Diploma thesis

The role of xenobiotic receptors in the regulation of autophagy

written by

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List of abbreviations

AF-2	Activation function 2
AMBRA1	autophagy/beclin-1 regulator
AMP	Adenosine monophosphate
AMPK	5' AMP-activated protein kinase
ATG10	Autophagy related gene 101
ATG101	Autophagy related gene 101
ATG12	Autophagy related gene 12
ATG13	Autophagy related gene 1
ATG14L	Autophagy related gene 14 like
ATG16L	Autophagy related gene 16 like 1
ATG4B	Cysteine protease ATG4B
ATG5	Autophagy related gene 5
ATG7	Autophagy related gene 7
ATG8	Autophagy related gene 8
BCL-2	B-cell lymphoma 2
BECN1	Beclin 1
BIF1	Endophilin-B1
BRCA1	Breast and ovarian tumor suppressor breast cancer
BRD4	Epigenetic reader bromodomain-containing protein 4
CAR	Constitutive androstane receptor
CD	Cluster of differentiation
cDNA	Complementary desoxyribonucleic acid
CITCO	6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime
class I PI3-Akt	Class I phosphatidylinositol-3-kinase (PI3)-Protein Kinase B
COUP-TF	chicken ovalbumin upstream promoter transcription factor

CPTI	mitochondrial carnitine palmitoyltransferase I
CYP3A4	cytochrome P450 gene
DMSO	Dimethylsulfoxid
DNA	Desoxyribonucleic acid
elF2	Eukaryotic initiation factor 2
FIP200	RB1-inducible coiled-coil protein 1
FXR	Farnesoid X receptor
GAAPDH	Glycerinaldehyd-3-phopshpate-dehydrogenase
GFP	Green fluorescent protein
GR	Glucocorticoid receptor
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C
HDAC	Histone deacetylases
HNF-4	Hepatocyte nuclear factor-4
HRE	hormone responive elements
hsp90	Heat shock protein 90
IRE-1	Serine/threonine-protein kinase/endoribonuclease inositol-requiring enzyme 1
KEAP1	Kelch-like ECH-associated protein 1
KRAS	Kirsten rat sarcoma virus
LBD	Ligand binding domain
LC3	Microtubule-associated proteins 1A/1B light chain 3A
LC3-I	LC3 without precursor form
LC3-II	LC3-I conjugated with PE
MDR1	multidrug resistance protein 1
MHC	Major histocompatibility complex
mRNA	Mesenger ribonucleic acid
mTOR	Mammalian target of rapamycin

mTORC1	mTOR signaling complex 1
NADPH	Nicotinamide adenine dinucleotide phosphate
NAFLD	Non-alcoholic-fatty-liver-disease
NRF2	Nuclear factor erythroid 2-related factor 2
NRs	Nuclear Receptors
OATP2	Solute carrier organic anion transporter family member 1B1
P-p70S6K	Ribosomal protein S6 kinase beta-1
p62	Sequestosome-1
PCN	pregnenolone-16-α-carbonitrile
PE	Phosphatidylethanolamine
PINK1	PTEN-induced kinase 1
PPAR-α	Perixosome proliferator-activated receptor
PPAR-γ	Perixosome-proliferator-activated receptor γ
PVDF	Polyvinylidenfluorid
PXR	Pregnane X receptor
qPCR	Quantitative polymerase chain reaction
RFP	Red fluorescent protein
RNA	Ribonucleic acid
RXR	9-cis retinoic acid receptor
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SQSTM1	Sequestosome-1
SXR	xenobiotic sensing nuclear receptor
TCPOBOP	3,3′,5,5′-Tetrachloro-1,4-bis(pyridyloxy)benzene
TFEB	transcription factor EB
TR	thyroid receptor
UGT1A1	Uridinephosphate glycoronyltransferase
ULK1	Unc-51 like autophagy activating kinase
UVRAG	UV radiation resistnace-associated gene protein

VDR	Vitamin D receptor
VPS34	class III phosphatidylinositol-3-kinase

Abstract

Autophagy is a cell component-digesting process, which is crucial in maintaining cell homeostasis and it is inevitably involved in the pathophysiology of many diseases. Its regulation is complex and targeting autophagy pharmacologically remains challenging.

Nuclear receptors (NRs) are a ligand sensitive class of proteins which work as transcription factors and operate together with other proteins to regulate the expression of specific genes by directly interacting with the DNA.

Xenobiotic receptors represent a special subgroup of NRs whose primary function is to anticipate the presence of foreign toxic substances, i.e. xenobiotics, in order to manage the upregulation of the expression of proteins which are engaged in the detoxification of these substances. Xenobiotic receptors compromise the pregnane X receptor (PXR) and the constitutive androstane receptor (CAR). So far there has not been published reliable data concerning the connection between xenobiotic receptors and autophagy. We, however, propose and provide evidence which proves that autophagy is also integrated in xenobiotic response mechanisms. Preliminary work from our lab indicates that activation of the PXR through agonist-treatment with rifampicin shows induction of autophagy in human samples. Rifampicin is a common antibiotic, whose use is limited due to its wide range of side effects including liver toxicity. Thus, the purpose of this work was to show that autophagy induction by PXR activation can be reproduced via treatment with less toxic PXR agonists such as hyperforin, the well-tolerated ingredient of the antidepressant St. John's Wort.

We could show an induction of autophagy related markers after hyperforin treatment in HepG2 cells and we confirmed induction of autophagic flux in HepG2 cells in immunofluorescence microscopy using GFP-RFP-LC3 constructs.

Moreover, our experiments show that this mechanism appears to be evolutionarily conserved because we were able to reproduce autophagy induction in mice treated with a PXR-agonist at least on protein basis. All experiments were also performed by agonist activation of the constitutive androstane receptor (CAR). Neither in rodents nor in humans CAR seems to be implicated in the induction of autophagy and was therefore not further investigated.

The main finding of this work is that activation of the xenobiotic receptor PXR leads to upregulation of autophagy-related markers and that this mechanism can be utilized by different available and tolerable PXR agonists and it is also conserved in mice.

Zusammenfassung

Die Autophagie ist ein unumgänglicher Prozess, bei dem Zellorganellen abgebaut werden. Sie dient zur Aufrechterhaltung der Zell-Homöostase und spielt darüber hinaus pathophysiologisch bei der Entstehung vieler Krankheiten eine maßgebliche Rolle. Ihre Regulation ist sehr komplex und es ist daher schwierig, die Autophagie pharmakologisch zu beeinflussen.

Kernrezeptoren sind Transkriptionsfaktoren, die erst durch die Bindung eines Liganden die Expression ihrer Zielgene durch direkte Interaktion mit der DNA regulieren.

Xenobiotica-Rezeptoren sind eine spezielle Subgruppe, deren primäre Funktion darin liegt, in Gegenwart von körperfremden, toxischen Stoffen, sog. Xenobiotica, aktiv zu werden, um in weiterer Folge für die Hochregulation von Proteinen zu sorgen, die an der Ausscheidung jener Stoffe beteiligt sind. Zu Xenobiotica-Rezeptoren zählen unter anderem der Pregnane X Receptor (PXR) sowie auch der Constitutive Androstane Receptor (CAR). Bis zum jetzigen Zeitpunkt gibt es keine Daten, die einen Zusammenhang zwischen Xenobiotica-Rezeptoren und der Autophagie nahelegen. Vorbestehende Daten aus unserem Labor zeigen, dass es zu einer Induktion der Autophagie kommt, wenn menschliche Proben mit dem PXR-Agonisten Rifampicin behandelt werden. Rifampicin ist ein weitverbreitetes Antibiotikum, das jedoch durch weitreichende Nebenwirkungen, wie unter anderem durch Lebertoxizität, gekennzeichnet ist.

Insofern war es das Ziel dieser Arbeit zu beweisen, dass die Autophagie auch durch die Behandlung mit weit weniger toxischen PXR-Agonisten, wie zum Beispiel Hyperforin, dem Wirkstoff des Antidepressivums Johanniskraut, induziert werden kann. Unsere Versuche zeigten nach einer Behandlung mit Hyperforin eine signifikante Hochregulation von Autophagie-Markern in HepG2-Zellen und wir konnten eine Induktion des Autophagie-Flusses in HepG2-Zellen mit Hilfe von Immunfluoreszenz nachweisen.

Überdies konnten wir zeigen, dass dieser Mechanismus evolutionär konserviert zu sein scheint, weil wir zumindest auf Protein-Basis nachweisen konnten, dass es zu einer Hochregulation von Autophagie-Markern kommt, wenn man Mäuse mit PXR-Agonisten behandelt.

Alle Experimente wurden auch mit CAR Agonisten durchgeführt, jedoch konnte weder in Mäusen noch in menschlichen Proben eine Hochregulation von Autophagie-Markern nachgewiesen werden.

Die Konklusion dieser Arbeit ist, dass durch die Behandlung mit PXR-Agonisten gewisse Autophagie-Marker hochreguliert werden und dass dieser Mechanismus auch in Mäusen erhalten ist.

1. Introduction

1.1 What is autophagy?

1.1.1 Overview on autophagy

Autophagy is a lysosomal self-digestion and degradation pathway, which is decisive for basal tasks of the cell like differentiation, development and homeostasis (1). It is a process in which the cell self-digests its own components to provide nutrients in harsh environmental conditions (2). It helps to adapt to a broad variety of stressors and diseases such as cancer, neurodegeneration, infections, liver diseases and diverse other pathologies (3). Furthermore, it is an effective opportunity for the cell to get rid of superfluous and damaged organelles, misfolded proteins or even invaded microorganisms. It occurs at low basal levels in all cells to perform basic homeostatic functions, whereas it can promptly be upregulated when cells suffer from deficiency of nutrients e.g. in starvation conditions, or when cells have to get rid of damaged cell components and organelles, for example during infection or protein aggregate accumulations. Therefore it is not surprising, that autophagy is evolutionary highly conserved and could even be found in yeast (4), where the basic characterization of the crucial elements of autophagy has been studied since the 1970ies (5).

Autophagy can be subdivided into three different types: microautophagy, macroautophagy and chaperon-mediated autophagy (6). While each type is morphologically different, all three end up in the delivery of cargo to the lysosome for degradation and recycling.

Briefly, microautophagy is characterized by the fact that invaginations or protrusions of the lysosomal membrane are used to capture cargo. Chaperone mediated autophagy (CMA) makes use of chaperones to identify cargo proteins that are marked through a particular pentapeptide motif. In the case of macroautophagy de novo synthesis of double membrane vesicles takes place (7). (Figure 1 illustrates the principle steps and differences of the three types of autophagy.)

This thesis will focus on macroautophagy (herein simply referred to as autophagy) (3, 8).

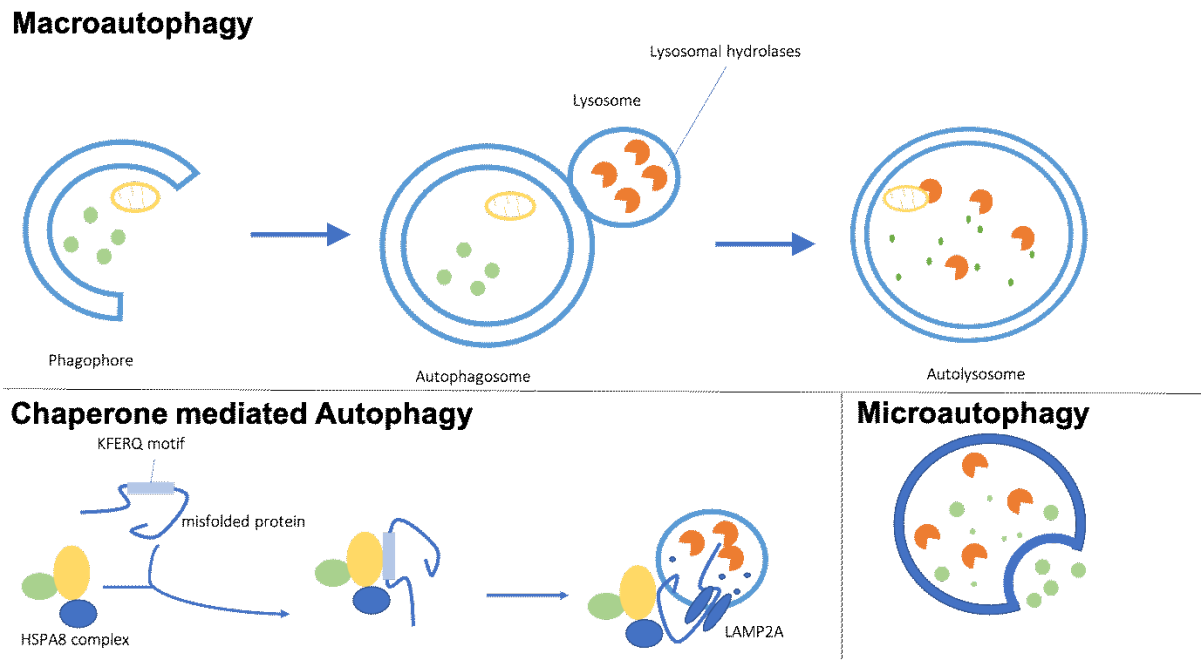

Figure 1. Different types of autophagy

Macroautophagy is marked through formation of cytosolic double membranes in order to segregate and transport cargo to the lysosome. Chaperone mediated autophagy is characterized by the fact that a certain targeting motif (KFERQ) and the heat shock 70 kDA protein 8 (HSPA8) is needed to degrade misfolded cytosolic proteins. The lysosomal-associated membrane protein 2A (LAMP2A) is needed for binding. Microautophagy is associated with direct uptake of cargo through invagination of the lysosomal membrane (adapted from (9)).

1.1.2 Principle steps of macroautophagy

The process of autophagy implies several highly regulated steps consisting of the interaction of multiple proteins at each single step. During the first "Initiation" step a cup shaped isolation membrane which is called phagophore is built. The second step consists of membrane elongation of the previously built membrane until a mature autophagosome is generated in the maturation step. During these steps cargo for final degradation will already be engulfed. Currently, it is not fully understood how cargo is selected for inclusion into the autophagosome. While for some organelles such as mitochondria or lipid droplets this process appears to be at least in part selectively regulated (10), for many proteins it still appears to be a random process. The autophagosome is not equipped with specific enzymes to degrade its cargo, therefore it has to fuse in a next step with lysosomes, which supply hydrolases and also the acidic pH required for optimal breakdown. Figure 2 illustrates the single steps in the process of autophagy. Every individual step is tightly regulated (11). There are "classical" as well as alternative regulators of autophagy.

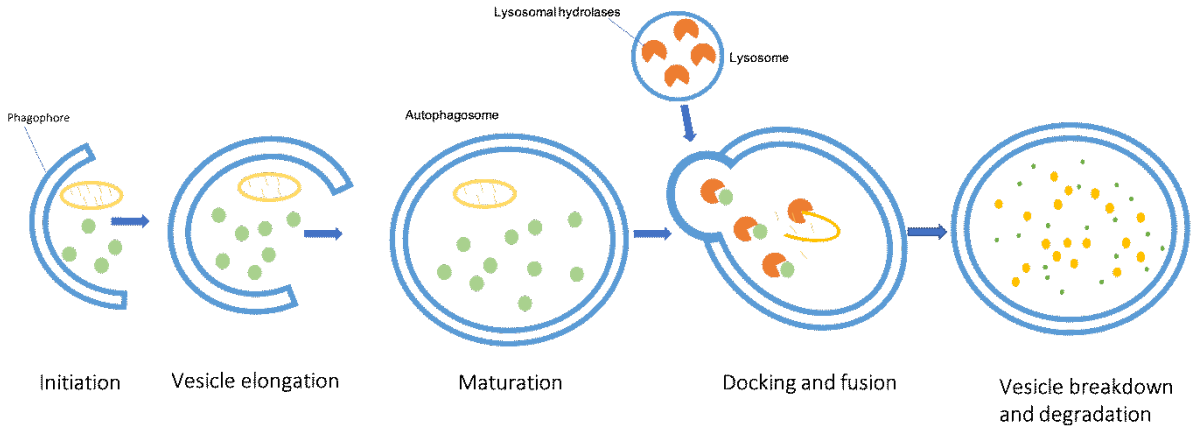

Figure 2. Different steps of macroautophagy

The pathway of autophagy implies several steps, ranging from initiation, which includes the formation of an isolation membrane (phagophore), to vesicle elongation, autophagosome maturation and cargo sequestration. At the end there is autophagosome-lysosome fusion which finally leads to cargo degradation (adapted from (11)).

1.1.3 Classical regulation of autophagy

Autophagy is a cytoprotective mechanism (7), therefore it is regulated by sources essential for cell survival, such as nutritional and energy status, oxygen concentrations as well as cell density and hormones (3).

In more detail, one of the essential controllers of autophagy is the mammalian/molecular target of rapamycin, mTOR kinase (12). mTOR is the prototypical sensor of the feeding state and is activated by nutritional components such as certain amino acids (13). In addition, mTOR is activated by class I phosphatidylinositol-3-kinase (PI3)-AKT signaling molecules as a reaction to growth-factor signaling (11). The mTOR protein compromises the mTOR-signaling complex 1 (mTORC1), which further encompasses the regulatory-associated protein of mTOR, G protein beta subunit-like protein and proline-rich AKT/PKB substrate 40kDa (11).
mTORC1 on the other hand is involved in the regulation of the mTOR substrate complex, which consists of ATG13, mammalian uncoordinated-51-like protein kinase ULK1, FIP200 and ATG101 (11). The activated mTOR signaling cascade is a powerful inhibitory signal, which blocks autophagy in presence of abundant nutrients and growth factors, like insulin (14).
Upon decreased cellular energy levels, as for example upon glucose starvation, autophagy also can be induced by the cell homeostasis regulatory kinase 5’ AMP-activated protein kinase

(AMPK) which senses low ATP:AMP ratios. AMPK inactivates mTORC1 and phosphorylates ULK1 which in term leads to induction of the autophagic machinery (15).
Another regulation pathway consists of the Beclin 1-interacting complex. It is composed of Beclin 1, class III PI3-kinase (VPS34) and ATG14L. Phosphatidylinositol-3-phosphate is generated via this complex, which further promotes autophagosomal membrane nucleation. This regulatory pathway is the target of many interacting factors, such as UVRAG and BIF1, as well as autophagy/beclin-1 regulator (AMBRA1) and Rubicon (11).

For autosomal elongation and autophagosome formation two ubiquitin-like conjugation systems are required. A distinction is drawn between the ATG5-ATG12 conjugation system on the one hand and the LC3-ATG8 conjugation system on the other hand.
The ubiquitin-like protein ATG12 connects with ATG5 along with ATG7 and ATG10 enzymes. Then it forms a complex with ATG16L1, which takes part in the elongation of the autophagic membrane (11).
For the second conjugation system the ubiquitin-like protein microtubule–associated protein 1 light chain 3 (LC3) is needed. The protease ATG4B cleaves the precursor form of LC3 to LC3-I. With the help of the enzymes ATG7 and ATG3, LC3-I gets conjugated with phosphatidylethanolamine (PE). The result is called LC3-II (PE-conjugated-form), which represents a key regulator role in autophagosome formation (11). LC3 I and II are also the classical experimental readout markers for autophagy studies.

There are several other regulators of autophagy induction like the before mentioned low energy sensor (AMPK), the endoplasmic reticulum (ER) stress sensors IRE-1 and eukaryotic initiation factor 2a (elF2a), BCL-2 family members governing mitochondrial outer membrane permeabilization, stress-activated kinase c-JUN, tumor suppressor p53, and calcium (3). This bundle of sensors is one reason why the autophagic pathway reacts highly responsive to the nutrient status as well as cellular and organelle stress. Moreover, these regulators allow intrinsic cellular adaptation to overcome unfavorable conditions. It is, however, challenging to target these pathways extrinsically or pharmacologically, respectively.
Figure 3 gives an overview on different signals which can modulate autophagy.

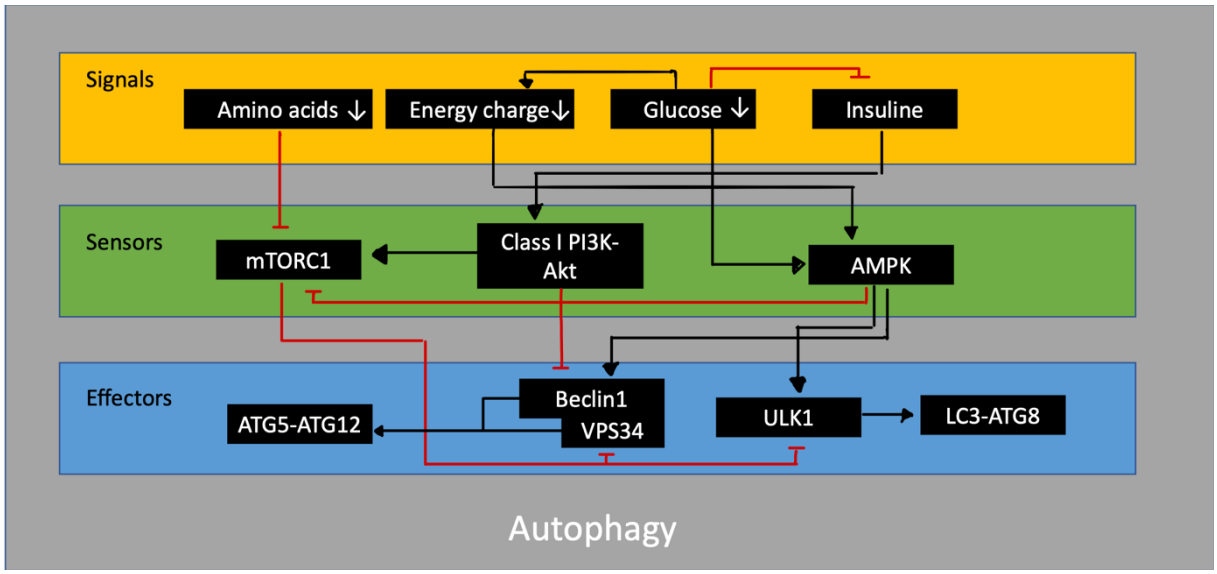

Figure 3. Overview on how autophagy is regulated

Understanding the regulation of autophagy is challenging, because so many different pathways are involved. This figure gives a simplified summary of the different steps and players which participate in the regulation of autophagy. It is structured in 3 dimensions. Molecular complexes like mTORC1, Class I PI3K-Akt and AMPK sense changes in the energy and nutritional status e.g. via sensing amino acid- or glucose-levels and create an effector-based answer which finally activates (→) or inhibits (--|) downstream autophagy molecules, e.g. via phosphorylation. See text for further information (adapted from (16)).

1.1.4 Regulation of autophagy by transcription factors

In contrast to these aforementioned kinase pathways for autophagy regulation, only little is known about transcriptional regulation of autophagy (17). The best characterized transcription factor to regulate autophagy is the transcription factor EB (TFEB). TFEB is the main controller of lysosomal biogenesis. It has been shown that during starvation (i.e. the physiological signal to start autophagy) both compartments, autophagosomes as well as lysosomes, expand in order to enhance degradation processes. Starvation-induced autophagy is transcriptionally regulated and coordinated by TFEB by driving expression of both autophagy and lysosomal genes, because cells that lack TFEB have an impaired physiological response to starvation and autophagy induction (18).

Moreover, it has recently been demonstrated that the epigenetic reader bromodomain-containing protein 4 (BRD4) in conjunction with the methyltransferase G9a contribute to downregulations of a transcriptional program of autophagic genes (19).

However, there is also evidence, that nuclear receptors (NRs), which are specialized transcription factors that can directly be activated by ligands, transcriptionally regulate autophagy (20).

Regulation of autophagy by NRs is of potential pharmacological interest, because NRs work, in contrast to many other transcription factors or the classical kinase regulators of autophagy, directly and thus they are easier to target pharmacologically. This would make NRs likely pharmacological candidates to address autophagy (for more information see 1.4.5).

1.2 Role of autophagy in physiology and pathology

1.2.1 Physiological functions of autophagy

Autophagy plays an important role for the cell to survive under conditions of stress, because it is primarily responsible for the cell to maintain cellular integrity by regenerating metabolic precursors and clearing subcellular debris (11). The most obvious physiological function of autophagy in the response to starvation and self-digestion is regulated by sensing cellular nutrient (i.e. mTOR pathway) and energy levels (i.e. AMPK pathway). The main outcome is that cell components are randomly engulfed in autophagosomes and degraded enzymatically in autophagolysosomes to deliver the cell with amino acids, lipids or carbohydrates which than can be channeled into downstream energy producing cycles (i.e. beta oxidation, glycolysis, citrate cycle, etc.) (10). Beside this prototypical function of autophagy there exist several other, more specific functions of autophagy, which will be briefly depicted in the following sections.

1.2.1.1 Role of autophagy for oxidative stress

The role of autophagy for oxidative stress involves p62. p62 is a protein, which gets phosphorylated through oxidative stress. Phosphorylated p62 binds to Kelch-like ECH-associated protein 1 (KEAP1) with high affinity (21). KEAP1 is an regulator of the ubiquitin ligase complex for nuclear factor erythroid 2-related factor 2 (NRF2) (22). Phosphorylated p62 and the KEAP1 complex are degraded by autophagy and thus KEAP1-driven ubiquitylation of NRF2 is suppressed which enables NRF2 to translocate to the nucleus in turn to induce a battery of genes with the ability to protect the cell from oxidative stress containing NAD(P)H dehydrogenase quinone 1 and glutathione S transferase (9, 23-25).

1.2.1.2 Role of autophagy for lipid metabolism

Autophagy has also been identified to be implicated in regulation of lipid metabolism (26). In mouse liver-tissue it was demonstrated that autophagosomes are formed to crop out a part of a lipid droplet and finally merge with a lysosome to provide free fatty acids for β-oxidation (27). This process is referred to as lipophagy. The control of the basic mechanism by which these lipid droplets are marked for selective autophagy still needs to be elucidated (9, 27).

In addition to this autophagy is engaged in the elimination of other polyubiquitinated protein aggregates (called aggregophagy), whose amount may increase during aging, stress and disease due to perturbations in protein structure or folding (28).

1.2.1.3 Role of autophagy for organelle degradation

Moreover, autophagy is engaged in the turnover of mitochondria and other organelles such as endoplasmatic reticulum, mitochondria and perixosomes (29, 30).

Parkin-mediated mitophagy for instance is one of the best described examples of selective autophagy. In this process depolarized mitochondria turns out to be particularly surrounded by autophagosomes and is further eradicated (31), while the E3-protein ubiquinase parkin and its interaction with the serine/threonine-protein kinase PINK1 works as marker for the autophagosomal machinery to start its degradation (9, 32).

1.2.1.4 Role of autophagy for immunity and inflammation

The autophagic pathway furthermore takes part in immune responses by eradicating viruses and intracellular bacteria (i.e. xenophagy) (33-35).

It has been shown that autophagy plays a role in the suppression of inflammation because studies have elucidated that knockdown of autophagy-related protein beclin 1 or autophagy-related protein 7 in immortalized human hepatocytes is associated with inhibition of HCV growth due to increased expression of interferon-β (36).

Autophagy is also associated with adaptive immune responses. Normally major-histocompatibility-complex (MHC) class II molecules are involved in the presentation of the products of lysosomal proteolysis to CD4+ T- cells. There is however evidence that also autophagosomes fuse with MHC class II loading compartments. In context with influenza it could even be shown that targeting of influenza matrix protein 1 to autophagosomes leads to firmly increased MHC class II presentation to CD4+ T cell clones (37). Autophagy also contributes to self-tolerant T-cell generation, as genetic intervention in autophagy related genes

in thymic epithelial cells leads to decreased self-tolerance which results in severe colitis and multi-organ inflammation (38).

1.2.2 Pathological aspects of autophagy

Because of the widespread physiological functions of autophagy, one can expect this highly conserved metabolic pathway to be also involved in pathophysiological processes such as cancer, cardiovascular diseases, metabolic diseases, infectious diseases, pulmonary diseases and both liver diseases and neurodegenerative diseases.

1.2.2.1 Role of autophagy in liver disease

Malfunction of autophagy contributes to several liver diseases, such as viral hepatitis and hepatocellular carcinoma (9), where autophagy plays sort of quality control. The role of autophagy for quality control and disease development becomes particularly apparent in the case of α1-antitrypsin deficiency, too.

About 1 in 2000-5000 individuals is affected by this genetically determined disease (39). In α1-antitrypsin deficiency in adults destruction of lung tissue and pulmonary emphysema develops, because the inhibitory effect of the remaining α1-antitrypsin concentration is too low to suppress proteolytic degradation of the lung connective tissue matrix by the neutrophil elastase (39).

Many patients with severe α1-antitrypsin deficiency with pulmonary emphysema tend to be homozygous for the SERPINA1 Z allele and the α1-antitrypsin levels in the blood are reduced to 10-15% (39, 40). While the damage in the lung is due to a loss-of-function mutation, liver cirrhosis is owed to a gain-of-function effect (9).

Due to the toxic gain-of-function mutation in α1-antitrypsin Z, physiological protein folding is impaired and consequently the normally secreted hepatic protein forms abnormal aggregated polymers within the hepatocyte (41). This process called loop-sheet polymerisation can cause liver cirrhosis (42) and represents one of the leading causes of liver transplantation in early childhood. Wild-type α1-antitrypsin is relieved by the proteasome-pathway, mutant α1-antitrypsin Z is assumed to be eradicated by autophagy (39, 41). In addition to this, α1-antitrypsin Z degradation shows to be decreased in autophagy-defective cell lines, assuming that autophagy plays an inevitable role in the pathogenesis of α1-antitrypsin deficiency (43).

The process of autophagy is also involved in pathogenesis of non-alcoholic-fatty-liver-disease (NAFLD). NAFLD is a widely spread disease which is consecutive to obesity, insulin resistance, the metabolic syndrome and type 2 diabetes. Approximately 30% of the American population shows to be affected by NAFLD according to actual data with similar rates in Europe (44).

Autophagy in hepatocytes works as defense mechanism in the prevention of NAFLD, because lipophagy is involved in selective degradation of cytoplasmic lipid droplets (9). Mouse studies have shown that in models of obesity autophagy, particularly Atg7, appears to be suppressed (45). Moreover, impaired autophagy in mouse liver leads to prominent accumulation of p62 and consecutive NRF2 activation is highly suspicious for autophagy to play a role in the pathogenesis of steatohepatitis und alcoholic hepatitis (9).

p62 has shown to be a major component of inclusion bodies in hepatocytes, also known as Mallory-Denk bodies, which have been found in patients with steatohepatitis and alcoholic hepatitis (46, 47). Moreover, elevated levels of p62 with consecutive NRF2 activation are associated with viral hepatitis, hepatic fibrosis and NAFLD, conditions which consequently lead to liver cirrhosis and HCC formation (48-51). Moreover, pharmacological targeting of autophagy by rapamycin improves liver steatosis (9).

On the other hand, there is evidence that loss of certain key players of the autophagic pathway in cultured mouse stellate cells reduces fibrogenesis and matrix accumulation (52). Hence autophagy in stellate cells contributes to contrary effects concerning the progression of NAFLD compared to autophagy in hepatocytes (9).

A further very interesting issue is that in context of cholestatic liver diseases and high bile acid levels autophagy seems to be impaired, due to transcriptional mediated inhibition of autophagy by FXR (53). More studies have to be done to elucidate further mechanisms of the role of autophagy in cholestatic liver diseases (see 1.4.5 for further information about the interplay of NRs and autophagy).

In chronic hepatitis-B-virus infection, the autophagic machinery is used for viral replication. HBV chronically infects approximately 350 million people globally. It has been proven that autophagosome formation in HepG2 cells is induced by transfection of HBV genomic DNA, however without up-regulation of autophagic protein degradation. The induction of autophagosomes depends on the interaction of HBx, which is a regulatory protein of HBV.

In this context, HBx is responsible for up-regulation of class III phosphatidylinositol-3-kinase, an enzyme crucial for the initiation of autophagy (54).
In addition to hepatitis B, it is established that proteins involved in autophagy, like Beclin-1, ATG4B, ATG5B and ATG12 are needed for translation of invading RNA of hepatitis C virus (HCV) and thus for initiation of HCV replication in cell culture (55).

1.2.2.2 Role of autophagy in cancer

The role of autophagy in cancer is complex and highly context dependent. Autophagy can either inhibit or promote tumorigenesis or cancer cell proliferation. Autophagy was formerly anticipated to be a clear tumor suppressive mechanism. This assumption seemed to be supported by early data, which had shown that the indispensable autophagy gene BECN1 (codes for Beclin 1) was mono-allelically lost on 40% to 75% of human prostate, breast and ovarian cancers (56). Thus, BECN1 was obviously thought to be a tumor suppressor gene. But continuing research has shown that loss of BECN1 cannot be disconnected from the loss of breast and ovarian tumor suppressor breast cancer 1 (BRCA1) because BECN1 is located in proximity to BRCA1 on human chromosome 17q21, revealing that BECN1 is not a tumor suppressor in most human cancers at all (57).
Recently, it could have even been shown that in mouse models for hereditary breast cancer p53-activation and reduced tumorigenesis develop through allelic loss of BECN1, which is exact the opposite result, if BECN1 is expected to act as a tumor suppressor (58).
In some context autophagy may work as a tumor promoter because cancer cells bank on autophagy as energy source. Due to the tumor-defining deregulation of proliferation, cancer cells bear increased metabolic and biosynthetic demands. It could have been shown that mitochondrial metabolic defects in tumors lead to an increased susceptibility to stress due to autophagy deficiency, especially in RAS-driven cancers. These tumor cells are dependent on mitochondria to sustain growth, hence autophagy is crucial, since defective autophagy causes the accumulation of abnormal mitochondria (59). There is also evidence that in K-ras^{G12D}-driven non-small cell lung cancer autophagy preserves metabolism and survival, because loss of autophagy suppresses tumorigenesis (60). The fact that loss of autophagy prevents development of pancreatic ductal adeno-carcinoma in mice with a pancreas-specific activated oncogenic KRAS allele, proves that the role of autophagy in tumor cells is strongly context-dependent (61).
The dual role model of autophagy in carcinogenesis seems to fit perfectly when it comes to hepatocellular carcinoma (HCC). Persuasive evidence for the tumor suppressor function of

autophagy is the finding that mice with deletion of Atg5 and Atg7 show development of multiple liver adenomas. There are also studies demonstrating that accumulation of p62 due to defective autophagy results in tumor development and progression (62), but if Atg7 and p62 are deleted simultaneously the size of liver adenomas is significantly reduced (63). Taken together, these findings strongly point out the crucial role for autophagy as a tumor suppressor. In contrast, it has been shown that mRNA of LC3 is considerably higher in HCC tissues than in non-tumor parenchymal cells (64). Further studies are essential to fully understand the complex function of autophagy concerning tumorigenesis in HCC (65).

1.2.2.3 Neurodegenerative Diseases

Neurodegenerative diseases are characterized by hereditary or sporadic loss of neural functions. Mutations and defective clearance mechanisms often lead to mitochondrial dysfunction and the accumulation of protein aggregates. Neuronal loss itself is linked to aggregation of misfolded proteins (66). It is therefore not far-fetched that the highly conserved process of autophagy is also involved in the pathogenesis of these diseases. Neurodegenerative diseases which combine features like intracellular protein aggregation and protein misfolding are called proteinopathies, like Alzheimer's disease, Parkinson's disease and Huntington's disease. The broad majority of proteinopathies include a genetic component and the dysfunction of the products of these genes is strongly linked to impaired autophagic function (67).

It has to be mentioned though that autophagy dysfunction is not the single mechanism underlying the pathogenesis of neurodegenerative diseases, it is rather an attendant aspect of the pathogenesis of these diseases. In mouse models it could have been shown that pharmacological stimulation of autophagy is able to lighten the symptoms related to neurodegeneration (68).

Huntington's disease is associated with aggregation of abnormal proteins with polyglutamine-rich extensions (69). Recently published results have elucidated that autophagy is involved in degradation of these proteins, since inhibition of autophagy leads to extensive accumulation (70).

Alzheimer's disease is defined by aberrant intracellular deposition of hyperphosphorylated forms of microtubule-associated protein tau, consequently causing the arrangement of neurofibrillary tangles and the accumulation of beta amyloid peptide in neural plaques (71). Studies have been published, which identified autophagic vacuoles as a major pool of intracellular beta amyloid (72).

1.3 Role of autophagy as therapeutic target

There are several obstacles which need to be considered when autophagy needs to be therapeutically targeted. First, the contribution of autophagy to disease pathogenesis is not always fully understood, particularly when it comes to translation from basic science to human diseases. More studies, addressing the exact role of autophagy in initiating, maintaining or resolving diseases are required.

Second, the amount of possible therapeutics with clinical efficacy is limited.

Third, compounds which are able to influence autophagy lack specificity. To adequately address points two and three, studies are required which identify (i) more easily drugable autophagy pathways or (ii) drugs with more comfortable safety profiles and specific actions.

From a teleological point of view, it appears most straight forward to target autophagy by interfering with its main regulative pathways. In many experimental models sirolimus, an immunosuppressive and anticancer drug with a broad range of use, has been used to enhance autophagy because of its inhibition of the mTOR-pathway (11). Until now there have been assessed multiple targets within the pathway including serine/threonine protein kinases ULK1 and ULK2, VPS34 as well as interactions within the Beclin 1 complex 180, ATG7 and ATG4B — the protease that processes pro-LC3 (light chain 3) (10).

Pharmacologic enhancement of autophagy with AMPK activators (e.g. metformin, salicylates, and others (73)) also seems promising to ameliorate special diseases like for instance infectious or neurodegenerative diseases.

Also, histone deacetylases (HDAC), which may act as controllers in the initiation of autophagy and also influence autophagic flux, are in the pharmacological pipeline. HDACs act as chromatin modifiers that modulate gene expression but also fulfill a broad range of functions outside the nucleus by deacetylating non-histone target proteins (74).

The first approved agent which was used to block autophagy was chloroquine, a drug once utilized against malaria and arthritis (75). Chloroquine inhibits lysosomal acidification and is therefore causative for inhibition of autophagy (76).

Targeting autophagy may also be a feasible therapeutic solution in the fight against cancer. But also, in this field of research the vast complexity of metabolic pathways in which autophagy is embedded makes pharmacological interventions exceedingly difficult.

As mentioned above, autophagy is involved in both tumor suppression as well as tumor cell survival (77). Consequently, one can state two strategies concerning anticancer treatment. The first is induction of autophagy with consequent enhancement of its tumor suppression attributes, while the other consists of inhibition of autophagy with subsequent induction of apoptosis (78),

increased chemosensitization and decreased resistance to other anticancer agents (10). Targeting cancer by modulating autophagy will, however, very likely depend on the type of cancer and the stage of disease, i.e. early pro-autophagic versus late anti-autophagic strategies.

Currently there are running clinical trials to examine the practicability of autophagy as a target in liver disease. One of the most advanced clinical phase 2 trials is the use of carbamazepine to treat alpha-1-antitrypsin deficiency with liver disease (ClinicalTrials.gov Identifier: NCT01379469). However, it has to be declared that a better understanding of the mechanisms underlying autophagy with regards to disease pathogenesis is needed to identify new targets for therapeutic approaches.

1.4 Nuclear Receptors

1.4.1 What are Nuclear Receptors?

Nuclear receptors (NRs) compromise a ligand sensitive class of proteins which work as transcription factors and they operate together with some different proteins in order to coordinate the expression of specific genes by directly interacting with the DNA (79).
Basically, if a NR is activated by an agonistic ligand it starts gene transcription of a distinct set of genes or, if a NRs is blocked by an antagonistic ligand, it blocks specific gene transcription (80).
The class of NRs compromises 48 members including amongst others receptors for endocrine ligands, bile acids, various lipids and also xenobiotics (81). They are inevitably involved in metabolic adaptation and also contribute to cell homeostasis, therefore they are also implicated in the pathogenesis of various diseases (82).
Accordingly, they are used as therapeutic targets in a broad range of diseases as well as in metabolic imbalances. Some of the most acknowledged examples of NRs concerning modification of diseases are the estrogen and the progesterone receptor and their aspect in breast cancer, as well as the constitutive androgen receptor and its role in hepatocellular cancer. (83-85).
With regards to modern human medicine, also the vitamin D receptor (VDR), the glucocorticoid receptor and the thyroid receptor have to be mentioned because they are addressed for a broad range of health issues to mediate the effects of vitamin D, glucocorticoids and thyroid hormones, respectively (86).

Other NRs as drug targets include the peroxisome proliferator-activated receptor α (PPAR-α) targeted by fibrates to treat hyperlipidaemia or the peroxisome-proliferator-activated receptor γ (PPAR-γ) targeted by glitazones to treat Diabetes mellitus (87, 88).

However, approximately half of the NRs are classified as orphan receptors because of the current lack of well characterized ligands. By far not all effects of NR-activation have been established yet (89). For some of the former orphan receptors new ligands have been established in recent past. These are now categorized as adopted orphan receptors. Figure 4 shows the bundle of nuclear receptors next to their typical ligands.

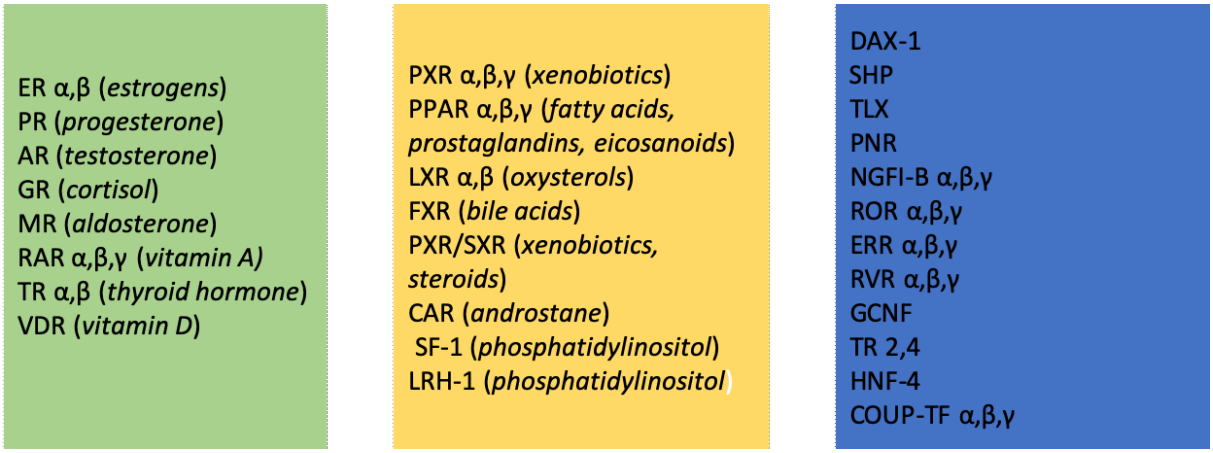

Figure 4. Nuclear receptors and their ligands in brackets (adapted from (90)).

This figure shows all nuclear receptors classified into Endocrine Receptors (green box), Adopted Orphan Receptors (yellow box) and Orphan Receptors (blue box).

1.4.2. Structure of Nuclear Receptors

Overall, NRs show a comparable structure. NRs contain a DNA-binding domain (DBD), which is highly conserved and also termed as C-Region, as well as a domain responsible for binding ligands (LBD), which is also called E-region (91). The DBD shows barely variation among different species and contains two small protein structural motifs called zinc fingers which are responsible for binding to specific sequences of DNA called hormone response elements (HRE) (92).

The ligand-binding domain is fairly conserved in sequence but highly conserved in structure between the various NRs. It contributes to the formation of the ligand pocket which binds the specific ligand. It also contains the activation function 2 domain (AF-2) which compromises a

ligand dependent action as well as a dimerization interface (93). The DBD and the LBD are connected through the D-region, also called hinge-region, which is very flexible and may affect intracellular trafficking and subcellular distribution. NR-activity is further modulated by the A/B-region, the N-terminal regulatory domain, which compromises the activation function 1 domain (AF-1) whose action is thought to be ligand-independent. The A/B-region highly differs in sequence between the numerous nuclear receptors. The A/B-, DNA-binding (C), and ligand binding (E) domains reveal to be structurally stable whereas the remaining regions may be conformationally flexible (94, 95). Figure **5** shows the basic structure of a typical NR.

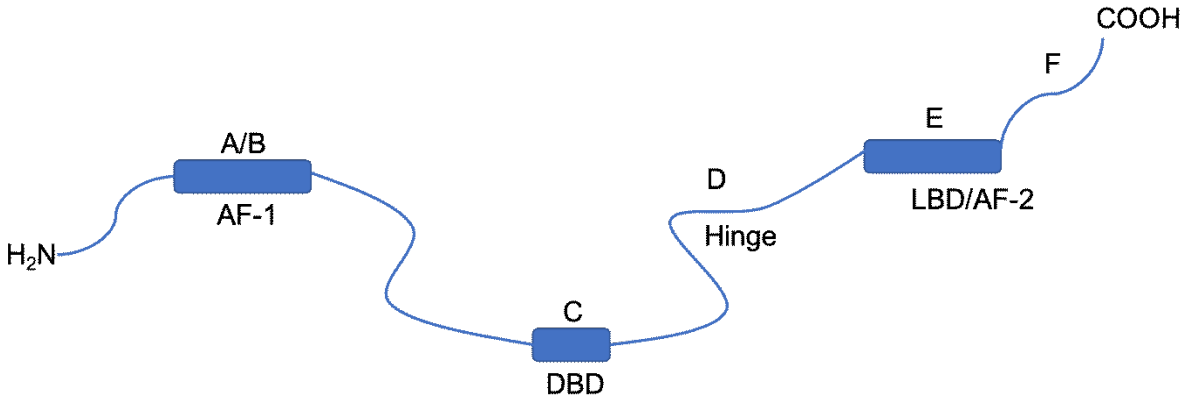

Figure 5. Arrangement of a typical nuclear receptor

Nuclear receptors contain a domain responsible for Ligand-binding (LBD, E-region) to bind their distinct ligand, the DNA binding domain (DBD, C-region) do bind to their hormone responsive element, the A/B-region, as well as the D-region (Hinge region) and the F-region (adapted from (86).

1.4.3 Classification of Nuclear Receptors

NRs can be distributed into two different types based on their binding aspect to DNA and dimerization-behavior with other NRs. Type I NRs are found in the cytosol while type II NRs persist in the nucleus (96).

Additionally, two further classes have been identified, type III which is a modification of type I as well as type IV, which binds DNA as monomer (93). After a ligand binds to a type I NR, homo-dimerization with an identical NR and translocation from the cytoplasm to the nucleus take place (93). Subsequently, the homo-dimer binds a specific sequence of DNA called the hormone response elements (HRE). The HRE is made up of two NR-specific half-sites on the DNA, and the second half-site consists of a sequence which is inverted from the first (inverted repeat) (93).

Type I NRs comprise for instance the glucocorticoid receptor, the estrogen receptor, the progesterone receptor and the androgen receptor (97).

Type II NRs reside in the nucleus and also bind their ligand in the nucleus, where they form a heterodimer with their typical NR partner, the retinoid X receptor (RXR) (82). Type II NRs include as examples the thyroid hormone receptor (TR) or the constitutive androgen receptor (CAR), as well as the pregnane X receptor (PXR) (98).

Type III NRs are quite comparable to type I NRs, the only difference is the fact that type III NRs bind to direct repeats instead of inverted repeat HREs. Type III NRs compromise among others the hepatocyte nuclear factor-4 (HNF-4) or the chicken ovalbumin upstream promoter transcription factor (COUP-TF). Type IV NRs bind to the DNA as monomers. This group includes as an example the steroidogenic-factor 1 (SF-1) (92).

Recent advances in NR research, however, have shown that various NRs may show a different binding behavior depending on the context and, for example, may bind as monomer, homo-dimer or heterodimer and may also use inverted, everted or direct repeats.

Coregulatory proteins play a key factor role during the process of NRs-mediated gene transcription. One can differentiate between co-activators and co-repressors, which either facilitate or inhibit the transcription of the associated target gene into mRNA. Co-activators, for instance, often bear histone acetyltransferase activity, which decreases the association of histones to DNA, and hence promotes gene transcription (99).

Figure 6 shows the mechanism of action of typical NRs.

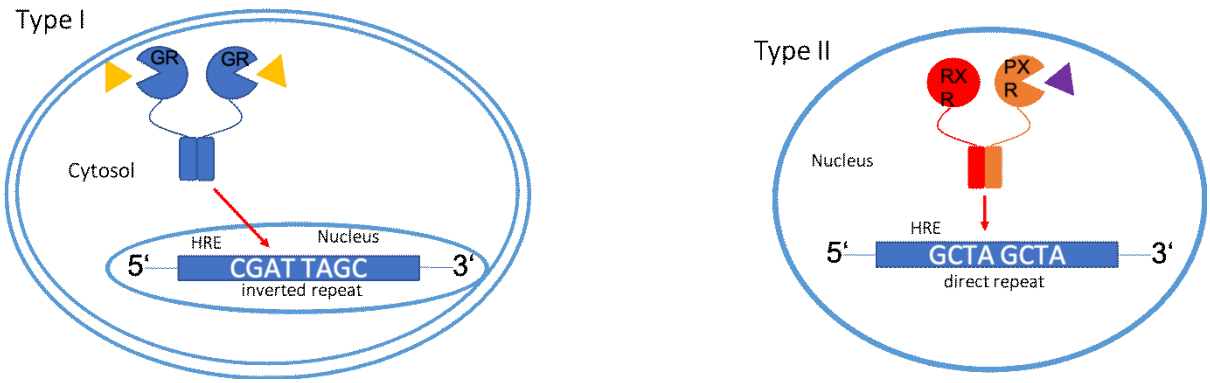

Figure 6. Typical mechanism of action of a nuclear receptor

This figure shows the typical mechanism of action of Type I and Type II NRs. As an example for type I the glucocorticoid receptor (GR) is shown. After ligand binding homodimerization takes places and the complex moves into the nucleus to bind its hormone responsive element (HRE). The HRE consists of specific DNA-sites, in this case they are inverted repeats. As an

example for type II NRs, the pregnane X receptor (PXR) is shown, which forms a heterodimer with the retinoid X receptor directly in the nucleus after ligand binding. For further information see text above.

1.4.4 Xenobiotic-Receptors

Because of the abundant number of genes that are regulated by NRs, they carry wide-ranging functions all over the organism and therefore they exert a profound impact on homeostasis. Figure 7 shows a functional classification of NRs.

A special task force of NRs is called "xenobiotic-receptors". The primary function of this subgroup is to anticipate the presence of external toxic substances, i.e. xenobiotics, in order to manage the upregulation of the expression of proteins which are engaged in the detoxification and clearance of these substances from the body (100). These NRs are mainly responsible for the upregulation of phase I, phase II and phase III detoxification reactions (101). The xenobiotic NRs are divided into PXR and CAR.

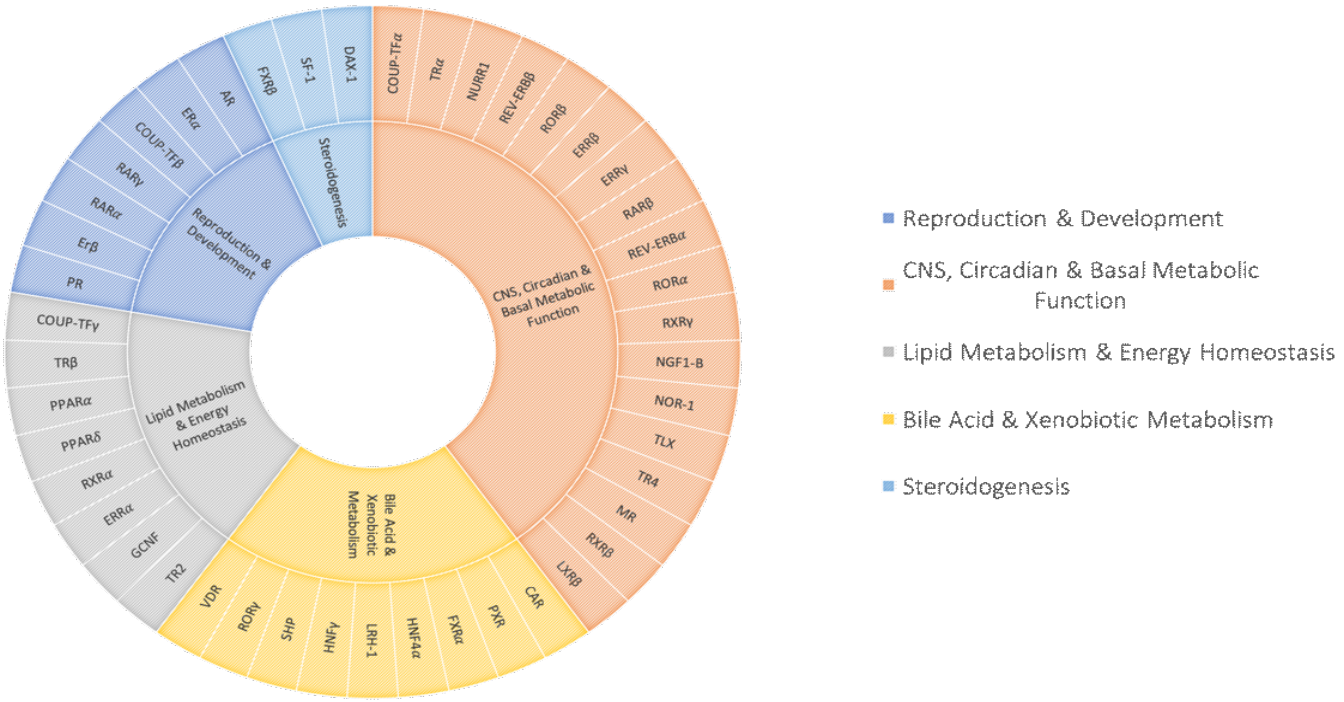

Figure 7. The Circle of Nuclear Receptor Physiology (adapted from Bookout et al. (102))

This figure shows the involvement of all NRs in different categories of the basic homeostasis. They are categorized into Reproduction & Development; CNS, Circadian & Basal Metabolic Function; Lipid Metabolism & Energy Homeostasis; Bile Acid & Xenobiotic Metabolism and Steroidogenesis.

1.4.4.1 PXR

The pregnane X receptor (PXR) senses steroids and xenobiotics. This protein is encoded by the nuclear receptor subfamily 1, group I, member 2 – gene (NR1I2) (103). Its main gene target is one of the cytochrome P450 genes, CYP3A4, which is robustly upregulated by PXR. PXR is a type II NR which means it binds to the HRE of the CYP3A4 promoter as a heterodimer with 9-cis retinoic acid receptor (RXR) (104). PXR is activated by a considerably large number of both exogenous and endogenous chemicals. Among these one can find several steroids like progesterone, antibiotics (rifampicin, rifaximin), as well as antimycotics, bile acids, and also hyperforin (a constituent of the herbal antidepressant St. John's Wort) (100). Also, meclizine shows to be a PXR-agonist (105).

On the other hand, there are very few known antagonists of PXR. Ketoconazole stands alone beside SPA70, which was identified in the recent past (106, 107).

Beside the primary target CYP3A4, which is a crucial phase I oxidative enzyme, PXR is additionally responsible for the upregulation of the expression of phase II conjugating enzymes such as the glutathione-S-transferase as well as phase III transport uptake and efflux proteins such as OATP2 and MDR1 (108-110). Less well-known functions include regulation of lipid metabolism. Studies have shown that activation of PXR through the rodent PXR agonist pregnenolone-16-α-carbonitrile (PCN) leads to a significant downregulation of the mitochondrial carnitine palmitoyltransferase I (CPT I) on mRNA-basis in wild-type but not in PXR -/- mice (111).

1.4.4.2 CAR

The constitutive androstane receptor (CAR) is encoded by the NR1I3 gene (112). Its main function is comparable to the function of PXR – it is responsible for binding endobiotic and xenobiotic substances to further regulate the clearance of these substances (113).

CAR represents a special form of NRs since despite the absence of ligands this receptor is constitutively active. If a ligand binds to CAR, the protein translocates from the cytosol to the nucleus, where it sticks to its specific HRE. The binding process takes place both as a monomer and together with RXR as heterodimer. CAR-mediated genes include among others members of the CYP2C and CYP2B subfamilies (114).

In the cytosol phosphorylated CAR forms complex together with the heat shock protein 90 (hsp90) and the cytoplasmic CAR retention protein, both examples for co-repressors (115).

For CAR activation dephosphorylation is required (90). In mice a direct specific activator of CAR is TCPOBOP (3,3′,5,5′-Tetrachloro-1,4-bis(pyridyloxy)benzene), while in human cells CITCO (6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde-O-(3,4-dichlorobenzyl)oxime) is a CAR specific agonist (116, 117). Phenobarbital, a sedative used in the past, acts on both human and murine CAR as agonist. A natural ligand for CAR in human may be unconjugated bilirubin (118).

1.4.5 Role of Nuclear-Receptors in autophagy

There is only limited data about the role NRs play in regard to autophagy regulation and the long-term transcriptional regulation of autophagy has not been accurately elucidated, yet. It has been shown that the peroxisome proliferator activated receptor-α (PPAR-α) and farnesoid X receptor (FXR) both take part in the regulation of autophagic flux in mice (20). FXR as well as PPAR-α is activated in the fed and fasted liver, respectively. In fed state conditions, autophagy normally is suppressed. This suppression can be reversed by pharmacological activation of PPAR-α, inducing autophagic lipid degradation, i.e. lipophagy. This hypothesis is confirmed by the fact that the effect is lost in PPAR-α-knockout mice, which are partially defective in fasting-mediated induction of autophagy (20).

On the other hand, induction of autophagy shows to be significantly suppressed due to pharmacological targeting of FXR, and this response is also found to be absent in FXR knockout mice, respectively (20).

So far there has no data been published, that links xenobiotic receptors and autophagy.

1.4.6 Hypothetical Role of Xenobiotic receptors for autophagy

The currently available data concerning the physiological role of autophagy clearly implies that the primary function of autophagy is to promote health and longevity of the cell (3). The activation of autophagy due to starvation, nutrient depletion and oxidative stress has been elucidated precisely but is that already the end of the road?

Currently, there is no data indicating that autophagy is linked to xenobiotic responses. However, teleologically it would make sense to couple xenobiotic defence mechanisms with mechanisms to maintain cell homeostasis and energy balance, i.e. to link xenobiotic metabolism and autophagy.

The CAR- and PXR- mediated transcriptional regulation of phase-I, phase-II and phase III enzymes is thoroughly described but regulation of autophagy-related genes has not been studied or reported yet.

This potential connection between xenobiotic metabolism and autophagy is intriguing because it would also immediately imply an even more relevant question. Could this pathway be utilized pharmacologically to target autophagy?

1.5 Pre-existing results

Data from studies which have been previously done in our lab strongly indicate that hepatic autophagy is induced through treatment with the potent PXR-agonist rifampicin in human patients. To verify this, a Western blot of liver tissue from 4 control patients and 8 patients, who received rifampicin during a previous study was performed (study protocol adapted from (119)). We observed that the autophagic markers LC3-I and LC3-II showed to be significantly increased in the liver samples of patients that were treated with rifampicin compared to the control samples (Figure 8).
This experiment was followed by an experiment with PXR knockout cells to determine the dependence of autophagic induction through rifampicin on PXR (Figure 9).

Those results were interpreted as induction of autophagic flux due to treatment with rifampicin. Since rifampicin is a strong agonist of PXR, the findings create the suspicion, that PXR is involved in the induction of autophagy.

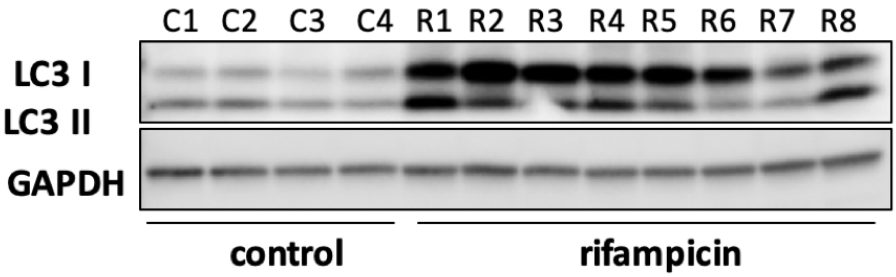

Figure 8. Western Blot of human liver specimens after rifampicin treatment

Significant increase of both LC3-I and LC3-II is shown in the intervention samples (R1-R8), while no change is observed in the control samples (C1-C4) compared to the loading control GAPDH. As control placebo treatment was used. Figure provided by Katrin Panzitt, unpublished results.

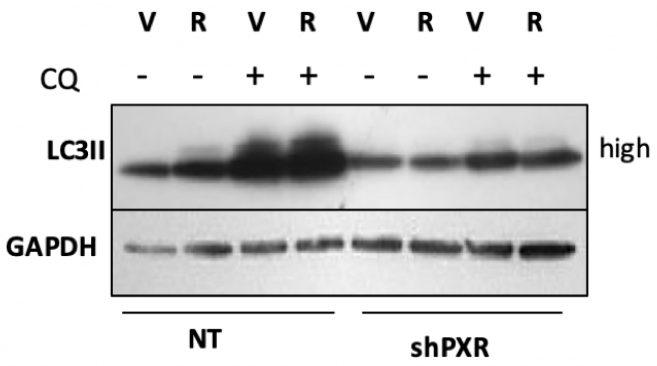

Figure 9. Western Blot of shPXR cells and normal type (NT) cells treated with rifampicin and DMSO

The NT cells showed significant upregulation of LC3II after treatment with rifampicin, while this upregulation was not detectable in the knockout-cells. Figure provided by Katrin Panzitt, unpublished results.

2. Hypothesis and specific aims

Preexisting results in this Lab show that patients treated with the potent PXR agonist rifampicin have increased markers of autophagy in liver biopsy specimen. These effects of rifampicin can also be reproduced *in vitro* in HepG2 cells and appear to depend on PXR, since PXR-silenced HepG2 cells lack the autophagic induction. A major drawback of rifampicin, however, is its well-known hepatotoxicity.

The aim of these studies therefore was to test if (i) other, potentially less hepatotoxic, PXR ligands also induce autophagy and if (ii) these effects are also conserved in other species, i.e. in mice in our experiments.

We hypothesize, that the PXR ligand hyperforin will also induce autophagy in a similar fashion to rifampicin and that the autophagy inducing effects of PXR activation are conserved from man to mice, underscoring autophagy as a more general mechanism of xenobiotic receptor regulation.

3. Material and methods

3.1 Mice

Mouse livers from previous studies from Wagner et. al. (120) were used to investigate the effects of PXR and CAR ligand activation. Mice were six to eight-week-old and originally derived from the Institute of Laboratory Animal Research, Medical University Vienna, Himberg, Austria. The materials, Pregenolone-16α-carbonitrile (PCN) and 3,3′,5,5′-Tetrachloro-1,4-bis(pyridyloxy)benzene (TCPOBOP), were purchased from Sigma. PCN is a strong rodent PXR ligand and TCPOBOP is a robust ligand for rodent CAR. Mice were treated in the morning at the same time points over a period of 3 days with intraperitoneal injections of TCPOBOP with a concentration of 3mg/kg body weight, dissolved in 100µl cornoil and with PCN with a concentration of 75mg/kg body weight, dissolved in 100µl dimethylsulfoxide (DMSO). As control, 100µl of DMSO for comparison of the PCN group and 100µl of cornoil for comparison of the TCPOBOP group were intraperitoneally injected in the same manner. 24h after the last application, the livers were excised and aliquots were frozen in liquid nitrogen for further protein preparation (120).

3.2 Cells and cell culture conditions

The human liver cancer cell line HepG2 was utilized for *invitro* studies. We received the HepG2 cells from ZMF cell culture core facility. For experiments cells were counted with trypan blue (Sigma) and the TC20 Automated Cell Counter (BioRad). After counting, 3x 10^5 cells were seeded into 6-well dishes. The cells were cultivated in dishes with DMEM media (GIBCO) enriched with 10% fetal bovine serum and incubated with 5% CO2. 24h post seeding the respective treatment was started. Cells were then used for RNA or protein-isolation.

3.2.1 Cell-treatment

After removal of the media and washing in phosphate buffered saline (PBS), cells were incubated for 24h with the vehicle DMSO and the PXR ligands (rifampicin, hyperforin, meclizine or ketoconazole). We used an end-concentration of 30 µM for ketokonazole dissolved in DMSO. For meclizine we also used 30 µM dissolved in DMSO. Ketokonazole and meclizine had to be warmed to dissolve. For rifampicin we used an end-concentration of 25 µM. For hyperforin we used a concentration of 1 µM dissolved in DMSO. Treatment was continued for 24 hours. Each experiment was performed in duplicates or triplicates.

3.4 RNA-isolation

RNA was isolated from HepG2 cells and mouse liver tissue by use of 500µl (HepG2 cells) and 1 mL (mouse liver tissue) TRIzol Reagent (Thermo Fisher). The tissue was homogenized with a Magnalyzer (Roche). Cells were washed and then TRIzol was directly added to the culture dish to lyse the cells. Afterwards the lysate was pipetted up and down several times for further homogenization. Subsequently 1/5 volume of chloroform (e.g. 100µl /200µl respectively) for lysis was added and incubated for 2-3 minutes. After this the samples were centrifuged for 15 minutes at 12000g at 4°C. The mixture separated into an upper colourless aqueous phase, an interphase and a lower red phenol-chloroform phase. The RNA-containing aqueous phase was transferred to a new tube. It was then diversed with an equal amount of isopropanol in order to precipitate the RNA. We cooled down the probes to 4°C for 15 min and then they were centrifuged for 10 minutes at 12000g. The next step was to discard the supernatant and to resuspend the remaining pellet in up to 500µl of 70% Ethanol. The sample was afterwards vortexed and centrifuged at 7500g at 4°C and then air dried before it was resuspended in 20-50µl of aqua dest., in order to solubilize the RNA. Thereafter, the sample was incubated at 65°C for 15 minutes and was put on ice for at least 30 min. As last step the RNA concentration was

spectrometically quantified using Thermo Scientific's NanoDrop2000c. Quality control was ensured by 260/280 ratio and a ratio of approx. 2.0 was considered as good quality material.

3.5 cDNA-synthesis

We used the Superscript II Reverse Transcriptase Kit (Invitrogen) to reverse transcribe 1µg of RNA into cDNA. The mastermix was prepared, which contained 4µl 5x Buffer; 2µl 0,1M DTT; 0,3µl dNTP; 0,5l dNTP; 0,75µl Superscript; 0,75µl RNAse inhibitor and 2,7µl Aqua dest. as well as 0,4 µl random hexamers. After preparation we added the mastermix to each tube. For reverse transcription we used the following protocol: 90 min of 42°C and 15 min of 70°C. As a last step, we cooled it down to 4°C to store the cDNA at 4°C until further qPCR.

3.6 Real time qPCR

cDNA was diluted with water 1:5. Of the 1:5 diluted cDNA 2,5µl were used as a template per qPCR reaction. 1.25µl of forward and reverse primer respectively, were put together with 5µl Sybr Green master mix (Invitrogen), which consisted of the polymerase and the nucleotides. For qPCR a Roche Lightcycler was used under the following conditions: 95°C for 10 minutes for initial denaturing; 40 cycles of 95°C for 10 seconds for further denaturing, 60°C for 1 minute for primer annealing and amplification. 96-Well PCR-plates were used (Roche). For calculation we used the ΔΔCT-method in Excel. Pipetting was performed in triplicates or duplicates and duplicates with Cp cycle deviation >0,5 were excluded. The housekeeping gene for normalization was 36b4. In order to design primers, I worked with Primer 3 software (http://bioinfo.ut.ee/primer3-0.4.0). We agreed upon primer specifications with an annealing temperature of around 60°C, the primer size between 20 and 25 nucleotides, the PCR product length was set between 75 and 130 nucleotides and the primer GC% optimum at 50%.

Human primers utilized for real time qPCR

Name	Forward	Reverse
CYP3A4	gatgaagaatggaagagattacgat	cctcagatttctcaccaacaca
UGT1A1	tctggctgtttagaagtgacttt	cattaatgtaggcttcaaattcct
TFEB	acctgtccgagacctatggg	cgtccagacgcataatgttgtc

Mouse primers utilized for real time qPCR

Name	Forward	Reverse
atg5	gacagatttgaccagttttgggc	gggtttccagcattggctctatc
atg10	aacagccaaggaatgttctgat	catgtggtgtcaaggtctcatt
lc3a	ttggtcaagatcatccggc	gctcaccatgctgtgctgg
lc3b	cccaccaagatcccagtgat	ccaggaacttggtcttgtcca
uvrag	gtctacctggatgggctgaa	tcttctgtgcgtttggatga
wipi	ccgctatcaccttcaactcc	cctcgggaacagagaacact
atg9b	atgccctcttcacatccatc	agaagcaacatcagccctgt
atg16I	ttgacccagaaactacaagcag	gactatcattccacgcacca
atg16II	ctgtgtggatgtggtgaagg	cccaaccgagtgacctctt
p62	tcggaagtcagcaaacctg	tgtgtccagtcatcgtctcc

3.7 Protein isolation

For extraction of protein from cells Cell Extraction Buffer (Invitrogen) was used. After removal of the medium, cells were washed with PBS. Subsequently cell extraction buffer was added. Then the cells were scraped with a plastic cell scraper and afterwards collected in microfuge tubes. The tubes were hereinafter centrifuged at 13.000 rpm for 20 min at 4°C. The supernatant was collected in a fresh tube and the pellet was removed. For protein quantification BCA Protein Assay Kit (Thermo Scientific) was applied. Samples were diluted 1:25 with homogenization buffer. The homogenization buffer contained 42,8g saccharose in 400ml Aqua dest. with 5mL HEPES-KOH and 1 mL 0,5M EDTA diluted in 500mL of Aqua dest. Reagent A and Reagent B of the protein quantification kit were diluted 50:1. Then, 25µl of the diluted samples and 200µl Reagent A+B were pipetted into a 96-well microplate (Thermo Scientific) and incubated at 37°C for 30-45min in the SPECTROstar (Omega). The absorbance was then measured at 562nm. For the standard curve Albumine Standard (BSA) from the kit was used in concentrations of 1000µg/ml, 500µg/ml, 250µg/ml and 125µg/ml. For the calculation of the final protein concentration the data was imported into Excel in order to calculate how the samples had to be diluted for loading and blotting. The samples were diluted with different amounts of sample buffer to ensure the same amount of protein in every sample. Dilution was calculated so that the range of the amount of loaded protein was between 7µg and 50µg, depending on the experiment.

3.8 Western blot

The procedure of Western blotting involves three main steps. It is composed of a gel electrophoresis in order to separate denaturated proteins by length of the polypeptide. Afterwards an electrophoretic transfer onto a nitrocellulose or PVDF membrane is done, which is finally followed by an immunostaining procedure to be able to visualize a certain protein on the blot membrane (121).

Concerning these studies sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed. Polyacrylamide gels with a concentration of 12,5% were made according to established protocols (122). The samples were loaded with amounts of protein between 7µg and 50µg. There was a bench-mark ladder (Invitrogen) used to determine the molecular weight of the loaded proteins. After the electrophoresis a transfer onto PVDF membranes was performed. The primary method for this was a semi-dry electroblot-transfer. After this, equal protein loading was visualized through staining of the membranes with Ponceau S. Thereafter blocking of non-specific binding was achieved by placing the membranes in 5% non-fat dry milk in tris-buffered-saline containing a 0,1% concentration of detergent Tween 20 (TBST) for 30-60 min. Next, the membranes were incubated overnight with the primary antibody with a concentration between 1:1000 and 1:5000 diluted in non-fat dry milk in TBST. The membranes were incubated at temperatures of about 4 degrees. On the next day the membranes underwent a washing procedure consisting of 3 rounds of 5 minutes washing with TBST. Then the membranes were incubated for 1h with horseradish peroxidase-conjugated anti-mouse/anti-rabbit IgG-antibodies to detect the immunocomplexes. As last step colorimetric and chemiluminescent detection was performed on a ChemiDoc Touch Imaging System (BioRad). To ensure equal loading the housekeeping **β**-Actin and GAPDH were used in addition to Ponceau S.

3.9 Assays to detect autophagy

There is no single test or "gold standard" experiment for the detection of autophagic activity. Autophagy guidelines suggest performing several experiments to investigate autophagy (123). For monitoring autophagic activity we used the following methods: Immunoblotting to visualize LC3 turnover, degradation of the autophagic substrate p62 as well as a fluorescent-tagged LC3 flux assays (124).

Since LC3 is the only established protein that is associated with all different types of autophagic membranes, which compromise the phagophore (isolation membrane), the autophagosome and

the autolysosome, the detected amount of LC3-II shows a direct correlation with the number of autophagosomes and is therefore a good marker for induction of autophagy. However, LC3-II can also be increased in the case of blocked autophagy, when autophagosomes are not being degraded properly. Therefore, simple LC3-II measurements have to be combined with additional tests.

Levels of p62 can be used for monitoring autophagic flux. The main function of p62 lies in linking ubiquitinated proteins to the autophagic machinery in order to facilitate their clearance in the lysosome. The amount of detected p62 is generally considered to correspond inversely with autophagic activity, as p62 itself is largely removed by autophagy. So p62 accumulation is used as a marker for suppression of autophagy (124).

Additionally, the subcellular distribution of LC3 to the autophagosomal membrane can be observed through indirect immunofluorescence or by examining the signal of fluorescent protein tagged to LC3 (e.g., green fluorescent protein (GFP)-LC3). An advanced method is dual labeling of LC3 with GFP and red fluorescent protein (RFP), which in addition to subcellular localization also provides information on fusion with lysosomes (see results section below for detailed description of this assay) (124).

3.10 Statistics T-Test

To determine if two sets of data were significantly different, we used t-Test. We repeated the experiments at least twice using a minimum of two replicates. I used Excel to perform statistical analysis. Data were analyzed as mean and standard deviation of mean. We considered a p-value of <0.05 as statistically significant.

4. Results and figures

4.1 Aim I: To test if other PXR-ligands also induce autophagy

In our preexisting results we have shown that the prototypical human PXR ligand rifampicin induces autophagy. We therefore were interested if this effect is specific to rifampicin only or if PXR ligands tend to induce this response in general. In addition, a major drawback of rifampicin – in terms of clinical utility – is its potential hepatotoxicity. Therefore, it would also be interesting to learn, if other less toxic PXR ligands can recapitulate the effects of rifampicin on autophagy.

4.1.1 Testing different concentrations of other PXR-ligands

Although rifampicin has now been used over the last five decades as antibiotic, a considerable challenge concerning rifampicin is its rare but well-established liver toxicity and its wide range of side effects. Rifampicin induced PXR-mediated autophagy would therefore eventually not be applicable for therapeutic long-term use, particularly not for long-term treatment such as in a1-AT deficiency. We therefore made a literature-based research to find potentially less hepatotoxic PXR-agonists, which could hypothetically be used for therapeutic issues. The research revealed the PXR agonists hyperforin and meclizine as well as the PXR antagonist ketoconazole to be potential candidates, which are approved for clinical use.

Since PXR is a transcription factor, our initial step was to test out the different ligands with different concentrations and use established PXR-dependent genes as readout in qPCR assays. As readout genes we examined the PXR-related genes CYP3A4 and UGT1A1, as well as the potential new PXR target gene TFEB, to determine if the master regulator of lysosomal biogenesis is involved, too.

As positive control for transcriptional PXR effects we used rifampicin. Figure 10 summarizes the effects of the different compounds and concentrations. For hyperforin the lowest concentration of 0,5µM worked best, as we examined a 3-fold induction of CYP3A4, and a slight up-regulation of UGT1A1. For meclizine as well the best results were accomplished with the lowest tested concentration of 10µM, as shown by a 2-fold up regulation of CYP3A4. Higher concentrations resulted in a decrease of CYP3A4, which we attributed to potential toxic effects since cells started to die in the dishes (measured by optical judgment of floating cells).

Ketokonazole did not show the expected antagonistic transcriptional effects at lower concentrations and appeared to be toxic at higher concentrations. As expected, the positive control rifampicin showed a robust increase for CYP3A4 (3-fold and 4-fold) with the smaller and the medium concentrations, respectively. For UGT1A1, rifampicin-treatment lead to a robust 3-fold upregulation with the medium concentration.

We concluded that all three compounds had transcriptional effects at the lowest concentrations on the prototypical PXR target gene CYP3A4. For further studies we focused on rifampicin as positive control and hyperforin as second PXR ligand, since hyperforin is widely used without significant side effects as antidepressant extracted from St. John's wort.

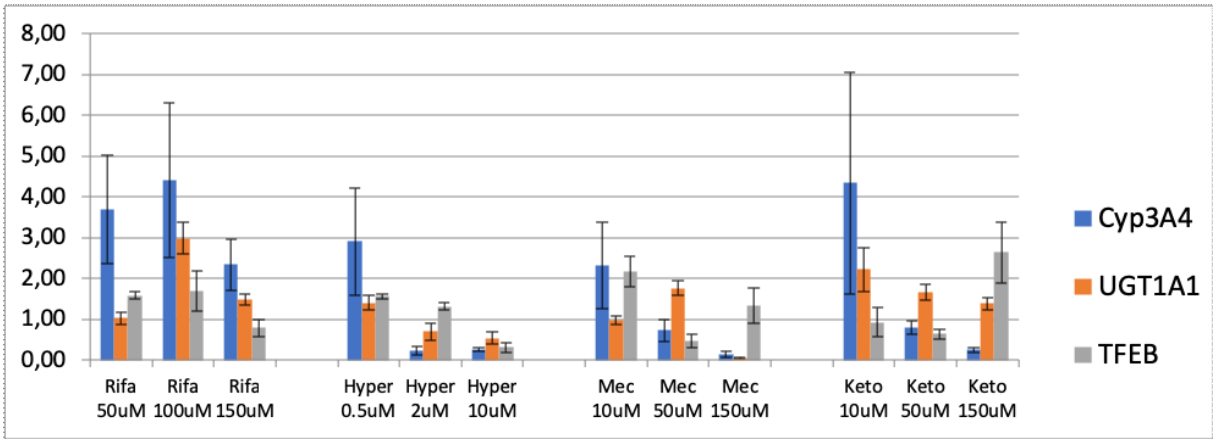

Figure 10. Different concentrations of PXR agonists on mRNA-basis

The smaller concentrations showed the most promising results, with a 2-4-fold increase of CYP3A4. For UGT1A1 a 3-fold increase could be detected.

4.1.2 Induction of autophagy related markers by PXR-agonists in human HepG2 cells

The next step was to determine if treatment with the optimal tested concentrations leads to an induction of autophagy related markers on protein basis. We therefore performed a Western Blot to detect increases in LC3I and LC3II, after treatment with hyperforin. To ensure equal loading we used GAPDH as loading control. As positive control rifampicin was used again. The substances were diluted in DMSO, which was therefore used as vehicle control. We observed a slight upregulation of LC3-II (Figure 11) in cells treated with hyperforin, which directly correlates with the number of autophagosomes, as mentioned above (see 3.9). As positive control we detected a robust upregulation of LC3-II as a result of treatment with rifampicin.

The conclusion from this experiment is that the activation of PXR with the agonist hyperforin leads to induction of autophagosome formation. However, to determine if autophagic flux is really induced or if autophagy is potentially blocked (also resulting in increased in LC3-II) additional autophagic flux experiments need to be done.

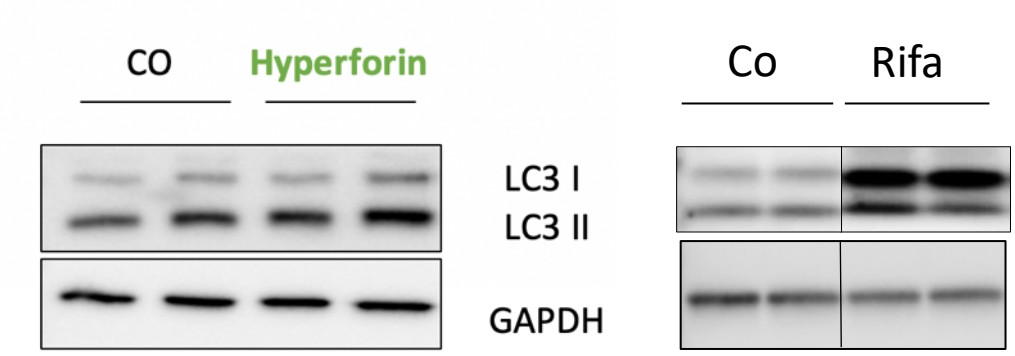

Figure 11. Western Blot of autophagy related markers after treatment with PXR agonists hyperforin and rifampicin

Treatment with hyperforin (1 µM) and rifampicin (25 µM) increases the autophagosome related marker LC3-II in comparison with the vehicle control DMSO. (Right blot: all samples ran on the same gel; different unrelated samples in between the control and rifampicin were cut out.)

4.1.3 Induction of autophagic flux by hyperforin treatment as revealed by immunofluorescence microscopy

The next step was to confirm the results of our Western blots with an independent immunofluorescent autophagic flux essay, because simple upregulation of LC3-II does not necessarily mean induction of autophagy. LC3 induction could also potentially occur if autophagy is blocked further downstream of the initiation and elongation steps. We therefore performed an independent autophagic flux experiment by transfecting a tandem fluorescent-tagged LC3 plasmid (pGFP-RFP-LC3) into HepG2 cells. The main principle behind this method is that upon induction of autophagy, LC3-II becomes inserted into the autophagosomal membrane which may then fuse with the lysosome. The advantage of this method is that we were able to determine whether autophagosome-lysosome-fusion occurs in the cell or not, as the GFP fluorescent signal extinguishes due to the low-pH-mediated degradation inside the lysosomes, while RFP preserves its fluorescence activity in acidic compartments. Hence,

yellow puncta show colocalization of GFP and RFP indicating that the autophagosome has not yet fused with a lysosome. Red puncta on the other hand, i.e., an RFP signal without GFP, stand for delivery of LC3 into lysosomes, which indicates the formation of autolysosomes (125). Figure 12 is a representative picture. As negative control for autophagy induction we used medium-fed cells, which inhibits autophagy.

In the fed state we observed uncountable green and yellow puncta, which indicate that there is no induction of autophagy. The positive control was rifampicin in the fed state. We examined loads of red puncta in the rifampicin treated cells. Also, the hyperforin treated fed-state cells revealed a significant number of red puncta, i.e. autophagosome-lysosome fusion. We concluded, that treatment with hyperforin leads to induction of autophagic flux.

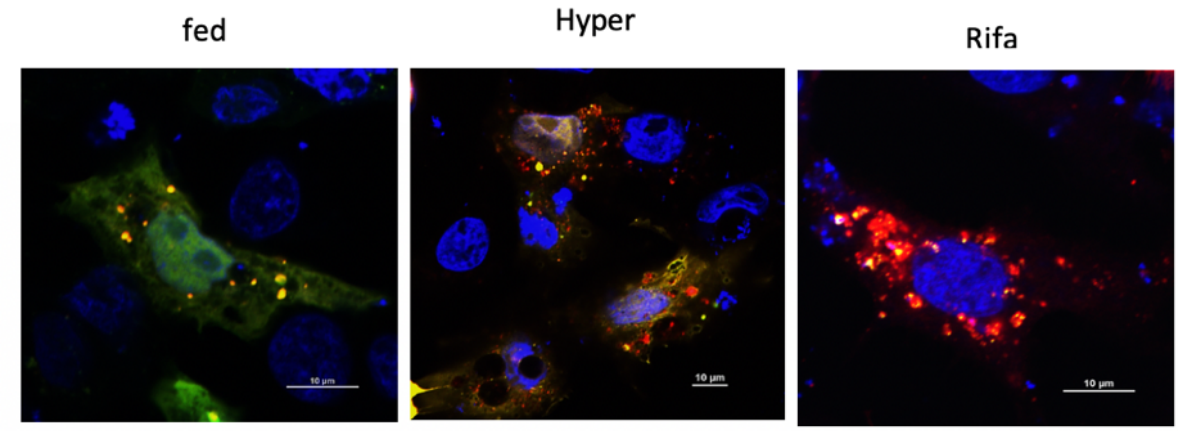

Figure 12. Induction of autophagic flux through PXR-agonist treatment in GFP-RFP-tandem-immunofluorescence

The green and yellow puncta in the fed state indicate that autophagy is inhibited, whereas red spots stand for autophagosome-lysosome fusion through treatment with hyperforin (Hyper) and rifampicin (Rifa) (for further information, see text above).

4.1.4 No changes in autophagy related markers after treatment of HepG2 cells with CAR agonist CITCO

Next, we wanted to examine if the second xenobiotic receptor, CAR, is also able to induce autophagy (similar to PXR) when CAR is targeted with the potent human in vitro CAR agonist

CITCO. Therefore, we performed another Western blot. As vehicle control DMSO was used. This time, however, we did not examine increased LC3-II levels and concluded that CAR does not affect autophagy at all (Figure 13).

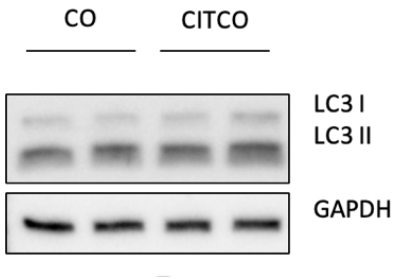

Figure 13. Western blot of LC3-I and LC3-II after treatment of HepG2 cells with CAR agonist CITCO

This blot shows no significant changes in LC3-II in comparison with the negative control (DMSO) after treatment with the potent human CAR agonist CITCO.

4.2 Aim II: To test if autophagy induction by PXR activation is evolutionarily conserved in mice

Although the ultimate goal in translational medicine should always be to show the relevance of experimental findings in human physiology, pathophysiology or therapy, it can sometimes be useful to underpin the importance of a finding by showing its evolutionary conservation across several species. In addition, finding an appropriate model, where disease relevant aspects can be studied in more detail and depth, which would otherwise not be possible in human studies or in *in vitro* models, can be helpful to understand functional consequences of a finding. In our specific case, we wanted to test if the human *in vivo* and *in vitro* findings are conserved in mice. This would imply that the finding is of broader significance since it has been evolutionarily conserved at least from mice to human. In addition, if PXR activation induces autophagy in mice too, rodent models could be used to test this mechanism in the treatment of various forms of neurodegenerative diseases and certain liver diseases where induction of autophagy might be useful. Even more, maybe the experiments could be expanded to test the consequence of PXR activation in tumorigenesis.

4.2.1. PXR activation induces markers of autophagy in mice

The xenobiotic receptors PXR and CAR represent an evolutionary highly conserved bundle of sensors for foreign and toxic substances in mammals. Human and mouse xenobiotic receptors are closely related. They exhibit amino acid sequence identity of about 95% (126). Our plan was to investigate if the process which we elucidated previously, i.e. the upregulation of autophagy related markers by xenobiotic receptor agonists, is also conserved in mice. We used liver specimens from mice which were treated with intraperitoneal injections of the rodent PXR ligand PCN and the CAR ligand TCPOBOP from previous studies (120). We performed a Western blot and we analyzed p62 and LC3 as read-out to determine if there was induction of autophagosome formation, in order to imitate our human experiments as accurately as possible. The control treatment was peritoneal injection with DMSO as control for PCN and corn-oil for TCPOBOP. We examined a significant increase of LC3 and a decrease of p62 due to treatment with the PXR agonist PCN in comparison with the control (C) (Figure 14).

To examine the involvement of the mTOR-pathway we used phosphorylated p70S6K as read-out. The phosphorylation of this enzyme is used as certification of activation of mTOR and thus correlated with inhibition of autophagy (127). We found unchanged levels of P-p70S6K, suggesting that mTOR is not changed. This indicates, that the PXR-dependent effects on autophagy do not necessarily involve the classical mTOR pathway.

The conclusion of this experiment was that PXR agonist treatment leads to an induction of autophagy and breakdown of p62 in mice too, i.e. the process may be evolutionarily conserved.

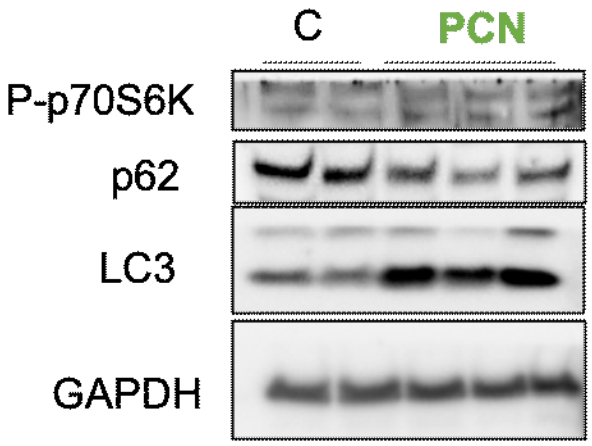

Figure 14. Induction of autophagy related markers through PCN treatment

Significant up-regulation of LC3 was examined, whereas p62 went down. The mTOR readout P-p70S6K did not show any changes. As loading control GAPDH was used. As control (C) DMSO was used.

4.2.2 CAR activation does not induce autophagy related markers in mice

We performed the identical experiment, but with material from TCPOBOP injected mice, to explore the effects of CAR activation in the regulation of autophagy in mice. Similar to the human *in vitro* experiments, the activation of CAR through TCPOBOP in mice did not lead to significant increase of LC3. Even more, we observed an accumulation of p62 (Figure 15). P-p70S6K was unchanged. This result indicates, that CAR activation is not inducing autophagy. The accumulation of p62 may rather point towards inhibition of autophagy.

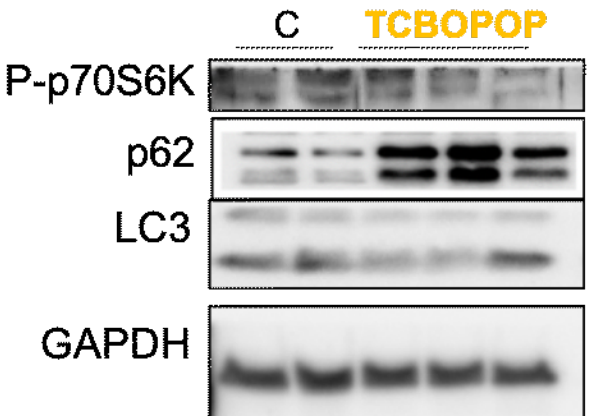

Figure 15. No induction of autophagy related markers through CAR activation

LC3 did not show to be increased, whereas p62 went up significantly. As loading control GAPDH was used. The mTOR readout P-p70S6K did not show significant changes either. As control treatment corn-oil was used.

4.2.3 No significant induction of autophagy related genes on mRNA basis through PXR agonist treatment in mice

Since NRs represent transcriptional regulators, the next logical step was to determine if PXR-activation would lead to any changes in mRNA expression of autophagy related genes. We used pieces of liver tissue from PCN treated mice (mentioned above) and isolated mRNA to examine a set of genes, that are involved in autophagy using qPCR. The genes investigated were atg5, lc3a, atg10, uvrag, wipi, atg9b, atg16I, atg16II and p62. Surprisingly, we were not able to detect a significant up-regulation of these genes (Figure 16) but rather a significant downregulation of some genes. The conclusion of this experiment was that intraperitoneal injection of PCN in mice does not result in up-regulation of autophagy-related genes at least in the conditions we used. However, we cannot dismiss the hypothesis that the effects on protein levels are still the consequence of transcriptional effects of PXR, since issues of timing and transcriptional counter-regulation may play an improtant role (see discussion below).

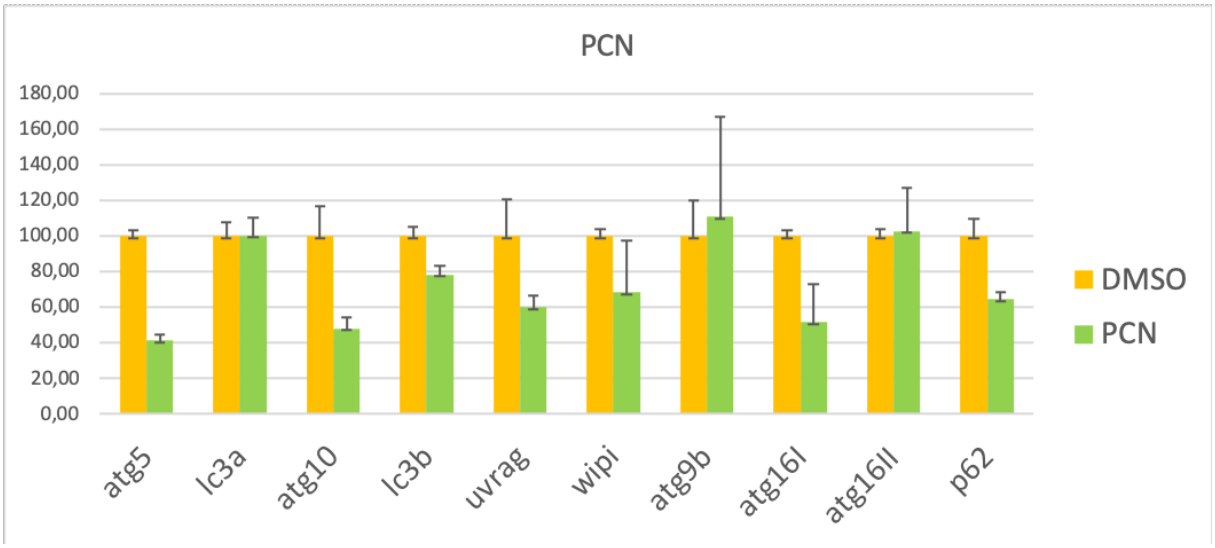

Figure 16. No significant up-regulation of autophagy related genes on mRNA basis

Treatment with PXR-agonist PCN did not lead to induction of autophagy related genes. Atg5, atg10, uvrag and atg16I rather showed a decrease.

5. Discussion

5.1 Overview, Implication and further studies

Autophagy is a process to maintain cell homeostasis and is involved in the pathophysiology of many diseases (11). Its regulation is complex and targeting autophagy pharmacologically remains challenging. We propose and provide evidence that autophagy is also integrated in xenobiotic response mechanisms, since the main human xenobiotic receptor PXR strongly induces autophagy.

The main finding of this work is that this mechanism can be utilized by different available and tolerable PXR agonists and is also conserved in mice. The findings open up new avenues to directly test the therapeutic potential of PXR activated autophagy in clinical trials and to perform additional in-depth physiological, pathophysiological and treatment studies in mouse models.

Studies, previously done in our lab, indicate that the treatment of human volunteers with the potent antimycobacterial agent rifampicin is followed by significant increase of the autophagy markers LC3-I and LC3-II in liver specimens. Up-regulation of LC3-II is an indicator of alteration in autophagic flux (124).

As rifampicin is known as a dominant agonist of the xenobiotic receptor PXR, our findings lead us to the assumption that xenobiotic receptors conceivably play a new and yet undiscovered key regulator role in the induction of autophagy.

The aim of our studies herein was to confirm this hypothesis and to extend preliminary results of one PXR agonist to other better tolerable and thus clinically more relevant agonists for potential long-term applications. Because of the well-established, though rare, hepatotoxicity of rifampicin, we used the less toxic ligand hyperforin, main ingredient of the antidepressant St. John's Wort, to elucidate the effects of xenobiotic receptors on autophagy, as to eventually apply our findings in a clinical and therapeutical context. Hyperforin would be an ideal drug candidate because of its primary antidepressive effect. In a 2015 meta-analysis it was shown that in treating depression it has superior efficacy compared to placebo while causing fewer adverse effects than other antidepressants (128). Literature studies revealed that hyperforin has an encouraging safety profile (129). These would be ideal prerequisites for the long-term

treatment of chronic diseases such as alpha 1-antitrypsin deficiency, as well as neurodegenerative diseases since PXR is also expressed in the brain (130).

We found that hyperforin significantly up-regulated LC3-II in our *in vitro* studies with human HepG2 cells. Since the accumulation of LC3-II could either be caused by an increase of autophagosome formation or due to blockage in the downstream steps of autophagy (124), we additionally performed experiments to investigate autophagic flux.

Since autophagy is a dynamic process that involves autophagosome biogenesis, maturation, fusion with lysosomes and following breakdown of substrates inside the lysosomes (11), a single essay which proves evidence of accumulation of LC3-II would not be able to show stringently that autophagy has been induced (124).

Single analyses are not able to show differences between autophagy upregulation and degradation inhibition. So at the moment a combination of immunoblotting LC3 and p62 and a RFP-GFP-LC3 tandem fluorescent protein assay is considered to measure autophagy most accurately (131).

Also, the results of the other *in vitro* studies we obtained on protein basis turned out to be promising, i.e. the decrease of p62 as well as the observation of autophagic flux in tandem fluorescence microscopy. Yoshii et al. however, declare that actually there is no method established yet to properly monitor autophagy in humans at all (131).

Disregarding this, our studies obtained almost the strongest yet available evidence for induction of autophagic flux, at least on protein basis.

Nonetheless, there are some limiting points concerning the interpretation of our results. We did not apply lysosomal enzyme inhibitors such as pepstatin A or chloroquine for determination if the degradation of p62 is really lysosome-dependent. Further studies have to be done to examine the difference in the amount of p62 and LC3-II between samples with and without lysosomal enzyme inhibitors to consequently obtain at least well-grounded evidence.

An important question is, whether these effects truly depend on PXR. Therefore, a logical experiment would have been to use PXR knockdown cells. We performed these experiments and silenced PXR using shRNA as shown in the preexisting results with rifampicin.

We, however, were not able to recapitulate a reliable PXR knockdown in additional studies were we also wanted to test the effect of hyperforin (results are not shown). Further studies definitely need to address this question, eventually using more sufficient CRISPR/Cas9 mediated knockdown-cell-lines.

In line with our *in vitro* studies we found that PXR activation in mice also induced autophagy. The murine PXR agonist PCN increased LC3 and decreased p62 in our Western blots with material of murine liver specimens. This implies that xenobiotic receptor (i.e. PXR) activated autophagy is evolutionarily conserved from mice to human. The important consequence of this finding is that pre-clinical interventional studies can be done in mouse models in order to test PXR activation in several disease models. Additional studies may clarify if this process is evolutionarily even further conserved down to worms such as C.elegans, which also express xenobiotic receptors (132).

A possible explanation for the induction of autophagy via the activation of PXR may be the complex crosstalk between Nuclear Factor-kappaB (NFkB) and PXR. It could have been demonstrated that at least in certain cancer cells NFkB negatively regulates autophagy (133). Zhou et al. on the other hand could show evidence, that the activity of NFkB is reduced after PXR agonist treatment in particular cell lines (134). A potential conclusion of these findings could be that PXR activation leads to the loss of the negative regulation of NFkB and therefore autophagy is induced. Further studies should be done to elucidate this complex crosstalk, i.e. to examine NFkB expression levels in our samples.
Another attractive possibility for the mechanism of PXR-induced autophagy is the observed induction of TFEB in preliminary experiments (not shown, personal communication with K. Panzitt). Induction of TFEB is required for lysosomal biogenesis to increase the number of lysosomes for the fusion with autophagosomes. In addition, TFEB transcriptionally induces several other autophagy related genes (18).
Taking into consideration the current evidence we do not think that PXR activation directly regulates autophagy as for example mTOR or other kinases do. We rather think that activation of PXR provides the building blocks for autophagy formation and processing by enhancing transcription of autophagy-related genes. Once autophagy is initiated by classical stimuli such as fasting, autophagy may get superboosted via PXR activation because of extended availability of building blocks to execute autophagy. This hypothesis definitely has to be specifically tested in further experiments and more mRNA expression analysis.

Our studies on the constitutive androgen receptor (CAR) did not show effects of this xenobiotic receptor for autophagy induction neither in human cells nor in mouse experiments. However, we only tested one agonist in our cell line studies so it could be quite possible that other agonists in human may show effects.

5.2 Major Limitations of our studies and experiments

Probably the most considerable issue within our findings is the fact that we were not able to show any significant changes on mRNA-basis after xenobiotic receptor agonist treatment in mice. This remains a controversy to our initial hypothesis of a transcriptional regulation of autophagy by the xenobiotic receptor PXR. There is room for discussion that our findings on protein basis are perhaps induced by completely different mechanisms, far away from our initial expectations.

One explanation, however, could be a simple timing issue and mRNA could have already been degraded after the last PCN injection in mice, while protein still remains. Clearly, *in vivo* and *in vitro* experiments addressing time courses would help to clarify this speculation. It is likely that autophagy gets "overboosted" over a prolonged treatment-time with PXR agonists which may lead to a counter-regulative downregulation of autophagy-genes. In this respect, it is important to note that continuous autophagy can result in apoptosis and thus limiting autophagy is also essential for cell survival.

Another possible explanation is a mechanistic one and may include the interaction between PXR and the forkhead box O protein family (FOXO). The main purpose of the FOXO protein lies in the regulation of genes involved in cell growth and differentiation. Furthermore, the FOXO proteins control cellular stress response and antioxidant defense. Biotransformation of xenobiotics commonly is associated with generation of reactive oxygen species (ROS), therefore FOXO proteins are involved in the transcription of genes involved in the clearance of ROS (135, 136).

There are two interesting facts, that were elucidated recently. First, the FOXO protein family promotes autophagy by enhancing the expression of ATG genes, notably those associated with phagophore and autophagosome formation (137, 138). Second, xenobiotic receptors, especially PXR/RXR as heterodimer, were established to act as corepressors of FOXO activity on its DNA binding elements (139).

Maybe the interplay between PXR, the FOXO proteins and the transcriptional regulation of ATG genes could be a possible explanation for the failure of our gain of function studies showing an up-regulation in mRNA-levels of autophagy related genes, even though this clue has only been conclusively proved in cardiomyocytes rather than in hepatocytes.

Existing data show that there is generally poor correlation reported between mRNA-levels and levels of protein, which in term supports our hypothesis (140). Greenbaum et al. further stated, that at least three reasons for that fact have to be taken into consideration:

i.) There are so many complex post-transcriptional mechanisms not fully established yet, which are involved in turning mRNA into protein.
ii.) There may be a substantial difference in the *in vivo* half-lives of proteins.
iii.) There is a compelling amount of bias in both protein and mRNA experiments that restrict our ability to get clear cut results.

In summary, it has been declared that most probably the concentration of proteins and their interactions are the true causative forces in the cells, since mRNA expression values have shown to be rather correlative than causative in a broad range of applications (140).

Our results indicate that, using our experimental approach, mTOR is not affected. However, limited reports indicate at least indirect involvement of the mTOR pathway. It has been shown that rifampicin leads to an inhibition of the rapamycin induced autophagy. Rapamycin induced autophagy is dependent on a certain protein phosphatase (PP2A), whose activity is reduced by rifampicin (141). Also, the FOXO protein family may play a crucial role. Chen et al. established that upon stress conditions FOXO is responsible for the inhibition of mTORC1 (142). If this process may be promoted due to ROS generation during xenobiotic biotransformation, i.e. treatment with xenobiotic receptor agonists, this could eventually lead to induction of the autophagic cascade due to repressed mTORC1-activity. More studies have to be done, to address this issue.

In conclusion, the xenobiotic response receptor PXR is involved in the induction of autophagy. PXR-mediated autophagy induction can easily be achieved by hyperforin, which is the main active compound of St. John's Wort and moreover clinically well tolerated. The results are of clinical relevance since this fact can be immediately utilized in pharmacological context for the treatment of several diseases, where autophagy induction appears warranted.

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