exposed to Nrf2 activators, an increase of CBR3 on mRNA as well as on protein level could be detected (Ebert et al., 2010). From this observation it may be concluded that the lacking up-regulation of CBR3 on protein level is associated to the activation of the NFκB signaling pathway. It has frequently been observed that mRNA and protein levels of a certain gene do not correlate (Wang, 2008). Several mechanisms, including post-transcriptional regulation affecting mRNA stability, protein stability, and translation initiation, have been identified as possible reasons (Tian et al., 2004).

CBR3 mRNA levels greatly increased and remained elevated in HT-29 cells for several hours (Fig. 10 A-C). It may be possible that mechanisms being activated along with the NFκB pathway target the newly synthesized CBR3 protein for rapid degradation. Therefore, HCT116 cells that lack endogenous CBR3 were stably transfected with CBR3 and analyzed for CBR3 protein expression after TNF-α treatment. However, no substantial down-regulation of the recombinantly expressed CBR3 was observable, suggesting that the unknown mechanism targets more likely the mRNA rather than the mature protein. These findings should be interpreted in light of the occurrence of polymorphisms that can result in the differences between endogenously and recombinantly expressed CBR3 proteins.

Micro RNAs (miRNAs) are evolutionarily conserved, endogenously produced regulatory RNAs that play important roles in the regulation of genes by targeting their 3′-UTR and inhibiting the translation of the mRNA. Two different mechanisms have been described by which miRNAs regulate gene expression. When near-perfectly complementary to its target mRNA, the miRNA triggers the degradation of the target mRNA. Consequently the down-regulation of gene expression occurs on both the mRNA and protein level. By contrast, a miRNA which is only partly complementary to its target mRNA will affect gene expression exclusively on protein level (Tomankova et al., 2010). Web applications for miRNA prediction (available at http://www.targetscan.org; Whitehead Institute for Biomedical Research) identified several miRNAs that potentially target the CBR3 mRNA. All of these miRNAs are only partially complementary to the CBR3 mRNA. Furthermore, miRNAs have been identified as important regulators in response to inflammation (Sonkoly and Pivarcsi, 2009). Although this needs further investigation, a miRNA-based regulation of CBR3 during inflammation could explain the phenomenon that the CBR3 protein level does not reflect the increase in CBR3 mRNA upon NFκB activation.

It can be further hypothesized that the enzymatic activity of CBR3 is somehow interfering with the inflammatory response. The increase of CBR3 protein after NFκB activation may be undesirable due to its proposed antioxidant function (see *2.3.4.2. Regulation of CBR3 expression by Nrf2*). Since the proper expression of proteins is strictly regulated, the cell may discard CBR3 protein or inhibit its synthesis to avoid detrimental effects that enhanced expression of CBR3 protein might otherwise cause. Interestingly, down-regulation of CBR3 protein has been reported by a study of Kang et al. (2009), who exposed human macrophages to the NFκB-activating agent oxLDL. In their proteomic analysis, the expression of CBR3 was found to be down-regulated even 2-fold compared to untreated cells (Kang et al., 2009). Moreover, CBR3 protein was down-regulated in cells overexpressing the anti-apoptotic protein Bcl-x_L (Espana et al., 2005). Thus, there may be a general mechanism not specific to carcinoma cell line HT-29 that blocks or even down-regulates the expression of CBR3 protein.

5.5. Functional analysis of CBR3 promoter

5.5.1. Reporter gene assay

The functional impact of NFκB binding sites in mediating the transcriptional regulation of CBR3 was investigated by performing reporter gene experiments with a series of progressive 5'-deletion constructs. Three putative NFκB binding sites are located at 1160 bp ($_{-1160}$NFκB), 593 bp ($_{-593}$NFκB), and 364 bp ($_{-364}$NFκB) upstream of the start codon (see *2.3.4.3. Hypothesis about involvement of NFκB in CBR3 regulation*). The reference construct encompassing 1297 bp of the 5'-flanking region of CBR3 ($_{-1297}$CBR3) contained all three NFκB binding sites. Progressive 5'-end deletions resulted in promoter constructs $_{-1130}$CBR3, $_{-393}$CBR3, and $_{-350}$CBR3 containing two, one, or no NFκB binding sites, respectively (Fig. 27 left).

The results of the reporter gene experiment demonstrated the considerable promoter activity of −1297 bp region of the *CBR3* gene in HepG2 cells (Fig. 27 right). Deletion of the −1297/−1130 bp region containing the $_{-1160}$NFκB binding site reduced reporter gene activity by 94 % when compared to the non-truncated reference construct $_{-1297}$CBR3. Further 5'-end truncation of up to −393 bp removed the second NFκB binding site ($_{-593}$NFκB) and resulted in the construct $_{-393}$CBR3 lacking luciferase activity. Interestingly, further deletion of 43 bp that removed the last NFκB binding site ($_{-364}$NFκB) increased the promoter activity. The resulting $_{-350}$CBR3 construct exerted even 21 % higher promoter activity than reference construct $_{-1297}$CBR3.

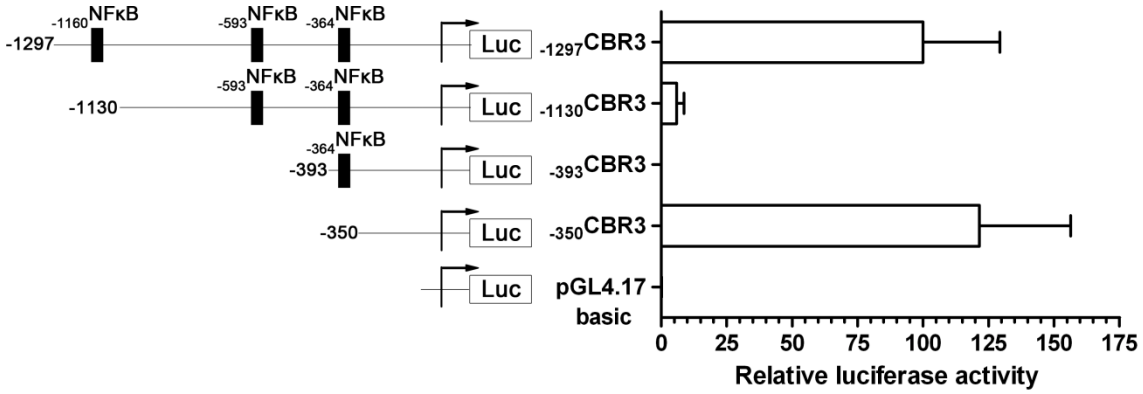

Fig. 27: Basal luciferase activity of CBR3 promoter constructs in HepG2 cells. The left panel shows the schematic illustration of each CBR3 promoter construct and its corresponding basal luciferase activity from the reporter gene experiment (right panel). Corrected luciferase activity values were expressed relative to the activity of the construct $_{-1297}$CBR3, which was set arbitrarily as 100 %. Each value represents the mean ± the standard deviation of one experiment performed in quadruplicate.

Next, we tested the ability of IL-1β to induce reporter gene activities of different CBR3 promoter constructs. Twenty-four hours after co-transfection with reporter constructs, the incubation with IL-1β (10 ng/ml) or serum-free medium without test compound was performed for 4 h. As presented in Fig. 28, IL-1β induced luciferase activity of the $_{-1297}$CBR3 construct by 12-fold, of the $_{-1130}$CBR3 construct by 9-fold, and of the $_{-350}$CBR3 construct by 3-fold. IL-1β treatment failed to induce the reporter gene activity of the $_{-393}$CBR3 construct.

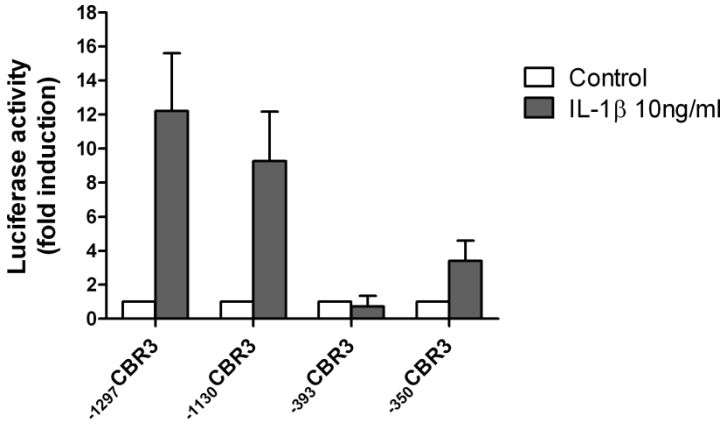

Fig. 28: Luciferase activity of CBR3 promotor constructs in response to IL-1β: HepG2 cells were co-transfected with CBR3 reporter constructs ($_{-1297}$CBR3, $_{-1130}$CBR3, $_{-393}$CBR3, and $_{-350}$CBR3 construct) and the normalizer plasmid pGL4.17. Twenty-four hours after co-transfection, cells were treated with IL-1β (10 ng/ml) or serum-free medium (control) for 4 h. For each construct, normalized luciferase activities were expressed as fold induction with respect to the values from control incubations, which were set arbitrarily to 1. Values are mean ± the standard deviation of one typical experiment performed in quadruplicate.

5.5.2. Summary and discussion of reporter gene assay

We investigated which of three putative NFκB binding sites ($_{-1160}$NFκB, $_{-593}$NFκB, and $_{-364}$NFκB) is responsible for the transcriptional regulation of CBR3 upon activation of NFκB. From the 1297 bp portion of the CBR3 promoter, three 5'-end progressive deletion constructs were cloned into a luciferase-reporter vector and their luciferase activities were determined in comparison to the whole promoter ($_{-1297}$CBR3 construct). The −1297 bp region of CBR3 promoter showed a significant promoter activity in HepG2 cells. Deletion of the region encompassing the $_{-1160}$NFκB binding site resulted in a construct ($_{-1130}$CBR3 construct) with greatly diminished reporter gene activity. Furthermore, deletion of the segment containing the two NFκB binding sites $_{-1160}$NFκB and $_{-593}$NFκB, completely abolished basal luciferase activity.

Together, the $_{-1160}$NFκB binding site seems to be crucial for CBR3 transcription. Only minor contribution may be assigned to $_{-593}$NFκB since the $_{-1130}$CBR3 construct exerted only negligible reporter activity when compared to the construct $_{-1297}$CBR3. Furthermore, our results indicated the presence of a negative regulatory element in the 393/350 bp region because the deletion of the 43-bp segment clearly increased the luciferase activity. Noteworthy, the resulting $_{-350}$CBR3 construct exerted the highest constitutive promoter activity from the series of promoter constructs. This is in accordance with previous finding by Zhang and Blanco (2009) who found that $_{-313}$CBR3 promoter construct exhibits the highest constitutive promoter activity from seven promoter constructs tested in their study in HepG2 cells.

The second aim of this experiment was to evaluate the ability of the prototypical NFκB activator IL-1β to induce the reporter gene activity of the CBR3 promoter constructs. We found that $_{-1160}$NFκB and $_{-593}$NFκB binding sites may act as *bona fide* functional elements to activate NFκB-mediated gene transcription in the presence of NFκB activators. Interestingly, IL-1β treatment increased the reporter gene activity of construct $_{-350}$CBR3 although this promoter construct contains no NFκB binding site. However, the increase was considerably lower than the increase of luciferase activities of the constructs $_{-1297}$CBR3 and $_{-1130}$CBR3.

In conclusion, our findings show that the CBR3 promoter construct harbouring the $_{-1160}$NFκB binding site exhibits considerable constitutive reporter activity in HepG2 cells. Furthermore, the $_{-1160}$NFκB and $_{-593}$NFκB binding sites appear to be essential for the NFκB-dependent inducible expression of the *CBR3* gene. This is in agreement with an earlier study suggesting the presence of *cis*-regulatory upstream element in sequence between 2500 and 500 bp upstream the initiation codon (Warnatz et al., 2010). However, it should be kept in mind that the progressive deletion removes not only particular NFκB binding sites but also adjacent regions that may contain other regulatory elements. Therefore, the construction of different promoter constructs with mutated putative NFκB binding sites is necessary to substantiate the functional impact of particular NFκB binding sites.

6. Conclusion

The review of human CBRs constitutes an important part of this thesis. It summarizes the current findings of CBRs and provides the basis for their further investigations. From the theoretical part of this thesis it is obvious that the understanding of CBRs did continuously grow until the last decade, but it is still not complete. Especially the knowledge about the transcriptional regulation is very limited and the enzyme CBR3 is poorly characterized in general (Malatkova et al., 2010).

In the experimental part, we have investigated the possible involvement of the NFκB pathway in CBR3 regulation by use of various approaches. The obtained data provide interesting findings about transcriptional regulation of CBR3. At the beginning, the cell lines HT-29 and HepG2 were chosen as suitable models to investigate the regulation of CBR3. The following experiments confirmed that the CBR3 mRNA is highly inducible in these cell lines in contrast to the cells with higher basal levels of the CBR3 mRNA such as Caco-2 cells.

The most important finding of the study is that CBR3 mRNA expression is inducible in the human cancer cell lines HT-29 and HepG2 by NFκB-dependent mechanism. Treatment of the cells with the NFκB activators TNF-α, IL-1β, and LPS induced CBR3 mRNA expression differentially. In both HT-29 and HepG2 cells, the CBR3 mRNA expression was regulated in a time- and concentration-dependent manner after incubation with TNF-α (50 ng/ml) or IL-1β (1 and 10 ng/ml), respectively. The results obtained by sqPCR analysis were confirmed by qPCR. In HT-29 cells, TNF-α caused the highest up-regulation of CBR3 mRNA after 24 h (10.3-fold vs. control, qPCR). By contrast, in HepG2 cells, the strongest effect caused treatment with IL-1β (10 ng/ml) after 2 h (20.7-fold increase compared to control, qPCR) and decreased thereafter. Apparently, HT-29 and HepG2 cells responded differently with respect to both the degree of CBR3 mRNA inducibility and the kinetics of CBR3 mRNA expression. Our findings imply a cell-specific regulation of the CBR3 transcription. The vector-based overexpression of the NFκB subunits p65 and p50 confirmed that the effect on CBR3 mRNA level is mediated *via* the NFκB pathway. Concretely, the overexpression of the NFκB subunits alone or in combination significantly increased the level of the CBR3 mRNA.

Next, we carried out two approaches of NFκB inhibition. However, we did not observe any definite effect on CBR3 mRNA expression upon NFκB inhibition due to the limitations of methods used. The action of applied NFκB inhibitors was probably influenced by the activation of other pathways such as the Nrf2/ARE pathway. Further, we investigated the effect of siRNA-mediated gene silencing of p65, which is a direct method for the inhibition of the NFκB pathway, on CBR3 mRNA expression. However, the siRNA-mediated knockdown of p65 never exceeded 50 % which was not sufficient to observe a significant effect on constitutive or inducible expression of CBR3 mRNA. It is possible that the poor effectivity of p65 siRNA caused the failure of the experiment.

An interesting finding of this work was that the activation of the NFκB pathway clearly enhanced the CBR3 expression on mRNA level but this effect was not accompanied with an increased protein expression. However, it has frequently been observed that mRNA and protein levels of a certain gene do not correlate due to various reasons. According our results, an unknown mechanism targets more likely the CBR3 mRNA rather than the mature protein. It can be suggested that miRNA-based regulation of CBR3 is responsible for the lack of CBR3 protein induction.

The last aim of the experimental part was to investigate the functional impact of putative NFκB binding sites in mediating the transcriptional regulation of CBR3. Experiments with 5'-progressive deletion constructs pinpointed areas on the CBR3 promoter that may regulate its transcription. We demonstrated that the $_{-1160}$NFκB binding site may be crucial for CBR3 mRNA expression. Furthermore, the $_{-1160}$NFκB and $_{-593}$NFκB binding sites may act as *bona fide* functional elements to activate NFκB-mediated gene transcription in the presence of NFκB activators.

In conclusion, we provide for the first time clear evidence that the NFκB signaling pathway is another important player in the regulation of the *CBR3* gene on mRNA level (Malátková et al., 2012). Until today, the physiological role of CBR3 in the human body remains obscure. Therefore, the identification of the transcriptional regulation of CBR3 may help to elucidate its possible physiological function. Undoubtedly, the physiological reason and the underlying mechanisms that lead to the divergent expression of CBR3 mRNA (highly inducible) and protein (unchanged or down-regulated) deserve further investigations.

7. References

Agrawal,A., Cha-Molstad,H., Samols,D., and Kushner,I. (2003). Overexpressed nuclear factor-kappaB can participate in endogenous C-reactive protein induction, and enhances the effects of C/EBPbeta and signal transducer and activator of transcription-3. *Immunology* **108**(4), 539-547

Albano,C.B., Muralikrishnan,D., and Ebadi,M. (2002). Distribution of coenzyme Q homologues in brain. *Neurochem. Res.* **27**(5), 359-368

Atalla,A., Breyer-Pfaff,U., and Maser,E. (2000). Purification and characterization of oxidoreductases-catalyzing carbonyl reduction of the tobacco-specific nitrosamine 4-methylnitrosamino-1-(3-pyridyl)-1-butanone (NNK) in human liver cytosol. *Xenobiotica* **30**(8), 755-769

Ax,W., Soldan,M., Koch,L., and Maser,E. (2000). Development of daunorubicin resistance in tumour cells by induction of carbonyl reduction. *Biochem. Pharmacol.* **59**(3), 293-300

Bains,O.S., Karkling,M.J., Grigliatti,T.A., Reid,R.E., and Riggs,K.W. (2009). Two nonsynonymous single nucleotide polymorphisms of human carbonyl reductase 1 demonstrate reduced in vitro metabolism of daunorubicin and doxorubicin. *Drug Metab Dispos.* **37**(5), 1107-1114

Bains,O.S., Karkling,M.J., Lubieniecka,J.M., Grigliatti,T.A., Reid,R.E., and Riggs,K.W. (2010). Naturally occurring variants of human CBR3 alter anthracycline in vitro metabolism. *J Pharmacol Exp Ther* **332**(3), 755-763

Balcz,B., Kirchner,L., Cairns,N., Fountoulakis,M., and Lubec,G. (2001). Increased brain protein levels of carbonyl reductase and alcohol dehydrogenase in Down syndrome and Alzheimer's disease. *J. Neural Transm. Suppl* (61), 193-201

Ballard,D.W., Dixon,E.P., Peffer,N.J., Bogerd,H., Doerre,S., Stein,B., and Greene,W.C. (1992). The 65-kDa subunit of human NF-kappa B functions as a potent transcriptional activator and a target for v-Rel-mediated repression. *Proc. Natl. Acad. Sci. U. S. A* **89**(5), 1875-1879

Bang-Berthelsen, C., Bergholdt, R., Heding, P., Nerup, J., and Pociot, F. (2006). The role of CBR1 in the pathogenesis of type 1 diabetes. *42nd Annual Meeting of the European-Association-for-the-Study-of-Diabetes (EASD). Diabetologia* **49,** 254-255

Barouki,R., Coumoul,X., and Fernandez-Salguero,P.M. (2007). The aryl hydrocarbon receptor, more than a xenobiotic-interacting protein. *FEBS Lett.* **581**(19), 3608-3615

Basu,S. (2007). Novel cyclooxygenase-catalyzed bioactive prostaglandin F2alpha from physiology to new principles in inflammation. *Med. Res. Rev.* **27**(4), 435-468

Bateman,R.L., Rauh,D., Tavshanjian,B., and Shokat,K.M. (2008). Human carbonyl reductase 1 is an S-nitrosoglutathione reductase. *J. Biol. Chem.* **283**(51), 35756-35762

Ben-Av,P., Crofford,L.J., Wilder,R.L., and Hla,T. (1995). Induction of vascular endothelial growth factor expression in synovial fibroblasts by prostaglandin E and interleukin-1: a potential mechanism for inflammatory angiogenesis. *FEBS Lett.* **372**(1), 83-87

Bergholdt,R., Karlsen,A.E., Hagedorn,P.H., Aalund,M., Nielsen,J.H., Kruhoffer,M., Orntoft,T., Wang,H., Wollheim,C.B., Nerup,J. and others (2007). Transcriptional profiling of type 1 diabetes genes on chromosome 21 in a rat beta-cell line and human pancreatic islets. *Genes Immun.* **8**(3), 232-238

Blanco,J.G., Leisenring,W.M., Gonzalez-Covarrubias,V.M., Kawashima,T.I., Davies,S.M., Relling,M.V., Robison,L.L., Sklar,C.A., Stovall,M., and Bhatia,S. (2008). Genetic polymorphisms in the carbonyl reductase 3 gene CBR3 and the NAD(P)H:quinone oxidoreductase 1 gene NQO1 in patients who developed anthracycline-related congestive heart failure after childhood cancer. *Cancer* **112**(12), 2789-2795

Blanco,J.G., Sun,C.L., Landier,W., Chen,L., Esparza-Duran,D., Leisenring,W., Mays,A., Friedman,D.L., Ginsberg,J.P., Hudson,M.M. and others (2011). Anthracycline-Related Cardiomyopathy After Childhood Cancer: Role of Polymorphisms in Carbonyl Reductase Genes-A report From the Children's Oncology Group. *J. Clin. Oncol.*

Bolton,J.L., Trush,M.A., Penning,T.M., Dryhurst,G., and Monks,T.J. (2000). Role of quinones in toxicology. *Chem. Res. Toxicol.* **13**(3), 135-160

Bonizzi,G. and Karin,M. (2004). The two NF-kappaB activation pathways and their role in innate and adaptive immunity. *Trends Immunol.* **25**(6), 280-288

Breyer-Pfaff,U., Martin,H.J., Ernst,M., and Maser,E. (2004). Enantioselectivity of carbonyl reduction of 4-methylnitrosamino-1-(3-pyridyl)-1-butanone by tissue fractions from human and rat and by enzymes isolated from human liver. *Drug Metab Dispos.* **32**(9), 915-922

Breyer-Pfaff,U. and Nill,K. (2004). Carbonyl reduction of naltrexone and dolasetron by oxidoreductases isolated from human liver cytosol. *J. Pharm. Pharmacol.* **56**(12), 1601-1606

Bubici,C., Papa,S., Dean,K., and Franzoso,G. (2006). Mutual cross-talk between reactive oxygen species and nuclear factor-kappa B: molecular basis and biological significance. *Oncogene* **25**(51), 6731-6748

Burczynski,M.E., Lin,H.K., and Penning,T.M. (1999). Isoform-specific induction of a human aldo-keto reductase by polycyclic aromatic hydrocarbons (PAHs), electrophiles, and oxidative stress: implications for the alternative pathway of PAH activation catalyzed by human dihydrodiol dehydrogenase. *Cancer Res.* **59**(3), 607-614

Butler,J.E. and Kadonaga,J.T. (2002). The RNA polymerase II core promoter: a key component in the regulation of gene expression. *Genes Dev.* **16**(20), 2583-2592

Caiafa,P. and Zampieri,M. (2005). DNA methylation and chromatin structure: the puzzling CpG islands. *J. Cell Biochem.* **94**(2), 257-265

Carey,M. and Smale,S.T. (2000). *Transcriptional Regulation in Eukaryotes: Concepts, Strategies, and Techniques* 1st ed.; Cold Spring Harbor Laboratory Press: New York. ISBN 978-8087969537

Chantret,I., Barbat,A., Dussaulx,E., Brattain,M.G., and Zweibaum,A. (1988). Epithelial polarity, villin expression, and enterocytic differentiation of cultured human colon carcinoma cells: a survey of twenty cell lines. *Cancer Res.* **48**(7), 1936-1942

Chen,F.E. and Ghosh,G. (1999). Regulation of DNA binding by Rel/NF-kappaB transcription factors: structural views. *Oncogene* **18**(49), 6845-6852

Chen,Z., Kastaniotis,A.J., Miinalainen,I.J., Rajaram,V., Wierenga,R.K., and Hiltunen,J.K. (2009). 17beta-hydroxysteroid dehydrogenase type 8 and carbonyl reductase type 4 assemble as a ketoacyl reductase of human mitochondrial FAS. *FASEB J.* **23**(11), 3682-3691

Cheng,Q., Kalabus,J.L., Zhang,J., and Blanco,J.G. (2012). A conserved antioxidant response element (ARE) in the promoter of human carbonyl reductase 3 (CBR3) mediates induction by the master redox switch Nrf2. *Biochem. Pharmacol.* **83**(1), 139-148

Cheon,M.S., Dierssen,M., Kim,S.H., and Lubec,G. (2008). Protein expression of BACE1, BACE2 and APP in Down syndrome brains. *Amino. Acids* **35**(2), 339-343

Choi,J.Y., Barlow,W.E., Albain,K.S., Hong,C.C., Blanco,J.G., Livingston,R.B., Davis,W., Rae,J.M., Yeh,I.T., Hutchins,L.F. and others (2009). Nitric oxide synthase variants and disease-free survival among treated and untreated breast cancer patients in a Southwest Oncology Group clinical trial. *Clin. Cancer Res.* **15**(16), 5258-5266

Chung,H., Fried,J., and Jarabak,J. (1987). Irreversible inhibition of the human placental NADP-linked 15-hydroxyprostaglandin dehydrogenase/9-ketoprostaglandin reductase by glutathione thiosulfonate. *Prostaglandins* **33**(3), 391-402

Day,A., Dong,J., Funari,V.A., Harry,B., Strom,S.P., Cohn,D.H., and Nelson,S.F. (2009). Disease gene characterization through large-scale co-expression analysis. *PLoS. One.* **4**(12), e8491

Denison,M.S. and Nagy,S.R. (2003). Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. *Annu. Rev. Pharmacol. Toxicol.* **43**, 309-334

Doorn,J.A., Maser,E., Blum,A., Claffey,D.J., and Petersen,D.R. (2004). Human carbonyl reductase catalyzes reduction of 4-oxonon-2-enal. *Biochemistry* **43**(41), 13106-13114

Duax,W.L., Ghosh,D., and Pletnev,V. (2000). Steroid dehydrogenase structures, mechanism of action, and disease. *Vitam. Horm.* **58**, 121-148

Ebert,B., Kisiela,M., Malatkova,P., El-Hawari,Y., and Maser,E. (2010). Regulation of human carbonyl reductase 3 (CBR3; SDR21C2) expression by Nrf2 in cultured cancer cells. *Biochemistry* **49**(39), 8499-8511

El-Hawari,Y., Favia,A.D., Pilka,E.S., Kisiela,M., Oppermann,U., Martin,H.J., and Maser,E. (2009). Analysis of the substrate-binding site of human carbonyl reductases CBR1 and CBR3 by site-directed mutagenesis. *Chem. Biol. Interact.* **178**(1-3), 234-241

Ellis,E.M. (2007). Reactive carbonyls and oxidative stress: potential for therapeutic intervention. *Pharmacol. Ther.* **115**(1), 13-24

Endo,S., Matsunaga,T., Kitade,Y., Ohno,S., Tajima,K., El-Kabbani,O., and Hara,A. (2008). Human carbonyl reductase 4 is a mitochondrial NADPH-dependent quinone reductase. *Biochem. Biophys. Res. Commun.* **377**(4), 1326-1330

Espana,L., Martin,B., Aragues,R., Chiva,C., Oliva,B., Andreu,D., and Sierra,A. (2005). Bcl-x(L)-mediated changes in metabolic pathways of breast cancer cells: from survival in the blood stream to organ-specific metastasis. *Am. J. Pathol.* **167**(4), 1125-1137

Fan,L., Goh,B.C., Wong,C.I., Sukri,N., Lim,S.E., Tan,S.H., Guo,J.Y., Lim,R., Yap,H.L., Khoo,Y.M. and others (2008). Genotype of human carbonyl reductase CBR3 correlates with doxorubicin disposition and toxicity. *Pharmacogenet. Genomics* **18**(7), 621-629

Feldman,R., Luncsford,A., Heinrikson,R.L., Westley,J., and Jarabak,J. (1981). Glutathione-related inhibition of prostaglandin metabolism. *Arch. Biochem. Biophys.* **211**(1), 375-381

Filling,C., Berndt,K.D., Benach,J., Knapp,S., Prozorovski,T., Nordling,E., Ladenstein,R., Jornvall,H., and Oppermann,U. (2002). Critical residues for structure and catalysis in short-chain dehydrogenases/reductases. *J Biol. Chem.* **277**(28), 25677-25684

Forrest,G.L., Akman,S., Krutzik,S., Paxton,R.J., Sparkes,R.S., Doroshow,J., Felsted,R.L., Glover,C.J., Mohandas,T., and Bachur,N.R. (1990). Induction of a human carbonyl reductase gene located on chromosome 21. *Biochim. Biophys. Acta* **1048**(2-3), 149-155

Foster,M.W., Hess,D.T., and Stamler,J.S. (2009). Protein S-nitrosylation in health and disease: a current perspective. *Trends Mol. Med.* **15**(9), 391-404

Foster,M.W., McMahon,T.J., and Stamler,J.S. (2003). S-nitrosylation in health and disease. *Trends Mol. Med.* **9**(4), 160-168

Garattini,E., Gianni,M., and Terao,M. (2007). Retinoids as differentiating agents in oncology: a network of interactions with intracellular pathways as the basis for rational therapeutic combinations. *Curr. Pharm. Des* **13**(13), 1375-1400

Gaston,B., Reilly,J., Drazen,J.M., Fackler,J., Ramdev,P., Arnelle,D., Mullins,M.E., Sugarbaker,D.J., Chee,C., Singel,D.J. and others (1993). Endogenous nitrogen oxides and bronchodilator S-nitrosothiols in human airways. *Proc. Natl. Acad. Sci. U. S. A* **90**(23), 10957-10961

Ghosh,S. and Karin,M. (2002). Missing pieces in the NF-kappaB puzzle. *Cell* **109** Suppl, S81-S96

Gilmore,T.D. (1999). The Rel/NF-kappaB signal transduction pathway: introduction. *Oncogene* **18**(49), 6842-6844

Gilmore,T.D. (2006). Introduction to NF-kappaB: players, pathways, perspectives. *Oncogene* **25**(51), 6680-6684

Gonzalez-Covarrubias,V., Ghosh,D., Lakhman,S.S., Pendyala,L., and Blanco,J.G. (2007). A functional genetic polymorphism on human carbonyl reductase 1 (CBR1 V88I) impacts on catalytic activity and NADPH binding affinity. *Drug Metab Dispos.* **35**(6), 973-980

Gonzalez-Covarrubias,V., Lakhman,S.S., Forrest,A., Relling,M.V., and Blanco,J.G. (2006). Higher activity of polymorphic NAD(P)H:quinone oxidoreductase in liver cytosols from blacks compared to whites. *Toxicol. Lett.* **164**(3), 249-258

Gonzalez-Covarrubias,V., Zhang,J., Kalabus,J.L., Relling,M.V., and Blanco,J.G. (2009). Pharmacogenetics of human carbonyl reductase 1 (CBR1) in livers from black and white donors. *Drug Metab Dispos.* **37**(2), 400-407

Griffiths,A.J., Miller,J.H., Suzuki,D.T., Lewontin,R.C., and Gelbart,W.M. (2000). *An Introduction to Genetic Analysis* 7th; W. H. Freeman. ISBN 0-7167-3520-2

Han,Y. and Brasier,A.R. (1997). Mechanism for biphasic rel A·NF-kappaB1 nuclear translocation in tumor necrosis factor alpha-stimulated hepatocytes. *J. Biol. Chem.* **272**(15), 9825-9832

Han,Y., Meng,T., Murray,N.R., Fields,A.P., and Brasier,A.R. (1999). Interleukin-1-induced nuclear factor-kappaB-IkappaBalpha autoregulatory feedback loop in hepatocytes. A role for protein kinase calpha in post-transcriptional regulation of IkappaBalpha resynthesis. *J. Biol. Chem.* **274**(2), 939-947

Hayden,M.S. and Ghosh,S. (2004). Signaling to NF-kappaB. *Genes Dev.* **18**(18), 2195-2224

Hayden,M.S. and Ghosh,S. (2008). Shared principles in NF-kappaB signaling. *Cell* **132**(3), 344-362

Hecht,S.S. (1998). Biochemistry, biology, and carcinogenicity of tobacco-specific N-nitrosamines. *Chem. Res. Toxicol.* **11**(6), 559-603

Hoffmann,A. and Baltimore,D. (2006). Circuitry of nuclear factor kappaB signaling. *Immunol. Rev.* **210**, 171-186

Hoffmann,F. and Maser,E. (2007). Carbonyl reductases and pluripotent hydroxysteroid dehydrogenases of the short-chain dehydrogenase/reductase superfamily. *Drug Metab Rev.* **39**(1), 87-144

Honkakoski,P. and Negishi,M. (2000). Regulation of cytochrome P450 (CYP) genes by nuclear receptors. *Biochem. J* **347**(Pt 2), 321-337

Iino,T., Tabata,M., Takikawa,S., Sawada,H., Shintaku,H., Ishikura,S., and Hara,A. (2003). Tetrahydrobiopterin is synthesized from 6-pyruvoyl-tetrahydropterin by the human aldo-keto reductase AKR1 family members. *Arch. Biochem. Biophys.* **416**(2), 180-187

Ismail,E., Al-Mulla,F., Tsuchida,S., Suto,K., Motley,P., Harrison,P.R., and Birnie,G.D. (2000). Carbonyl reductase: a novel metastasis-modulating function. *Cancer Res.* **60**(5), 1173-1176

Jain,S., Chakraborty,G., Raja,R., Kale,S., and Kundu,G.C. (2008). Prostaglandin E2 regulates tumor angiogenesis in prostate cancer. *Cancer Res.* **68**(19), 7750-7759

Jarabak,J. and Harvey,R.G. (1993). Studies on three reductases which have polycyclic aromatic hydrocarbon quinones as substrates. *Arch. Biochem. Biophys.* **303**(2), 394-401

Jiang,D., Jarrett,H.W., and Haskins,W.E. (2009). Methods for proteomic analysis of transcription factors. *J Chromatogr. A* **1216**(41), 6881-6889

Jobin,C., Haskill,S., Mayer,L., Panja,A., and Sartor,R.B. (1997). Evidence for altered regulation of I kappa B alpha degradation in human colonic epithelial cells. *J. Immunol.* **158**(1), 226-234

Jornvall,H., Persson,B., Krook,M., Atrian,S., Gonzalez-Duarte,R., Jeffery,J., and Ghosh,D. (1995). Short-chain dehydrogenases/reductases (SDR). *Biochemistry* **34**(18), 6003-6013

Kadonaga,J.T. (2002). The DPE, a core promoter element for transcription by RNA polymerase II. *Exp Mol. Med.* **34**(4), 259-264

Kallberg,Y., Oppermann,U., and Persson,B. (2010). Classification of the short-chain dehydrogenase/reductase superfamily using hidden Markov models. *FEBS J.* **277**(10), 2375-2386

Kang,J.H., Ryu,H.S., Kim,H.T., Lee,S.J., Choi,U.K., Park,Y.B., Huh,T.L., Choi,M.S., Kang,T.C., Choi,S.Y. and others (2009). Proteomic analysis of human macrophages exposed to hypochlorite-oxidized low-density lipoprotein. *Biochim. Biophys. Acta* **1794**(3), 446-458

Kassner,N., Huse,K., Martin,H.J., Godtel-Armbrust,U., Metzger,A., Meineke,I., Brockmoller,J., Klein,K., Zanger,U.M., Maser,E. and others (2008). Carbonyl reductase 1 is a predominant doxorubicin reductase in the human liver. *Drug Metab Dispos.* **36**(10), 2113-2120

Kelner,M.J., Estes,L., Rutherford,M., Uglik,S.F., and Peitzke,J.A. (1997). Heterologous expression of carbonyl reductase: demonstration of prostaglandin 9-ketoreductase activity and paraquat resistance. *Life Sci.* **61**(23), 2317-2322

Kensler,T.W., Wakabayashi,N., and Biswal,S. (2007). Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. *Annu. Rev. Pharmacol. Toxicol.* **47**, 89-116

Kim,C.H., Kim,J.H., Lee,J., Hsu,C.Y., and Ahn,Y.S. (2003). Thiol antioxidant reversal of pyrrolidine dithiocarbamate-induced reciprocal regulation of AP-1 and NF-kappaB. *Biol. Chem.* **384**(1), 143-150

Krook,M., Ghosh,D., Stromberg,R., Carlquist,M., and Jornvall,H. (1993). Carboxyethyllysine in a protein: native carbonyl reductase/NADP(+)-dependent prostaglandin dehydrogenase. *Proc. Natl. Acad. Sci. U. S. A* **90**(2), 502-506

Kumar,A., Takada,Y., Boriek,A.M., and Aggarwal,B.B. (2004). Nuclear factor-kappaB: its role in health and disease. *J. Mol. Med. (Berl)* **82**(7), 434-448

Lakhman,S.S., Chen,X., Gonzalez-Covarrubias,V., Schuetz,E.G., and Blanco,J.G. (2007). Functional characterization of the promoter of human carbonyl reductase 1 (CBR1). Role of XRE elements in mediating the induction of CBR1 by ligands of the aryl hydrocarbon receptor. *Mol. Pharmacol.* **72**(3), 734-743

Lakhman,S.S., Ghosh,D., and Blanco,J.G. (2005). Functional significance of a natural allelic variant of human carbonyl reductase 3 (CBR3). *Drug Metab Dispos.* **33**(2), 254-257

Lal,S., Sandanaraj,E., Wong,Z.W., Ang,P.C., Wong,N.S., Lee,E.J., and Chowbay,B. (2008). CBR1 and CBR3 pharmacogenetics and their influence on doxorubicin disposition in Asian breast cancer patients. *Cancer Sci.* **99**(10), 2045-2054

Lamson,D.W. and Plaza,S.M. (2003). The anticancer effects of vitamin K. *Altern. Med. Rev.* **8**(3), 303-318

Lee,S.H. and Blair,I.A. (2000). Characterization of 4-oxo-2-nonenal as a novel product of lipid peroxidation. *Chem. Res. Toxicol.* **13**(8), 698-702

Lewin,B. (2007). *Genes IX* 9th ed.; Jones and Bartlett Publishers: Sudbury, MA. ISBN 978-0-7637-4063-4

Littarru,G.P. and Tiano,L. (2007). Bioenergetic and antioxidant properties of coenzyme Q10: recent developments. *Mol. Biotechnol.* **37**(1), 31-37

Lopez de,C.A., Marin,A., Idoate,M.A., Tunon,M.T., and Bello,J. (1999). Carbonyl reductase and NADPH cytochrome P450 reductase activities in human tumoral versus normal tissues. *Eur. J. Cancer* **35**(2), 320-324

Lushchak,V.I. (2011). Adaptive response to oxidative stress: Bacteria, fungi, plants and animals. *Comp Biochem. Physiol C. Toxicol. Pharmacol.* **153**(2), 175-190

Malátková, P., Ebert, B., Wsól, V., and Maser, E. (2012). Expression of human carbonyl reductase 3 (CBR3; SDR21C2) is inducible by pro-inflammatory stimuli. *Biochem Biophys Res Commun*. In press. doi: 10.1016/j.bbrc.2012.03.002.

Malatkova,P., Maser,E., and Wsol,V. (2010). Human carbonyl reductases. *Curr. Drug Metab* **11**(8), 639-658

Manabe,S., Gao,Q., Yuan,J., Takahashi,T., and Ueki,A. (1997). Determination of isatin in urine and plasma by high-performance liquid chromatography. *J. Chromatogr. B Biomed. Sci. Appl.* **691**(1), 197-202

Maser,E. (2006). Neuroprotective role for carbonyl reductase? *Biochem. Biophys. Res. Commun.* **340**(4), 1019-1022

Maser,E., Stinner,B., and Atalla,A. (2000). Carbonyl reduction of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) by cytosolic enzymes in human liver and lung. *Cancer Lett.* **148**(2), 135-144

Matsunaga,T., Shintani,S., and Hara,A. (2006). Multiplicity of mammalian reductases for xenobiotic carbonyl compounds. *Drug Metab Pharmacokinet.* **21**(1), 1-18

Medvedev,A., Igosheva,N., Crumeyrolle-Arias,M., and Glover,V. (2005). Isatin: role in stress and anxiety. *Stress.* **8**(3), 175-183

Meunier,V., Bourrie,M., Berger,Y., and Fabre,G. (1995). The human intestinal epithelial cell line Caco-2; pharmacological and pharmacokinetic applications. *Cell Biol. Toxicol.* **11**(3-4), 187-194

Meyer,U.A. (1996). Overview of enzymes of drug metabolism. *J Pharmacokinet. Biopharm.* **24**(5), 449-459

Minotti,G., Menna,P., Salvatorelli,E., Cairo,G., and Gianni,L. (2004). Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. *Pharmacol. Rev.* **56**(2), 185-229

Miura,T., Itoh,Y., Takada,M., Tsutsui,H., Yukimura,T., Nishinaka,T., and Terada,T. (2009a). Investigation of the role of the amino acid residue at position 230 for catalysis in monomeric carbonyl reductase 3. *Chem. Biol. Interact.* **178**(1-3), 211-214

Miura,T., Nishinaka,T., and Terada,T. (2008). Different functions between human monomeric carbonyl reductase 3 and carbonyl reductase 1. *Mol. Cell Biochem.* **315**(1-2), 113-121

Miura,T., Nishinaka,T., and Terada,T. (2009b). Importance of the substrate-binding loop region of human monomeric carbonyl reductases in catalysis and coenzyme binding. *Life Sci.* **85**(7-8), 303-308

Mizobuchi, H., Miura, T., Tsujikawa, K., Nishinaka, T., and Terada, T. (2007). Expression of Carbonyl Reductases in Fetal Human Brain, but Not in Adult. *13th International Symposium on Enzymology and Molecular Biology of Carbonyl Metabolism*. *Enzymology and Molecular Biology of Carbonyl Metabolism***,** 368-378

Monks,T.J., Hanzlik,R.P., Cohen,G.M., Ross,D., and Graham,D.G. (1992). Quinone chemistry and toxicity. *Toxicol. Appl. Pharmacol.* **112**(1), 2-16

Morgan,M.J. and Liu,Z.G. (2011). Crosstalk of reactive oxygen species and NF-kappaB signaling. *Cell Res.* **21**(1), 103-115

Motohashi,H. and Yamamoto,M. (2004). Nrf2-Keap1 defines a physiologically important stress response mechanism. *Trends Mol. Med.* **10**(11), 549-557

Musacchio,J.M., D'Angelo,G.L., and McQueen,C.A. (1971). Dihydropteridine reductase: implication on the regulation of catecholamine biosynthesis. *Proc. Natl. Acad. Sci. U. S. A* **68**(9), 2087-2091

Nguyen,T., Sherratt,P.J., and Pickett,C.B. (2003). Regulatory mechanisms controlling gene expression mediated by the antioxidant response element. *Annu. Rev. Pharmacol Toxicol.* **43**, 233-260

Ohara,H., Miyabe,Y., Deyashiki,Y., Matsuura,K., and Hara,A. (1995). Reduction of drug ketones by dihydrodiol dehydrogenases, carbonyl reductase and aldehyde reductase of human liver. *Biochem. Pharmacol.* **50**(2), 221-227

Ohkura-Hada,S., Kondoh,N., Hada,A., Arai,M., Yamazaki,Y., Shindoh,M., Kitagawa,Y., Takahashi,M., Ando,T., Sato,Y. and others (2008). Carbonyl Reductase 3 (CBR3) Mediates 9-cis-Retinoic Acid-Induced Cytostatis and is a Potential Prognostic Marker for Oral Malignancy. *Open. Dent. J.* **2**, 78-88

Oppermann,U. (2007). Carbonyl reductases: the complex relationships of mammalian carbonyl- and quinone-reducing enzymes and their role in physiology. *Annu. Rev. Pharmacol. Toxicol.* **47**, 293-322

Ota,T., Suzuki,Y., Nishikawa,T., Otsuki,T., Sugiyama,T., Irie,R., Wakamatsu,A., Hayashi,K., Sato,H., Nagai,K. and others (2004). Complete sequencing and characterization of 21,243 full-length human cDNAs. *Nat. Genet.* **36**(1), 40-45

Pahl,H.L. (1999). Activators and target genes of Rel/NF-kappaB transcription factors. *Oncogene* **18**(49), 6853-6866

Park,Y.S., Heizmann,C.W., Wermuth,B., Levine,R.A., Steinerstauch,P., Guzman,J., and Blau,N. (1991). Human carbonyl and aldose reductases: new catalytic functions in tetrahydrobiopterin biosynthesis. *Biochem. Biophys. Res. Commun.* **175**(3), 738-744

Pawliczak,R., Logun,C., Madara,P., Barb,J., Suffredini,A.F., Munson,P.J., Danner,R.L., and Shelhamer,J.H. (2005). Influence of IFN-gamma on gene expression in normal human bronchial epithelial cells: modulation of IFN-gamma effects by dexamethasone. *Physiol Genomics* **23**(1), 28-45

Persson,B., Kallberg,Y., Bray,J.E., Bruford,E., Dellaporta,S.L., Favia,A.D., Duarte,R.G., Jornvall,H., Kavanagh,K.L., Kedishvili,N. and others (2009). The SDR (short-chain dehydrogenase/reductase and related enzymes) nomenclature initiative. *Chem. Biol. Interact.* **178**(1-3), 94-98

Pilka,E.S., Niesen,F.H., Lee,W.H., El-Hawari,Y., Dunford,J.E., Kochan,G., Wsol,V., Martin,H.J., Maser,E., and Oppermann,U. (2009). Structural basis for substrate specificity in human monomeric carbonyl reductases. *PLoS. One.* **4**(10), e7113

Rady-Pentek,P., Mueller,R., Tang,B.K., and Kalow,W. (1997). Interindividual variation in the enzymatic 15-keto-reduction of 13,14-dihydro-15-keto-prostaglandin E1 in human liver and in human erythrocytes. *Eur. J. Clin. Pharmacol.* **52**(2), 147-153

Rahman,A., Anwar,K.N., True,A.L., and Malik,A.B. (1999). Thrombin-induced p65 homodimer binding to downstream NF-kappa B site of the promoter mediates endothelial ICAM-1 expression and neutrophil adhesion. *J Immunol.* **162**(9), 5466-5476

Rajaiya,J., Sadeghi,N., and Chodosh,J. (2009). Specific NFkappaB subunit activation and kinetics of cytokine induction in adenoviral keratitis. *Mol. Vis.* **15**, 2879-2889

Razani,B., Zarnegar,B., Ytterberg,A.J., Shiba,T., Dempsey,P.W., Ware,C.F., Loo,J.A., and Cheng,G. (2010). Negative feedback in noncanonical NF-kappaB signaling modulates NIK stability through IKKalpha-mediated phosphorylation. *Sci. Signal.* **3**(123), ra41

Ris,M.M. and von Wartburg,J.P. (1973). Heterogeneity of NADPH-dependent aldehyde reductase from human and rat brain. *Eur. J. Biochem.* **37**(1), 69-77

Russell,P.T., Eberle,A.J., and Cheng,H.C. (1975). The prostaglandins in clinical medicine. A developing role for the clinical chemist. *Clin. Chem.* **21**(6), 653-666

Schieber,A., Frank,R.W., and Ghisla,S. (1992). Purification and properties of prostaglandin 9-ketoreductase from pig and human kidney. Identity with human carbonyl reductase. *Eur. J. Biochem.* **206**(2), 491-502

Schlager,J.J. and Powis,G. (1990). Cytosolic NAD(P)H:(quinone-acceptor)oxidoreductase in human normal and tumor tissue: effects of cigarette smoking and alcohol. *Int. J. Cancer* **45**(3), 403-409

Sciotti,M.A., Nakajin,S., Wermuth,B., and Baker,M.E. (2000). Mutation of threonine-241 to proline eliminates autocatalytic modification of human carbonyl reductase. *Biochem. J.* **350**, 89-92

Senftleben,U., Cao,Y., Xiao,G., Greten,F.R., Krahn,G., Bonizzi,G., Chen,Y., Hu,Y., Fong,A., Sun,S.C. and others (2001). Activation by IKKalpha of a second, evolutionary conserved, NF-kappa B signaling pathway. *Science* **293**(5534), 1495-1499

Shapiro,H.K. (1998). Carbonyl-trapping therapeutic strategies. *Am. J. Ther.* **5**(5), 323-353

Shearer,M.J. and Newman,P. (2008). Metabolism and cell biology of vitamin K. *Thromb. Haemost.* **100**(4), 530-547

Sheng,H., Shao,J., Morrow,J.D., Beauchamp,R.D., and DuBois,R.N. (1998). Modulation of apoptosis and Bcl-2 expression by prostaglandin E2 in human colon cancer cells. *Cancer Res.* **58**(2), 362-366

Smale,S.T. (1997). Transcription initiation from TATA-less promoters within eukaryotic protein-coding genes. *Biochim. Biophys. Acta* **1351**(1-2), 73-88

Soldan,M., Netter,K.J., and Maser,E. (1996). Induction of daunorubicin carbonyl reducing enzymes by daunorubicin in sensitive and resistant pancreas carcinoma cells. *Biochem. Pharmacol.* **51**(2), 117-123

Sonkoly,E. and Pivarcsi,A. (2009). microRNAs in inflammation. *Int. Rev. Immunol.* **28**(6), 535-561

Strausberg,R.L., Feingold,E.A., Grouse,L.H., Derge,J.G., Klausner,R.D., Collins,F.S., Wagner,L., Shenmen,C.M., Schuler,G.D., Altschul,S.F. and others (2002). Generation and initial analysis of more than 15,000 full-length human and mouse cDNA sequences. *Proc. Natl. Acad. Sci. U. S. A* **99**(26), 16899-16903

Suto,K., Kajihara-Kano,H., Yokoyama,Y., Hayakari,M., Kimura,J., Kumano,T., Takahata,T., Kudo,H., and Tsuchida,S. (1999). Decreased expression of the peroxisomal bifunctional enzyme and carbonyl reductase in human hepatocellular carcinomas. *J. Cancer Res. Clin. Oncol.* **125**(2), 83-88

Tai,H.H., Ensor,C.M., Tong,M., Zhou,H., and Yan,F. (2002). Prostaglandin catabolizing enzymes. *Prostaglandins Other Lipid Mediat.* **68-69**, 483-493

Takenaka,K., Ogawa,E., Oyanagi,H., Wada,H., and Tanaka,F. (2005). Carbonyl reductase expression and its clinical significance in non-small-cell lung cancer. *Cancer Epidemiol. Biomarkers Prev.* **14**(8), 1972-1975

Tanaka,M., Bateman,R., Rauh,D., Vaisberg,E., Ramachandani,S., Zhang,C., Hansen,K.C., Burlingame,A.L., Trautman,J.K., Shokat,K.M. and others (2005). An unbiased cell morphology-based screen for new, biologically active small molecules. *PLoS. Biol.* **3**(5), e128

Testa,B. and Kramer,S.D. (2007). The biochemistry of drug metabolism--an introduction: Part 2. Redox reactions and their enzymes. *Chem. Biodivers.* **4**(3), 257-405

Thijssen,H.H., Vervoort,L.M., Schurgers,L.J., and Shearer,M.J. (2006). Menadione is a metabolite of oral vitamin K. *Br. J. Nutr.* **95**(2), 260-266

Thony,B., Auerbach,G., and Blau,N. (2000). Tetrahydrobiopterin biosynthesis, regeneration and functions. *Biochem. J.* **347 Pt 1**, 1-16

Tian,Q., Stepaniants,S.B., Mao,M., Weng,L., Feetham,M.C., Doyle,M.J., Yi,E.C., Dai,H., Thorsson,V., Eng,J. and others (2004). Integrated genomic and proteomic analyses of gene expression in Mammalian cells. *Mol. Cell Proteomics.* **3**(10), 960-969

Tomankova,T., Petrek,M., and Kriegova,E. (2010). Involvement of microRNAs in physiological and pathological processes in the lung. *Respir. Res.* **11**, 159

Triezenberg,S.J. (1995). Structure and function of transcriptional activation domains. *Curr. Opin. Genet. Dev.* **5**(2), 190-196

Umemoto,M., Yokoyama,Y., Sato,S., Tsuchida,S., Al-Mulla,F., and Saito,Y. (2001). Carbonyl reductase as a significant predictor of survival and lymph node metastasis in epithelial ovarian cancer. *Br. J. Cancer* **85**(7), 1032-1036

Usami,N., Kitahara,K., Ishikura,S., Nagano,M., Sakai,S., and Hara,A. (2001). Characterization of a major form of human isatin reductase and the reduced metabolite. *Eur. J. Biochem.* **268**(22), 5755-5763

Valasek,M.A. and Repa,J.J. (2005). The power of real-time PCR. *Adv. Physiol Educ.* **29**(3), 151-159

Vancurova,I., Wu,R., Miskolci,V., and Sun,S. (2002). Increased p50/p50 NF-kappaB activation in human papillomavirus type 6- or type 11-induced laryngeal papilloma tissue. *J Virol.* **76**(3), 1533-1536

Wang,D. (2008). Discrepancy between mRNA and protein abundance: insight from information retrieval process in computers. *Comput. Biol. Chem.* **32**(6), 462-468

Warnatz,H.J., Querfurth,R., Guerasimova,A., Cheng,X., Haas,S.A., Hufton,A.L., Manke,T., Vanhecke,D., Nietfeld,W., Vingron,M. and others (2010). Functional analysis and identification of cis-regulatory elements of human chromosome 21 gene promoters. *Nucleic Acids Res.* **38**(18), 6112-6123

Watanabe,K., Sugawara,C., Ono,A., Fukuzumi,Y., Itakura,S., Yamazaki,M., Tashiro,H., Osoegawa,K., Soeda,E., and Nomura,T. (1998). Mapping of a novel human carbonyl reductase, CBR3, and ribosomal pseudogenes to human chromosome 21q22.2. *Genomics* **52**(1), 95-100

Wen,J., You,K.R., Lee,S.Y., Song,C.H., and Kim,D.G. (2002). Oxidative stress-mediated apoptosis. The anticancer effect of the sesquiterpene lactone parthenolide. *J Biol. Chem.* **277**(41), 38954-38964

Wermuth,B. (1981). Purification and properties of an NADPH-dependent carbonyl reductase from human brain. Relationship to prostaglandin 9-ketoreductase and xenobiotic ketone reductase. *J. Biol. Chem.* **256**(3), 1206-1213

Wermuth,B., Bohren,K.M., and Ernst,E. (1993). Autocatalytic modification of human carbonyl reductase by 2-oxocarboxylic acids. *FEBS Lett.* **335**(2), 151-154

Wermuth,B., Bohren,K.M., Heinemann,G., von Wartburg,J.P., and Gabbay,K.H. (1988). Human carbonyl reductase. Nucleotide sequence analysis of a cDNA and amino acid sequence of the encoded protein. *J. Biol. Chem.* **263**(31), 16185-16188

Wermuth,B., Platts,K.L., Seidel,A., and Oesch,F. (1986). Carbonyl reductase provides the enzymatic basis of quinone detoxication in man. *Biochem. Pharmacol.* **35**(8), 1277-1282

Wirth,H. and Wermuth,B. (1992). Immunohistochemical localization of carbonyl reductase in human tissues. *J. Histochem. Cytochem.* **40**(12), 1857-1863

Xu,C., Li,C.Y., and Kong,A.N. (2005). Induction of phase I, II and III drug metabolism/transport by xenobiotics. *Arch. Pharm. Res.* **28**(3), 249-268

Zhang,D.D. (2006). Mechanistic studies of the Nrf2-Keap1 signaling pathway. *Drug Metab Rev.* **38**(4), 769-789

Zhang,J. and Blanco,J.G. (2009). Identification of the promoter of human carbonyl reductase 3 (CBR3) and impact of common promoter polymorphisms on hepatic CBR3 mRNA expression. *Pharm. Res.* **26**(9), 2209-2215

8. Supplements

Publications related to the topic of the thesis

1. **Malatkova,P.**, Maser,E., and Wsol,V. (2010). Human carbonyl reductases. *Curr. Drug Metab* **11**(8), 639-658. IF (3.896)

2. Ebert,B., Kisiela,M., **Malatkova,P.**, El-Hawari,Y., and Maser,E. (2010). Regulation of human carbonyl reductase 3 (CBR3; SDR21C2) expression by Nrf2 in cultured cancer cells. *Biochemistry* **49**(39), 8499-8511. IF (3.226)

3. **Malátková, P.**, Ebert, B., Wsól, V., and Maser, E. (2012). Expression of human carbonyl reductase 3 (CBR3; SDR21C2) is inducible by pro-inflammatory stimuli. *Biochem Biophys Res Commun*. In press. doi: 10.1016/j.bbrc.2012.03.002. IF (2.595)

Presentations related to the topic of the thesis

1. Maser E., Kisiela M., Malátková P., El-Hawari Y., Wsól V., Ebert B. (2010). Regulation of carbonyl reducing enzymes by Nrf2 and effect of proteasome inhibitors on CBR3, AKR1B10, AKR1B1 and AKR1C3 expression in human colon cancer cell lines. 15th Meeting of Enzymology and Molecular Biology of Carbonyl Metabolism, Lexington, USA

2. Malátková P., Ebert B., Maser E, Wsól V. (2012). Transcriptional regulation of human carbonyl reductase 3 (CBR3) via NFκB signaling pathway. 2. Postgraduální vědecké konference Farmaceutické fakulty Univerzity Karlovy, Hradec Králové

3. Malátková P., Ebert B., Wsól V., Maser E. (2012). Activation of NFκB regulates human carbonyl reductase 3 (CBR3; SDR21C2) RNA expression in cultured cancer cells. 16th Meeting of Enzymology and Molecular Biology of Carbonyl Metabolism, Plön, Germany. Submitted.

Publication 1

Malatkova,P., Maser,E., and Wsol,V. (2010). Human carbonyl reductases. *Curr. Drug Metab* **11**(8), 639-658

Publication 2

Ebert,B., Kisiela,M., Malatkova,P., El-Hawari,Y., and Maser,E. (2010). Regulation of human carbonyl reductase 3 (CBR3; SDR21C2) expression by Nrf2 in cultured cancer cells. *Biochemistry* **49**(39), 8499-8511

Publication 3

Malátková, P., Ebert, B., Wsól, V., and Maser, E. (2012). Expression of human carbonyl reductase 3 (CBR3; SDR21C2) is inducible by pro-inflammatory stimuli. *Biochem Biophys Res Commun*. In press. doi: 10.1016/j.bbrc.2012.03.002.