Diploma Thesis

Structure-activity relationship of primary and secondary bile acids on different isoforms of murine and human FXR

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Declaration of Academic Integrity

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Graz, 25.04.2023 Fabio Lampl m.p.

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Zusammenfassung (German)

Hintergrund

Im menschlichen Körper und im Körper der Maus kommen über Gallengänge aus der Leber und der Gallenblase verschiedene konjugierte und un-konjugierte (freie) Gallensäuren in den Gastrointestinaltrakt (GIT) und später wieder bei der Leber an. Diese können als Liganden an die jeweiligen Isoformen das Farnesoid X Rezeptors (FXR) wirken, was eine Veränderung der Genexpression und dadurch metabolische Änderungen zur Folge hat. FXR spielt auch eine wichtige Rolle in der Regulation der Gallensäuresynthese und in der Leberregeneration. Hauptsächlich werden die FXR Rezeptoren in der Leber und im GIT exprimiert, wo Gallensäuren sie aktivieren können. Im Vergleich zum Menschen hat die Maus auch einen anderen Pool an Gallensäuren, in welchem auch spezielle murine Gallensäuren vorkommen. Diese Diplomarbeit veranschaulicht nicht nur, dass humane FXR Isoformen eine höhere Affinität auf Chenodeoxycholsäure (CDCA) haben, als ihre murinen Gegenstücke, sondern auch, dass die rein murinen Gallensäuren keine FXR Aktivierung verursachen. Darüber hinaus wird in dieser Arbeit auf die Wichtigkeit des Gallensäuretransporters NTCP als eine Möglichkeit für Aufnahme hydrophiler Gallensäuren eingegangen, damit diese den nuklearen FXR Rezeptor durch die Zellmembran erreichen können. Des Weiteren gibt diese Arbeit Aufschluss über Induktions- und Hemmwirkung von Gallensäuren untereinander, welche einerseits über das Natrium-Taurocholat Cotransporting Polypeptid (NTCP), und andererseits noch über unerforschte Mechanismen stattfinden.

Methoden

In dieser Diplomarbeit wurde, die durch mehrere Gallensäuren hervorgerufene Induktion verschiedener Isoformen von FXR mittels Luciferase-Assays untersucht. Ziel war es, Unterschiede der Induktivität von den Isoformen zu quantifizieren. Zur Durchführung wurden verschiedene FXR-DNA Vektoren/Plasmide verwendet die zuvor in Bakterien vervielfältigt wurden. Anschließend wurden jene in U2OS Zellen transfektiert und mit verschiedenen Gallensäuren versehen (inkubiert). Zum Abschluss verabreichten wir den Zellen einen Luciferase Puffer und werteten die Ergebnisse aus.

Transfektionsqualität und Normalisierung der Daten wurde mittels Photometer mit Hilfe der Beta-Galaktosidase bestimmt.

Ergebnisse

Im Vergleich zu murinen Formen hatten die humanen FXR Isoformen im Durschnitt eine höhere Induktivität. Rein in Mäusen vorkommende Gallensäuren konnten die Basisaktivität in allen Luciferase-Assays nicht überschreiten. Trihydroxy-Gallensäuren wie Cholsäure konnten FXR nur stimulieren, wenn der NTCP Transporter zu den U2OS Zellen bei der Transfektion hinzugefügt wurde. Dadurch verursachten diese Gallensäuren ähnliche Aktivitätslevel wie Chenodeoxycholsäure, der anerkannteste physiologische Aktivator des humanen FXR Rezeptors. Wenn andere Gallensäuren mit den Trihydroxy-Gallensäuren hinzugefügt wurden, sank die Luciferase-Aktivität signifikant. Auf GW4064 (einem synthetischen Liganden) und Chenodeoxycholsäure hatte die Zugabe keinen Effekt. Das änderte sich, als konjugierte Gallensäuren wie Tauro-Ursodeoxycholsäure (TUDCA) als Antagonisten hinzugefügt wurden. Unter diesem Versuchsaufbau sorgte GW4064 für eine geringere FXR Induktion. Im Gegensatz dazu führte Ursodeoxycholsäure (UDCA) bei manchen Gallensäuren wie Chenodeoxycholsäure in geringen Dosierungen zu einer erhöhten Luciferase-Aktivität. Auf höheren Dosierungen wie 100 µm verschwand dieser synergistische Effekt.

Schlussfolgerung

Humane FXR Isoformen werden in vitro stärker von CDCA aktiviert als murines FXR. Rein murine Gallensäuren konnten FXR nicht stimulieren aber auch nicht direkt inhibieren. Damit hydrophilere Gallensäuren wie Cholsäure oder konjugierte Gallensäuren an FXR wirken können, müssen diese erst durch NTCP transportiert werden. Dieser Transport kann durch andere Gallensäuren teilweise kompetitiv inhibiert werden. Konjugierte Gallensäuren sorgen für einen hemmenden Effekt auf den synthetischen FXR Agonisten GW4064. Außerdem scheint die Gallensäure Ursodeoxycholsäure auf manche Gallensäuren wie Chenodeoxycholsäure synergistische Effekte zu haben, obwohl diese selbst kein FXR Aktivator ist.

Abstract

Background

In humans and mice, the secretion of conjugated and unconjugated bile acids (BA) from the liver reaches the GIT through bile ducts and the gallbladder and later reach the liver again. BAs act as ligands for the various isoforms of the Farnesoid X Receptor (FXR), which leads to changes in the gene expression and further metabolic alterations. FXR also plays an important role in the regulation of BA synthesis and liver regeneration. The receptor isoforms are mainly expressed in the intestine and in the liver, where BAs can activate them. In comparison to humans, mice have a different BA-pool, with more hydrophilic BAs. This diploma thesis demonstrates, that human FXR isoforms have a higher affinity to chenodeoxycholic acid (CDCA) than the murine counterpart. It also clarifies, that solely in rodents appearing BAs do not cause activation of FXR. Beyond that, this thesis sinks into the importance of the BA-transporter sodium-taurocholate cotransporting polypeptide (NTCP) as a possibility to transport hydrophilic bile acids in order to reach the nuclear FXR receptors through the cell membrane. Furthermore, this thesis gives insights about novel mechanisms involving uptake and competition of different Bas via NTCP and other not well investigated mechanisms.

Methods

In this study, the induction of FXR through several BA was tested and quantified using luciferase assays. U2OS cells were transfected with different plasmids of various FXR isoforms and incubated with different human and mouse bile acids, respectively. Finally, these cells were analyzed using a luciferase assay systems that allows to monitor gene regulation by the different isoforms. Data were normalized using beta-galactosidase co transfection.

Results

In comparison to their murine counterparts, human FXR isoforms had a higher efficacy towards CDCA. The solely in mice appearing BAs could not overcome baseline activity of all FXR-isoforms. Trihydroxy-BAs like cholic acid (CA) could only stimulate FXR

when NTCP was overexpressed in U2OS cells. In this combination those BAs caused similar FXR-induction like chenodeoxycholic acid (CDCA), the best physiological activator of FXR. When other BAs were added together with the trihydroxy-BA, luciferase activity decreased significantly. However, GW4064 and chenodeoxycholic acid were unaffected by the co-incubation with other BAs. When conjugated BAs such as tauroursodeoxycholic acid (TUDCA) were used as competitor, FXR induction by GW4064 was significantly impaired. Contrary, free ursodeoxycholic acid (UDCA) led to increased luciferase activity in combination with certain BAs such as chenodeoxycholic acid, in particular at lower doses. On higher dosages such as 100 µm, the synergistic effect was not significant anymore, most likely because FXR activity was already saturated.

Conclusion

Human FXR isoforms are more strongly activated by CDCA than their murine counterparts. Solely murine BAs are not able to stimulate mouse or human FXR, respectively. Hydrophilic BAs such as CA and conjugated BAs need to be transported by NTCP to activate the FXR receptor. This transport capacity can be partly inhibited by competition with other BAs. Conjugated BAs have inhibitory effects on the synthetic FXR agonist GW4064. Furthermore, free UDCA has synergistic effects on some BAs like CDCA, although this BA is not an FXR activator by itself.

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Abbreviations

ATP	Adenosintriphosphate
BA	Bile-Acid
BSEP	Bile Salt Export Pump
CDCA	Chenodeoxycholic Acid
ChREBP	Carbohydrate Responsive Element Binding Protein
CoA	Coenzyme A
CYP7A1	Cholesterol 7α-Hydroxylase
CYP8B1	12-alpha-Hydroxylase
CYP27A1	Sterol 27-Hydroxylase
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl-Sulfoxid
DNA	Deoxyribonucleic Acid
EcRE	Ecdysone Response Element
EDTA	Ethylenediaminetetraacetic Acid
FGF15/19	Fibroblast Growth Factor 15/19
FGFR4	Fibroblast Growth Factor Receptor 4
FXR	Farnesoid X ReceptorGF Germ Free
GIT	Gastrointestinal Tract
GUDCA	Glycoursodeoxycholic Acid
HDCA	Hyodeoxycholic Acid
IBABP	Ileal Bile Acid Binding Protein
LB	Luria Broth
LCA	Lithocholic Acid
LLTE	Leucine, Leucine, Threonine, Glutamate

MCA	Muricholic Acid
MCP-1	Monocyte Chemo-Attractant Protein-1
MDCA	Murideoxycholic Acid
mRNA	Messenger Ribonucleic Acid
MLTE	Methionine, Leucine, Threonine, Glutamate
MYTE	Methionine, Tyrosine, Threonine, Glutamate
MYTG	Methionine, Tyrosine, Threonine, Glycine
NAFLD	Non-Alcoholic Fatty Liver Disease
NTCP	Sodium-Taurocholate Cotransporting Poly-peptide
OATP1A2/1B1	Organic Anion Transporting Polypeptide 1A2/1B1
OCA	Obeticholic Acid
PBC	Primary Biliary Cholangitis
PBS	Phosphate Buffered Saline
PPARα	Peroxisome Proloferator Activated Receptor alpha
RIP-14	RNA Interacting Protein 14
RIP-15	RNA Interacting Protein 15
RNA	Ribonucleic Acid
SHP	Small Heterodimer Partner
SREBP	Sterol Regulatory Element Binding Prot.
TCA	Tauro-Cholic Acid
TCDCA	Tauro-Chenodeoxycholic Acid
TF	Transcription Factor
TMCA	Taurine Conjugated Muricholic Acid

TUDCA	Tauro-Ursodeoxycholic Acid
UDCA	Ursodeoxycholic Acid
VLDL	Very Low Density Lipoprotein
WT	Wild Type
αMCA	Alpha-Muricholic Acid
βMCA	Beta-Muricholic Acid
γMCA	Gamma-Muricholic Acid/Hyocholic Acid
ΩMCA	Omega-Muricholic Acid
7α-HOC	7-alpha-Hydroxycholest

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Introduction & Outline

Bile acids (BA) are physiological detergent molecules that facilitate the absorption of dietary lipids and vitamins in the gut.[1] In humans chenodeoxycholic acid (CDCA) is predominant whereas mice have more muricholic acids (MCA).[2] After being synthesized and conjugated in the liver to increase solubility, the BAs are transported into the gallbladder by the bile salt export pump (BSEP).[3] When the gallbladder contracts in response to dietary fat, BAs enter the enterohepatic circulation. The exocrine secretion is required for digestion, disposal of toxins and metabolic waste and a part of the innate immune system. Inside the intestine/colon they are bio-transformed by the gut microbiota to secondary BAs. Most BAs are brought back to the liver by transport systems in the ileum.[4] Inside the gut and at the liver BAs have a regulatory function on their synthesis and metabolism. This regulatory function is predominantly mediated by the bile acid-activated nuclear receptor farnesoid X receptor (FXR). This receptor is mainly found in the liver and the gastrointestinal tract (GIT) and appears in 4 different isoforms in humans and in rodents.[5] Those isoforms have different affinity to BAs and have diverse metabolic functions, changing the expression of specific genes. FXR additionally has an impact on lipid, glucose and energy homeostasis. In order to develop medication for the FXR-receptor, lots of research happening.[6]

1.1 Physicochemical properties of bile acids

BAs are essential for the solubilization and transport of dietary lipids and are the major, soluble end-products of cholesterol catabolism. During synthesis, they retain their steroid core structure, which consists of three cyclohexane rings and one cyclopentane ring, which are bonded together. Typical human and murine BAs have a 5β-hydrogen group resulting in a cis-configuration (bend) along the plane of the fused A and B rings. BAs differ by amount, position and configuration of hydroxyl

groups (position 6, 7 and 12), which are attached to the steroid core structure. The differences in hydroxyl groups in combination with the molecule's ionization status and the presence or absence of conjugation results in differences in water solubility of various BAs.[7]

Most BAs in multicellular organisms are conjugated with glycine or taurine. The conjugation with taurine or glycine causes a lower pKa. Therefore, conjugated BAs are strongly ionized and still soluble in acidic milieus, though unconjugated BAs are significantly better soluble in aqueous solutions at a higher pH value. Ionization even at low pH prevents passive back absorption during enterohepatic circulation and precipitation by low pH or divalent cations in the intestine.

In the biliary and gastrointestinal tract BAs occur in micelles. Due to the amphipathic property of BAs, they solubilize at a higher pH in a linear manner. Protonated BAs also have a higher solubility.[8]

1.2 Bile acids are synthesized in the liver

BAs are synthesized out of cholesterol in the liver. Two major bile acid biosynthetic pathways exist in the human body, with the classic pathway being the major bile acid synthetic pathway. It forms most of the BA pool. In this pathway, cholesterol gets converted to 7α-hydroxycholesterol (7α-HOC) by the rate-limiting enzyme cholesterol 7α-hydroxylase (CYP7A1). Further enzyme reactions bring forth the common precursor of cholic acid (CA) or CDCA: 7α hydroxy-4-cholesten-3-one synthesized by 3β-hydroxy-Δ5-C27-steroid dehydrogenase.[9] 12-alpha-hydroxylation by 12-alpha-Hydroxylase (CYP8B1) produces a precursor for CA.[10]

In mouse liver, most of the CDCA is converted to α-muricholic acid (αMCA) and ursodeoxycholic acid (UDCA) is converted into β-muricholic acid (βMCA) via hydroxylation of the 6-beta-position. This process is made by the cytochrome CYP2C70. In CYP2C70 knockout mice, no MCAs were found but higher proportions of CDCA and UDCA, respectively.[11] UDCA emerges from epimerization of CDCA´s 7α position to 7β. This process is done by oxidation of the 7α-hydroxyl group

followed by stereospecific reduction of the 7-keto group thus creating the 7β-hydroxyl group.[12] Compared to mice, human´s bile acid pool only has a small proportion of UDCA (4%).[13]

In the large intestine, the bile salt hydrolase removes the taurine or glycine conjugation of the primary bile acids. Next the 7-α-dehydroxylation process converts CA to deoxycholic acid (DCA) and converts CDCA to lithocholic acid (LCA).[14] This process is done by a certain spectrum of anaerobic gut bacteria. Additionally βMCA is transformed by the microbiome to omega-muricholic.acid (ΩMCA) and hyocholic acid (yMCA), whereas αMCA, and ΩMCA are converted to hyodeoxycholic-acid.[15,16]

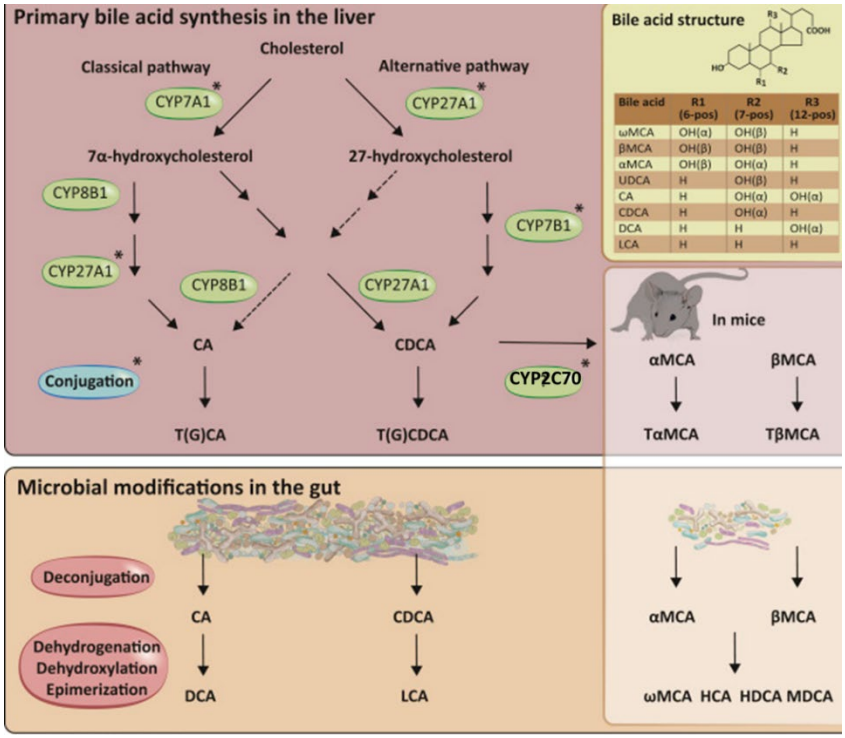

Figure 1 Primary Bile Acid Synthesis adapted and modified from Wahlström A. [17]

In humans, men produce about 30 percent more BAs than women and therefore have a greater BA pool, which is the opposite in mice. A positive correlation exists between the level of triglycerides and the amount of serum BAs.[18,19]

The primary bile acids in humans are CA and CDCA, whereas the primary bile acids in mice are CA, and muricholic acids (α-MCA and β-MCA), as well as UDCA. Therefore, the murine BA pool composition possess more hydrophilic properties.[20] Mice have a more taurine conjugated BA pool (95%), whereas in humans the glycine conjugation is more predominant.[21]

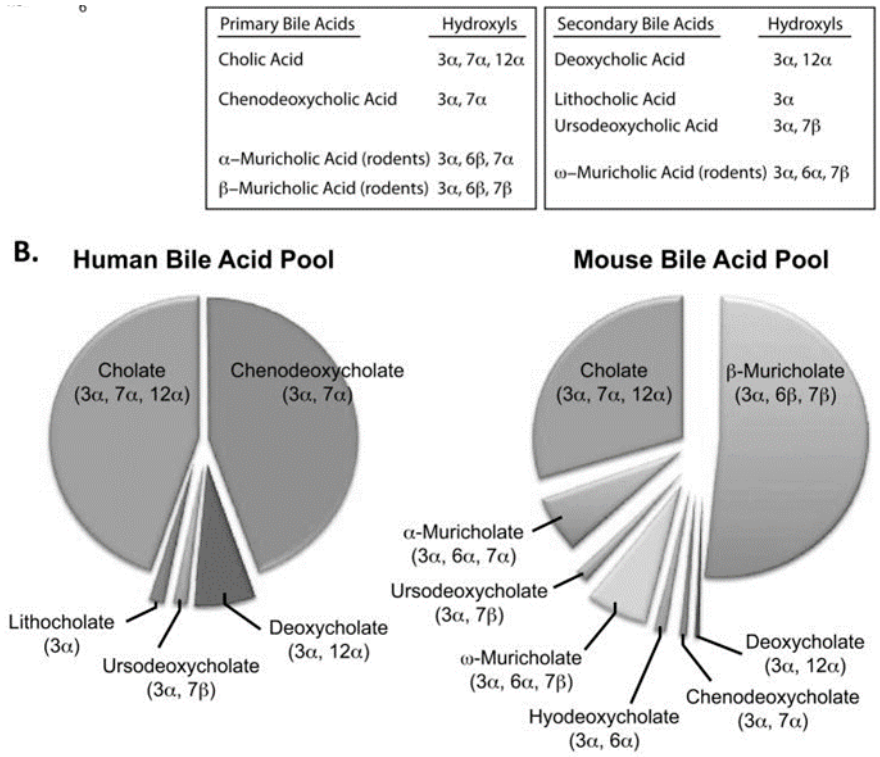

Figure 2 Bile Acid Pool of Humans and Mice. Human bile composition possesses much more CDCA whereas rodents have big amounts of βMCA. Figure adapted and modified from Valim T and Edwarts P [2]

1.3 The bile acid receptor farnesoid X receptor (FXR)

Nuclear receptors are a group of ligand-activated transcription factors that play important roles in embryogenesis, development, and metabolism.[45] Several researching teams reported the discovery of FXR as the nuclear receptor for bile acids. This breakthrough resulted in the denomination of BAs to endocrine

hormones. Rekindled BA research was the consequence and created a path for many future scientific discoveries in the field of metabolic research. Farnesoid X receptor (FXR) was first isolated in 1995. Other occurring names at that time were RXR-interacting protein 14 (RIP14) and RXR-interacting protein 15 (RIP15). The FXR receptor only becomes active with RXR as heterodimer.[46,47,48]

The idea of FXR being a key regulator of BA homeostasis came due to its similar properties to LXR, the feedforward activator of BA synthesis, and their high expression in liver and intestine.[49] In 2002 and 2003 four conserved FXR isoforms that differed in the N-terminal activation domain or the hinge region (between the DNA and the ligand binding domain) were found in hamsters, and later in mice and humans.[50] The four isoforms have different binding affinities to the FXR responsive elements, with the α2 and α4 variations being the most affine.[51]

Inspecting differences between the molecular structure of humans and rodents, murine FXR consists of 11 exons and 10 introns, whereas human FXR has 12 exons and 11 introns. Transcription of FXRα1 and FXRα2 is initiated from exon 1 in both species. In the case of FXRα3 und FXRα4 transcription is set in motion from a different exon: exon 3 in mice and exon 4 in humans. Furthermore, a 12-base pair insert can be found between exon 5/6 in mice and exon 6/7 in humans. An alternative promoter is the deciding factor, whether FXRα1/2 or the FXRα3/4 isoforms are produced. The process of alternative splicing between exon 5/6 in mice and exon 6/7 in humans leads to the FXR isoforms that either possess (α1, α3) or lose (α2, α4) the four-amino acid Methionine, Tyrosine, Threonine and Glycine (MYTG) insert.[51]

FXR receptors are expressed in cells of the liver and the GI-tract, but also appear in the kidney and adrenal glands. FXR is located at the nucleus of the cell and binds to RXR when activated.[52]

When a ligand binds on its helices, a change in conformation happens and the helix stabilizes. It causes the activation of the response element and the nuclear receptor recruits diverse coactivators that modify chromatin and activate several transcription factors.[53]

1.4 FXR isoforms differentially control gene expression

Structural changes as a result of alternative splicing processes lead to differences in function of the nuclear receptor´s isoforms. Every isoform has distinct gene expression. This results in each variant of FXR controlling different metabolic pathways.[60] There is an abundant expression of all FXR-isoforms in the liver. Isoforms FXRα3 and FXRα4 are expressed highly in the ileum, moderately in kidney and only very little in stomach, duodenum, and jejunum. FXRα1 and FXRα2 are located in moderate levels in adrenal glands and ileum. If FXRα1 or FXRα2 are stimulated with the potent agonist CDCA, BSEP activity rises more in the human α2 isoforms compared to the α1 variant. The isoforms are recruited to different response elements, in particular IR1a or IR1b to trans-activate BSEP. Mouse FXRα1 and FXRα2 had relatively the same gene expression of BSEP and presented lower levels.[61,62] In addition, small heterodimer partner (SHP), the CYP7A1 controlling protein, is induced at similar levels by all FXR isoforms using NIH3T3 cells.[63] In case of hepatocellular carcinoma, especially the FXRα2 expression can significantly decrease at the liver, thus leading to cholestasis due to diminished BSEP transactivation and BA accumulation.[64] Hereditary cholestasis also leads to reduced FXR expression via a mutation of the ATP8B1 gene.[65]

FXRα2 has a stronger impact on trans-repressing the key enzyme Cyp8B1 than FXRα4**.**[66] Possible reasons for the different regulation of FXR-regulated genes are partly due to differences in amino acid structure. FXR isoforms lacking the MYTG motif (the FXRα2 and the FXRα4 isoform) regulate much larger gene sets than their MYTG-containing counterparts. Gene analysis revealed, that only around 170 genes are co-regulated by all four isoforms, whereas several thousand genes are modulated altogether.[67] Also the location of the receptors plays a role in the expression of specific genes. Certain tissue specific cofactors could determine the binding of FXR tissue-specific isoforms to certain groups of genes. It would explain the specific distribution of the FXR isoforms throughout the body, to express genes specifically needed at this location.[68]

1.5 Metabolic consequences of FXR activation

FXR is an important regulator of hepatic lipid metabolism. Its activation reduces hepatic fat accumulation and plasma triglyceride levels by inhibition of hepatic de novo lipogenesis and very low density lipoprotein (VLDL) production.[69] In Syrian hamsters, addition of CDCA to a high fructose diet attenuated triglyceride production and prevented the increase of plasma triglycerides and cholesterol levels in the blood.[70] Moreover, FXR represses the LXRα mediated induction of steroid response element binding protein 1-c (SREBP-1c) and thereby lipogenesis. It also inhibits the transcription factor (TF) carbohydrate response element binding protein (ChREBP) in a SHP depended manner.[71,72] FXR induces peroxisome proliferator activated receptor alpha (PPARα), a TF that works as a heterodimer with RXR. Activated, PPARα has positive influences on fatty acid oxidation, fatty acid uptake and fatty acid binding. The agonistic effect only appears in human samples and cannot be reproduced in mice.[73,74] Fibroblast growth factor 15/19 (FGF15/19) is induced as well. It controls the postprandial glucose metabolism, due to BA emission while eating. They have an impact on glycogen synthesis and repressing lipogenesis and hepatic glucose production. Transgenic mice overexpressing FGF19 had lower blood glucose and insulin levels and a higher glucose tolerance and insulin sensitivity in comparison to controls. FGF15/19 also promotes the filling of the gall bladder.[75, 76]

FXR has an impact on glucose metabolism and insulin sensitivity. Activation of FXR in wild type (WT) mice caused a repression of gluconeogenic genes and decreased serum glucose. When SHP was knocked out in those mice, the positive effects of FXR on glucose metabolism are absent, indicating that the FXR-SHP cascade plays a regulatory role.[77] Controversially some other studies with knockout mice, which lacked both, FXR and SHP had improved oral glucose tolerance and lower triglyceride accumulation in the liver.[78]

Liver protective effects of FXR are also described. FXR activation in mice having liver cirrhosis results in less fibrosis in liver tissues and blood vessels, due to

decreased expression of monocyte chemo-attractant protein-1 (MCP-1), a protein that is activated by tumor necrosis factor alpha. MCP-1 activates hepatic stem cells, which further cause the fibrosis.[79]

Several BA transporters are also regulated by FXR. Activation of FXR upregulates the ABC-transporter BSEP for BA efflux via heterodimer binding to certain elements. Conversely, it negatively affects the expression of sodium-taurocholate cotransporting polypeptide (NTCP), apical sodium dependent bile acid transporter (ASBT) and organic anion transporting polypeptide 1B1, who bring the BAs inside the cell. Thus, stimulation of FXR decreases the reuptake of BAs.[3,80]

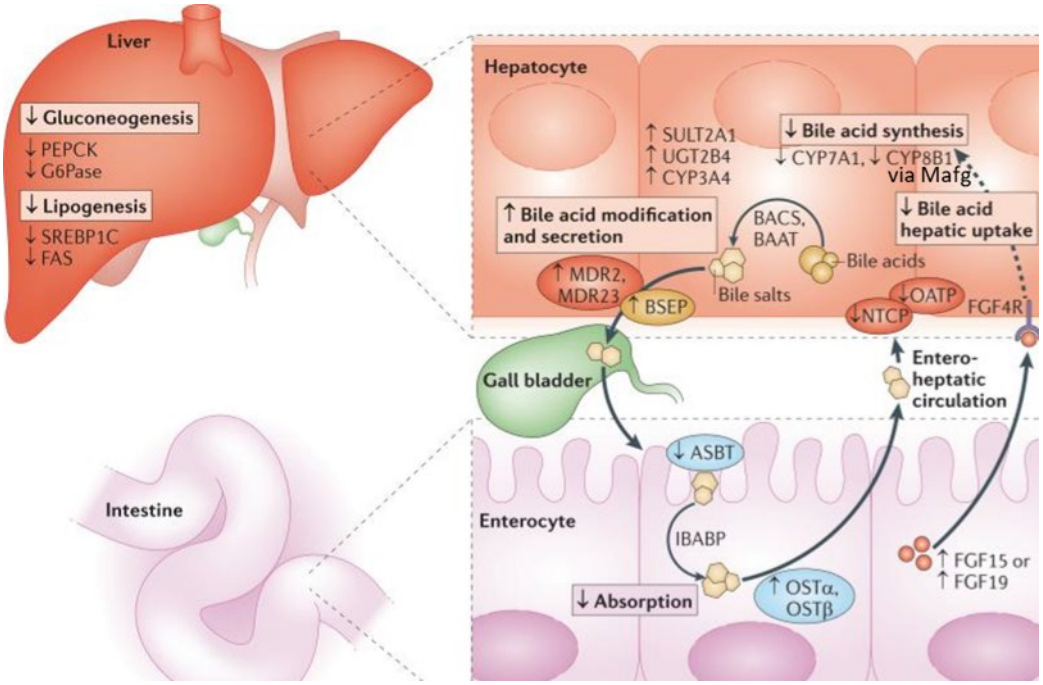

Figure 3 Coordinated effects of FXR on metabolism FXR´s effects on lipogenesis, gluconeogenesis and the expression of BA transporters adapted and modified by Calkin und Tontonoz [81]

1.6 Regulation of FXR activity

FXR is activated in different magnitude by various BAs. There are also differences in activity between the various isoforms of FXR. The best physiological activator is CDCA. Other BAs like LCA and DCA and their conjugates also activate FXR, but to a lesser extent.[54]

Nowadays there are also synthetically produced FXR agonists for in vitro experiments and medical treatment. One of the most important agonists are fexaramine and obeticholic acid (OCA), that have a strong selectivity to FXR. Their affinity to FXR is higher compared to natural compounds. OCA, like physiological BAs, is also metabolized into glycine and taurine conjugates and demonstrates very similar gene expression when it activates FXR. OCA does not have major toxic effects on the body.[55] Also GW4064 is of high importance. One of the greatest benefits of this agonist is its selectivity/fidelity for FXR, although it has been revealed that it has an effect on histamine receptors as well as some other G-Protein coupled receptors.[56]

Glycoursodeoxycholic acid (GUDCA) and tauroursodeoxycholic acid (TUDCA) have been identified as direct FXR antagonists. In luciferase assays, the addition of GUDCA or TUDCA suppressed the agonistic effects of CDCA on FXR on Caco-2 cells.[57] The same seems to be true for taurine conjugated MCAs.[58]

A further human antagonist of FXR is Guggulsterone, an extract of the guggul tree Commiphora mukul. Even though it works as an antagonist, Guggulsterone enhances transcription of BSEP.[59]

1.7 Regulation of Bile Acid Synthesis

One of the main regulators of BA synthesis is the Liver X Receptor (LXR). There are two isoforms of the receptor: LXR alpha that is located mainly in the liver and

induces the expression for lipogenesis genes, and LXR beta. Both function as heterodimers with the retinoid X receptor (RXR) and belong to the same family of nuclear receptors like FXR.[24,25] Ligands of the receptor are oxysterols, derivatives of cholesterol. Many genes involved in lipogenesis and BA synthesis are regulated via LXR, most importantly cholesterol 7α-hydroxylase, the key enzyme of the BA synthesis.[26]

The most important antagonist of BA synthesis is FXR. When FXR gets activated, SHP is expressed, which acts as corepressor to inhibit the CYP7A1 gene activation through the recruitment of several cofactors and methyltransferases.[27,28] FXR activation also results in the induction of the protein FGF15/19. It acts as an endocrine hormone to inhibit hepatic Cyp7a1 gene transcription via binding to the cell complex containing the fibroblast growth factor 4 receptor, a tyrosine kinase receptor, and Beta-Klotho, a structural member of the glycosidase I superfamily, on hepatocytes.[29]

A recently found mechanism of inhibiting CYP7A1 and CYP8B1 by FXR activation is via Zfp36l1. Zfp36l1 binds to elements of specific cytokine messenger RNAs (mRNA) in immune cells to promote mRNA degradation. Experiments with SHP knockout mice demonstrated that Zfp36l1 operates in a SHP-independent mechanism.[30]

Another key player in regulating BA-synthesis in humans and mice is Mafg. When the gene is upregulated by FXR, it represses CYP8B1, causing a shift in the BA-pool with a reduced amount of CA. CYP7a1 was unaffected by Mafg.[31]

1.8 Enterohepatic circulation and transport of bile acids

After synthesized and conjugated, BAs are excreted from the liver and are actively transported across the canalicular membrane into the gallbladder.[1] During digestion, in order to overcome the electrochemical gradient, the parenchymal liver cells use distinct sets of primary adenosine triphosphate (ATP) dependent transporters, which are secondary dependent on a Na+ gradient as well as tertiary transport

systems at the basolateral and canalicular membrane. Most conjugated BAs are transported against the concentration gradient into the bile by BSEP.[32] The role as major BA efflux pump was proven through the identification of mutations in patients with progressive familial intrahepatic cholestasis. Those patients possess less than one percent of bile acid concentration in the gallbladder and highly elevated plasma BA levels.[33] Expression of BSEP is mainly induced by FXR receptor via FXR/RXR heterodimer binding to the IR-1 element of the BSEP promotor. If FXR is mutated, transactivation of BSEP is not happening, confirming that the BA regulation of BSEP is FXR dependent.[34]

Inside the gallbladder, BAs are stored until they are needed for digestion. When food enters the gut, the hormone Cholecystokinin is released. This hormone causes the gallbladder to contract, letting the bile flow from the gall bladder over the cystic duct into the common hepatic duct.[35] At the same time, the sphincter oddi muscle of the gallbladder relaxes, releasing the bile into the duodenum. Inside the small intestinal tract, BAs serve as detergents to facilitate the emulsification of fatty acids and monoacylglycerols, enabling digestion and absorption of dietary lipids and fat-soluble vitamins.[1,36] Reaching the ileum, around 95 percent of the BAs are being reabsorbed and transported via the venous portal vessels back to the liver. Only 5 percent are excreted into the feces.[37] Mostly secondary BAs like LCA are excreted, while primary BAs are reabsorbed.[23] Inside the intestines, BAs have a regulatory function on the growth of the microbiome.[22]

Unconjugated BAs are generally considered to pass the plasma membrane by passive diffusion, while conjugated BAs need active transporters. In the terminal part of the ileum conjugated BAs are reabsorbed by ASBT (also SLC10A2) from the lumen across the apical brush border membrane. ASBT also appears in renal tubule cells, cholangiocytes, and the gallbladder.[32] Inside the intestinal cells BA are bound and further transported by the intestinal bile acid binding protein (IBABP).[36] This protein helps mediate the transcellular movement of the BAs to the basolateral membrane. BAs are then excreted into the portal circulation by the heterodimer organic solute transporters (OSTα and OSTβ). They comprise the major basolateral bile acid transport system in the intestine. They are also expressed in the liver and

in small fractions in the kidney. Like most of the other BA transporters, OSTs are regulated by FXR.[38,39]

For a successful reuptake, the liver has NTCP, which transports conjugated BAs with higher affinity than unconjugated BAs. Some BAs like CDCA and UDCA are not transported by NTCP.[40,41] NTCP is an electrogenic and membrane-bound transport-glycoprotein, which pumps two or more sodium molecules per molecule of solute. Highest expression of NTCP can be seen at the sinusoidal membrane for shifting BAs from the portal blood into the liver.[42] NTCP is regulated indirectly by FXR/SHP, and importantly during liver inflammation or cholestasis.[43] With regard to activation, fasting can induce NTCP.[44]

Additionally, there are also Na^+ independent transporters, which mainly serve for the uptake of unconjugated BAs, like the anion transporting polypeptides 1A2 and 1B1 (OATP1A2, OATP1B1). Those transporters are expressed on the hepatocyte sinusoidal membrane. They work via efflux of one hydrogen carbonate, or glutathione molecule in exchange for one conjugated BA molecule. But compared to NTCP, they are less effective at the transport of trihydroxy BAs, as shown in experiments with HeLa cells.[41]

1.9 Clinical research and medical applications

Due to beneficial effects of FXR stimulation in in vitro and in vivo experiments, there is a lot of research going on for several human diseases. Hopes are high for refining medical therapy for conditions like NAFLD, cholestasis, diabetes and various cancers. OCA and Fexaramine have also shown promising effects on obesity and diabetes, leading to induced browning and weight losses. This further emphasis the importance of BA research for the treatment of until now difficult to treat diseases.[82]

1.10 Aims and Hypothesis

The aims of this thesis are:

1) To study the activity of all murine and human isoforms of FXR in response to naturally occurring (un)-conjugated primary and secondary bile acids in a cell culture-based luciferase assay: We wanted to create a comprehensive overview of the activity of all FXR isoforms to various physiological relevant BAs. No prior paper has compared all isoforms at once before. We expected CDCA to be the best FXR activator in all isoforms. It is known that CDCA is also more affine to human FXR receptors than murine FXR.[83] We also wanted to compare the inductivity of OCA, the ethylated form of CDCA. To take things further, we planned on using the promotors of the following genes: BSEP, SHP and EcDysone Receptor, to have more comprehensive results. In addition, further attention was set on CA and MCAs, because of its high prevalence in both the human and murine BA pool.

2) FXR activation and competition (ligand & inhibitors): NTCP is able to transport most BAs through the cell membrane and has a high affinity for conjugated BAs.[41] Thus we suggested, that conjugated and some of the more hydrophilic BAs need this transporter in order to activate the nuclear receptor. Our research was also set on competing FXR agonists with possible FXR antagonists like UDCA, TUDCA and taurine conjugated muricholic acids (TMCA). We speculated that an antagonistic effect could be caused by a competition for the uptake from NTCP.

3) FXR mutations: The final aim was to explore, why FXRα2 has usually more activity than FXRα1. Based on their molecular structure, FXRα1 possesses a MYTG (Methionine, Tyrosine, Threonine, Glycine) amino acid sequence at the hinge region, which FXRα2 does not have. We created mutants of FXRα1, where the MYTG sequence was replaced with

different amino acid sequences. We wanted to clarify, if such mutations would change activity of the FXR-receptor or if the sequence only has a spacer function.[63]

Materials and Methods

All products were purchased just before the start of our experiments. The majority came from Sigma Aldrich, Cayman, Theromofischer and Invitrogen. The promotors used for our cell lines were a gift from Paul Dawson from UCLA, who deserves our gratitude.

1.11 Materials

Bile Acids

Product	Company	Quantity
αMCA	Cayman	1 mg
βMCA	Cayman	1 mg
CA	Sigma-Aldrich	100 g
CDCA	Sigma-Aldrich	5 g
DCA	Sigma-Aldrich	10 mg
γMCA	Sigma-Aldrich	1 mg
GW4064	Fa. Tocris	10 mg
HDCA	Cayman	1 g
LCA	Sigma-Aldrich	10 mg
MCA	Cayman	1 mg
MDCA	Cayman	1 mg
OCA	Sigma-Aldrich	1 mg
ΩMCA	Cayman	1 mg
TCA	Sigma-Aldrich	250 mg
TCDCA	Cayman	50 mg
TDCA	Sigma-Aldrich	1 g
TUDCA	Sigma-Aldrich	1 g
UDCA	Sigma-Aldrich	1 g

Promotor

Product	Company	Quantity
BSEP	Paul Dawson	1
ECRE-Lu	Paul Dawson	1
SHP	Paul Dawson	1

Cell Culture

Product	Company	Quantity
DMEM 1g Glucose Gibco	Thermofischer	500 mL
DMEM 4,5g Glucose	Thermofischer	500 mL
FPS	Thermofischer	500 mL
FPS-CS	Thermofischer	500 mL
Galactosidase	Adam Antebi gift	
Lipofectamine 3000	Invitrogen	0,75 mL
p3000	Invitrogen	0,75mL
PBS	Thermofischer	500 mL
Trypsin	Thermofischer	500 mL

Luciferase Assay Buffer

Product	Company	Quantity
2-Mercaptoethanol	Sigma-Aldrich	10 ml
2-Nitrophenyl Beta-D-galactopyranoside	Sigma-Aldrich	250 mg
5'-ATP-K2	Sigma-Aldrich	1 g
CoA Na2	Sigma-Aldrich	25 mg
D-Luciferin	Thermofischer	25 mg
EDTA	Sigma-Aldrich	100 mL
Mg Acetat Tetrahydrate	Sigma-Aldrich	50 g
Tricine	Sigma-Aldrich	25 g
Triton X	Sigma-Aldrich	100 mL

Beta Gal Buffer

Product	Company	Quantity
Betamercaptoethanol	Sigma-Aldrich	500 mg
KCl	Sigma-Aldrich	25g
MgCl	Sigma-Aldrich	100g
Natriumdihydrogenphosphate	Sigma-Aldrich	1kg
Natriumhydrogenphosphate	Sigma-Aldrich	500 mg
Nitrophenyl-Beta-D-Galactopyranoside	Sigma-Aldrich	250 mg

Plasmid Isolation

Product	Company	Quantity
New England Biolabs Monarch Mini Prep Kit	Thermo Fisher	1 set
pQ Labs X-Change Plasmid Kit	Thermo Fisher	1 set

1.12 Cell culture

U2OS cells (osteosarcoma cells, originating from bone tissue of a 15 years old girl) were maintained in DMEM (Dulbecco's modified eagle medium) media to which 50 mL with heat inactivated FBS (fetal bovine serum) (Gibco™) and 5 mL Penicillin-Streptomycin (10,000U-10,000 µg/ml) (Gibco™) were added. Cells were kept in a standard CO2 incubator at 37 °C with 1 g/L glucose and 10 % fetal bovine serum (FBS) supplemented with pen CO_2 atmosphere for approximately 48 hours until the cells were confluent. Cells were freshly plated for each experiment. In order to keep transfection rates high enough for the experiments, cell cultures were renewed every couple of weeks.

To keep the number of cells at the desired range of around 90 percent confluence and thus guarantee a successful transfection, they were controlled with a light microscope every day and got split if necessary with 10 mL of phosphate buffered saline (PBS). Next the buffer was aspirated and 4 mL of 0.25% Trypsin-0.53 mM Ethylenediaminetetraacetic acid (EDTA) were dispersed in the flask to bring the cells into solution and the bottle was incubated for around 10 minutes. To deactivate

the Trypsin, 10 mL of full medium were added. Next 2 mL of the solution were put into another bottle and diluted with 20 mL of the medium. The rest of the solution in the other bottle was pipetted into a falcon tube and the old flask was discarded. Subcultures were prepared according to the ATCC protocol.

1.13 Plasmid constructs

Every plasmid was verified by sequencing before application. Plasmid DNA was extracted from overnight bacterial cultures using the New England Bio Labs Monarch Kit according to the manufacturer's protocol. Glycerol stocks of 5-alpha competent E. coli containing the plasmid of interest were streaked on agar plates with antibiotics. After overnight incubation at 37 °C a single colony was picked and allowed to grow in a few mL of Luria Broth (LB) medium supplemented with the proper antibiotics overnight at 37 °C with vigorous shaking. On the following day, 2-4 mL bacterial culture was pelleted by centrifugation. The pellet was resuspended in the first buffer. Bacteria were lysed in alkali buffer and the lysate was neutralized. Plasmid DNA was purified with DNA binding columns and eluted in 35 µL pure water. Determination of concentration and purity check were performed using the Nanodrop 2000 (280/260 ratio around 1.8 and 260/230 ratio >1.8). Integrity of the plasmids was checked by gel electrophoresis. Insertion of our genes of interest was verified by sequencing.

For the plasmid extraction, instructions of the peq GOLD Xchange Plasmid Maxi Kit were followed. Briefly, glycerol stocks of 5-alpha competent E. coli containing the plasmid of interest were streaked on agar plates with antibiotics. After overnight incubation at 37°C a single colony was picked and allowed to grow in a LB medium. The starter culture was diluted 1:500-1000 in 250 mL LB medium and antibiotics for 8 hours at 37°C with vigorous shaking. On the following day, the bacterial culture was pelleted by centrifugation. The pellet was processed according to the manufacturer's instructions (essentially classical alkaline lysis followed by column purification). Plasmid DNA was dissolved in pure water and concentration was adjusted to 1 µg/µL.

1.14 Transient transfection

For this process we followed the Lipofectamine 3000 protocol (Invitrogen). After cell splitting, 100 µL of the solution in the falcon and 100 µL Tryptan blue were given into a microfuge tube and pipetted onto a cell counting plate with a density from about 25000 cells and put in 100 µL to 200 µL medium per well plate. Afterwards it was put on the 96-wells plate and we incubated for 24 hours. After incubation cells were transfected with a mixture consisting of 30 ng FXR receptor, 30 ng promotor, and 5 ng beta-galactosidase. In addition lipofectamine 3000 and p3000 reagent for a successful lipofection according to the manufacturers protocol. After 30 minutes of incubation, medium was exchanged for DMEM 1 % glucose containing 0.1% (w/v) bovine serum albumin (BSA) as new solution.

1.15 Cell culture treatment

After 24 hours incubation cells were treated with various BA concentrations. Those BA were diluted with Dimethyl-sulfoxide (DMSO) and put into an UV-bath to avoid clump formation before the treatment. DMSO served as baseline and GW4064 as a positive control. Later the Wells plate was put into the incubator for another 24 hours, before being evaluated.

1.16 Luciferase Assay

All BAs and derivatives were submitted to luciferase reporter assays to evaluate their capacity to activate the nuclear BA receptor FXR. Because of the high reactivity of the D-Luciferin substrate, the buffer was made just a few minutes before the assay and with the Luciferin as last substance. The first ingredient was 10x Core Buffer consisting of following substances: 30 mM Tricine, pH 7.8, 8 mM

Magnesiumacetat, 0.2 mM EDTA. The other substances in the buffer are 1 percent Triton-X-100, 0.5 mM D-Luciferin, 1.5 mM ATP, 0.5 mM Coenzyme A (CoA), 0.7 percent Beta- Mercaptoethanol. Distilled water was added to create a 5 mL solution. On the final step, the buffer was given into the wells of the treated cells. Luminescence was measured with lumistar luminometer.
We conducted the analysis with three run-throughs and a duration of one second per well, to get a good signal. Following the luciferase activities were normalized to the beta-galactosidase activity.

To control the efficiency of the transfection, Beta Galactosidase assays were performed with a Beta Galactase Buffer (60 mM Natriumhydrogenphosphate, 40 mM Natriumdihydrogenphosphate, 8 mM Kaliumchloride 0.8 mM Magnesiumchloride). After 0.5-3 hours of incubation, a stop buffer (0.04 percent of 2-Nitrophenyl-Beta-D Galactopyranoside and 0.3 percent Beta Mercaptoethanol) was added and photometric measurement was performed.

At the photo spectrometer values between 0.2 and 0.8 allow to suggest a successful transfection process. Any values below were regarded as background disturbances. Results were normalized to the photometer signal.

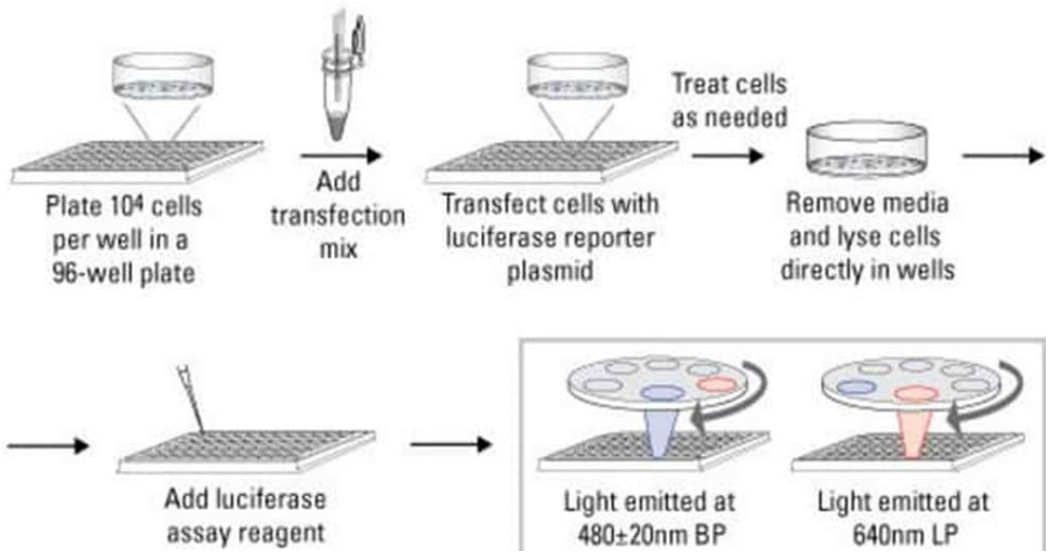

Figure 4 The procedure of Luciferase Assays Adapted and modified from Thermo Fisher Scientific

1.17 Mass Spectrometry

In order to control the purity of the BAs, we took advantage of the mass spectrometry. The results revealed little contamination of the BA leading to the assumption that the quality of the used BA was high enough to exclude false results due to impurity.

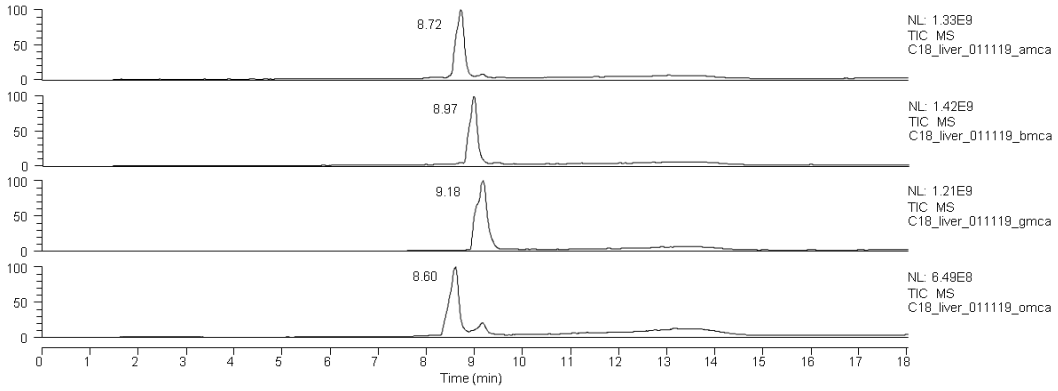

Figure 5 Mass Spectroscopy of BAs

1.18 Calculations

All experiments were done in triplicates. All data points are represented in arithmetic means +/- and standard deviation. Data were normalized to beta galactosidase activity. FXR activity was either depicted as fold change or efficacy. Fold change is the quotient of the luciferase signal between the BA activated FXR receptor and the FXR baseline activity. They are seen as integers. Efficacy is the quotient of the signal from the positive control GW4064 and a tested BA. Those values were depicted in percent.

Results

All experiments done for this diploma thesis used DMSO as a baseline of activity and GW4064 or OCA as a positive control. The following results are illustrated as fold changes normalized to DMSO or as an efficacy quotient of GW.

1.19 Human FXR isoforms are more active than their murine counterparts

The first experiments evaluated primary and secondary physiological BA´s efficacy for human and murine FXR isoforms. We investigated whether certain BAs show species or isoform specific effects on FXR activation in cell-based luciferase assays using the FXR response element of the murine promoter of BSEP. All FXR isoforms were stimulated with 100 µM of those BAs compared to saturated stimulation with 1 µM GW4064 (positive control). see **Table 1**

In the screen, CDCA led to the highest luciferase signal in both humans and rodents (after GW4064). In the human isoforms the signal was twice as strong compared to rodents. All isoforms had approximately the same efficacy with CDCA. The taurine conjugated form of chenodeoxycholic acid (TCDCA) could hardly induce FXR. All the other BAs had lower potency than CDCA by a margin. A difference between human and murine isoforms was not seen clearly. LCA and DCA induced activity was similar in all isoforms with the alpha 1 receptor being the strongest in humans and rodents. UDCA and CA had less activity in the FXRα2 and FXRα4 isoforms and their overall efficacy was low. The strongly hydrophilic trihydroxy-BAs αMCA and βMCA induced the murine α3 isoform the most. The other murine BAs γMCA, ΩMCA, hyodeoxycholic acid (HDCA) had their highest efficacy in the α1-isoforms and had a higher efficacy than αMCA and βMCA. But overall, the low efficacy of all murine BA, CA, and TCDCA speaks for no agonistic properties.

Bile Acid Screen Depicted As Efficacy Of GW4064									
Hydroxyposition	Bile Acid	mα1	mα2	mα3	mα4	hα1	hα2	hα3	hα4
Primary Bile Acids									
3α7α	CDCA	0.29	0.27	0.30	0.28	0.57	0.48	0.56	0.50
3α7α	TCDCA	0.11	0.10	0.12	0.04	0.09	0.13	0.07	0.04
3α7α12α	CA	0.13	0.06	0.14	0.04	0.10	0.07	0.09	0.05
Secondary Bile Acids									
3α7β	UDCA	0.13	0.07	0.17	0.06	0.10	0.07	0.10	0.07
3α	LCA	0.19	0.12	0.15	0.17	0.14	0.13	0.12	0.15
3α12α	DCA	0.19	0.16	0.14	0.06	0.13	0.11	0.08	0.09
Murine Bile Acids									
3α6β7α	αMCA	0.09	0.05	0.11	0.03	0.08	0.05	0.06	0.04
3α6β7β	βMCA	0.11	0.07	0.14	0.05	0.10	0.05	0.08	0.05
3α6α7α	γMCA	0.18	0.06	0.08	0.04	0.13	0.07	0.07	0.06
3α6α7β	ΩMCA	0.19	0.06	0.08	0.03	0.14	0.07	0.06	0.04
3α6β	MDCA	0.19	0.07	0.08	0.05	0.15	0.08	0.06	0.06
3α6α	HDCA	0.16	0.06	0.10	0.07	0.22	0.16	0.08	0.07

Table 1 BA Efficacy Screen with all FXR isoforms.

U2OS cells were first transfected with BSEP and one of the human or murine FXR isoforms in combination with 5 ng Beta Gal Reporter. After transfection cells were incubated with a vehicle (DMSO) or 100 µM of one of the primary or secondary physiological BAs. Luciferase signal was later measured and normalized to photometer measurements. This data represents average values of at least two independent experiments of luciferase reporter assays Unit is % of 1 µM GW4064 value for efficacy. Positions of the hydroxyl groups are depicted on the left site. CDCA is the best FXR ligand.

1.20 CDCA, TCDCA and OCA are strong agonists of human FXR

Based on research with the ligand binding domain of both human and murine FXR, it is well established, that murine FXR tends to bind CDCA with much less affinity than human FXR.[83] That was the case in our earlier experiment with the BSEP promotor. **Figure 6** In order to prove, that this effect is promotor independent, we changed to the SHP-promotor and incubated all FXR isoforms with CDCA. This time the human FXR isoforms α1 and α2 were higher in induction than rodent´s, but the values were almost identical with the α3 and α4 isoforms. **Figure 7**

Then the Ecdysone response element (EcRE) was used as a promotor for comparing the human and murine FXR alpha 2 isoforms. This promotor is not directly involved in the BA metabolism.[85] Ecdysone itself is a steroid hormone in Drosophila Melanogaster, which functions as a major inducing and coordinating signal for changing gene expression causing molding and metamorphosis in insects. Ecdysone receptor senses Ecdysone and binds to the ecdysone-receptor-response-element. FXR also binds to EcRE.[86,87] Like for the BSEP and SHP promotors, CDCA reached the highest efficacy right after GW4064. The human isoform had twice the efficacy of the murine isoform with CDCA. Second strongest in potency were CA and TCDCA. Murine BAs reached higher efficacy on the rodents FXRα2 receptor than the human FXRα2 receptor, except for αMCA, where the efficacy was the same in both species. Nevertheless, efficacy of murine BAs was negligibly small. **Figure 8**

Our next aim was to clarify, if the results would be similar with the semisynthetic CDCA derivate OCA, an ethylated version of CDCA.[84] BSEP served as a promotor. Concentrations of 1 µM and 10 µM OCA were compared to GW. With 1 µM, the murine isoforms were hardly activated at all. 10 µM OCA caused especially high receptor stimulation in the human isoforms α1 to α3. Rodent´s isoforms did not reach that level. OCA caused higher signal on FXRα1 and FXRα3. Following the trend with CDCA, the murine isoforms displayed less potency than human FXR. **Figure 9**

In our BA screen, TCDCA did not show high efficacy, as summarized in **Table 1**. We believed that this was due to its inability to bypass the outer cell membrane, because of its more hydrophilic properties with the taurine added to its structure. Thus, we added the bile acid transporter NTCP to our cell lines. Unlike before, efficacy rose up several times. Like with unconjugated CDCA, the human FXR isoforms reached much higher activity levels than their murine counterparts except for α3, where the values were almost identical. The FXRα4 isoforms had the most impactful change in both species. **Table 2**

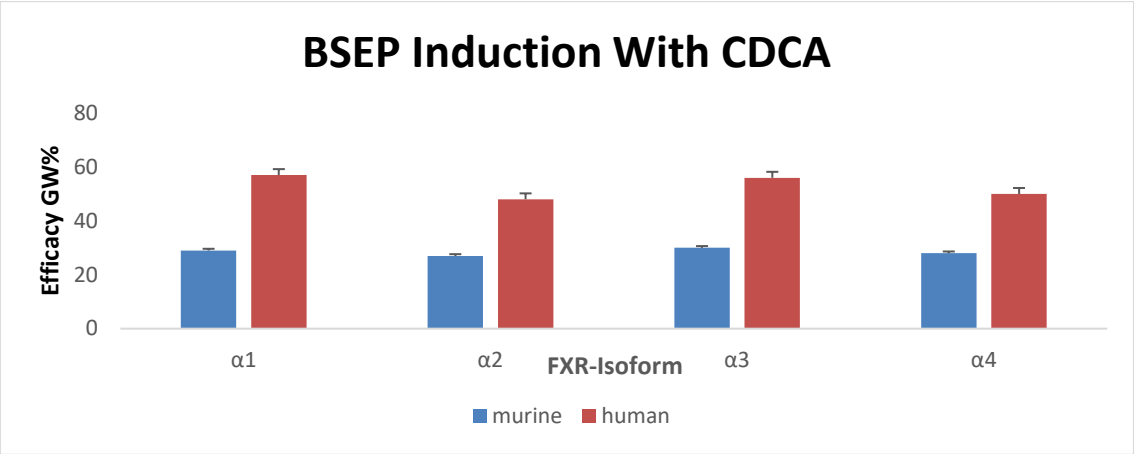

Figure 6 Human FXR is more sensitive to CDCA than rodent FXR

U2OS cells were first transfected with BSEP, and a human or murine FXRα2 isoform in combination with 5 ng Beta Gal Reporter. After transfection cells were incubated with a vehicle (DMSO) or CDCA at a concentration of 100 µM for 24 hours. Luciferase signal was later measured and normalized to photometer measurements. The values are depicted as the mean of two independent experiments +/- standard deviation of the quotient between CDCA and GW as efficacy in percent. The human isoforms show twice the activity as rodent´s.

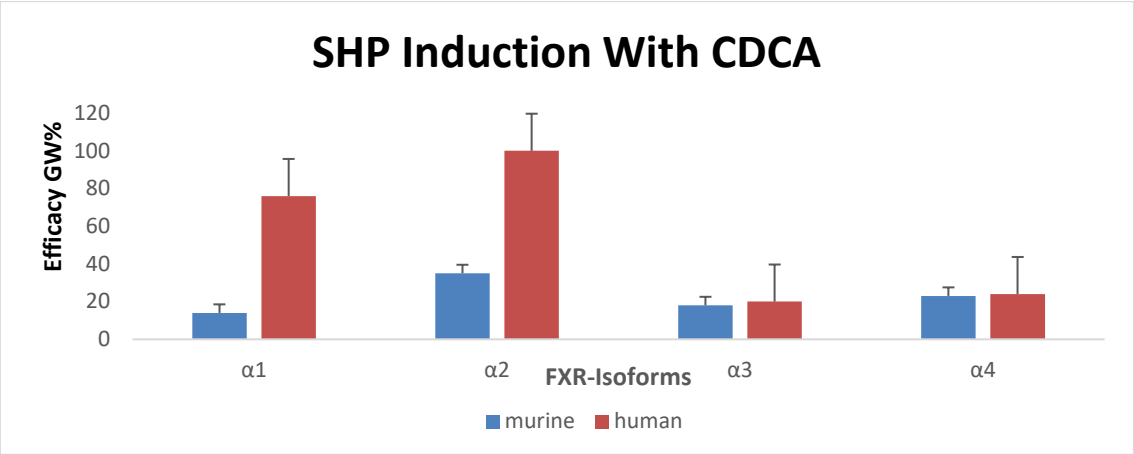

Figure 7 Comparison of SHP activity between FXR isoforms.

U2OS cells were first transfected with SHP promotor, 5 ng Beta Gal Reporter and an FXR isoform, then stimulated with a vehicle (DMSO) or CDCA at a concentration of 100 µM for 24 hours. Luciferase signal was later measured and normalized to photometer measurements. The values are depicted as the mean of two independent experiments +/- standard deviation of the quotient between CDCA and GW as efficacy in percent. Human isoforms FXR α1 and α2 have higher efficacy than the murine counterparts. The other isoforms have similar values.

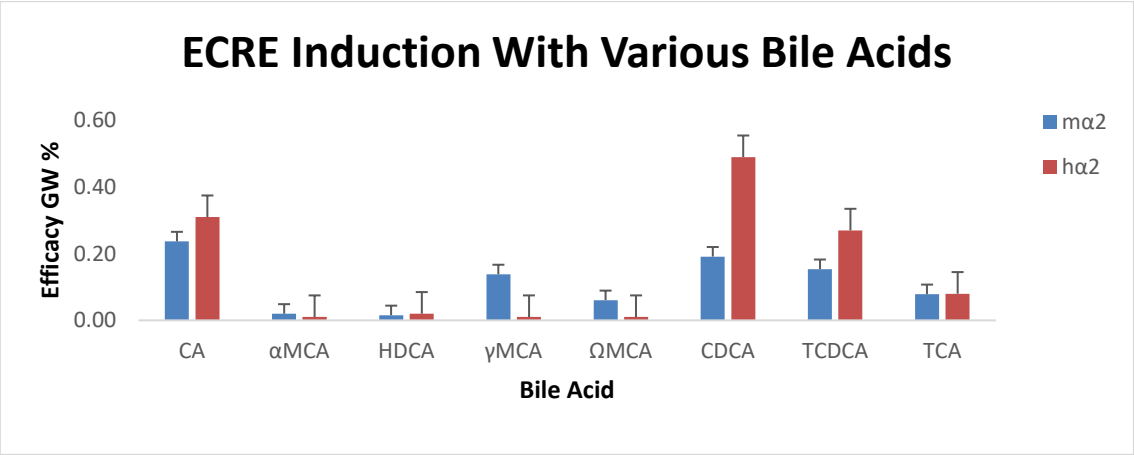

Figure 8 Comparison of Ecdysone Receptor activity under various BAs.

U2OS cells were first transfected with Ecdysone response element promotor, NTCP and a human (hα2) or murine (mα2) FXRα2 isoform in combination with 5 ng Beta Gal Reporter. After transfection cells were incubated with a vehicle (DMSO) or one of the indicated BAs at a concentration of 100 µM for 24 hours. Luciferase signal was later measured and normalized to photometer measurements. The values are depicted as the mean of two independent experiments +/- standard deviation of the quotient between BA and GW as efficacy in percent. The human FXRα2 isoform is more active with the Ecdysone promotor than rodent´s

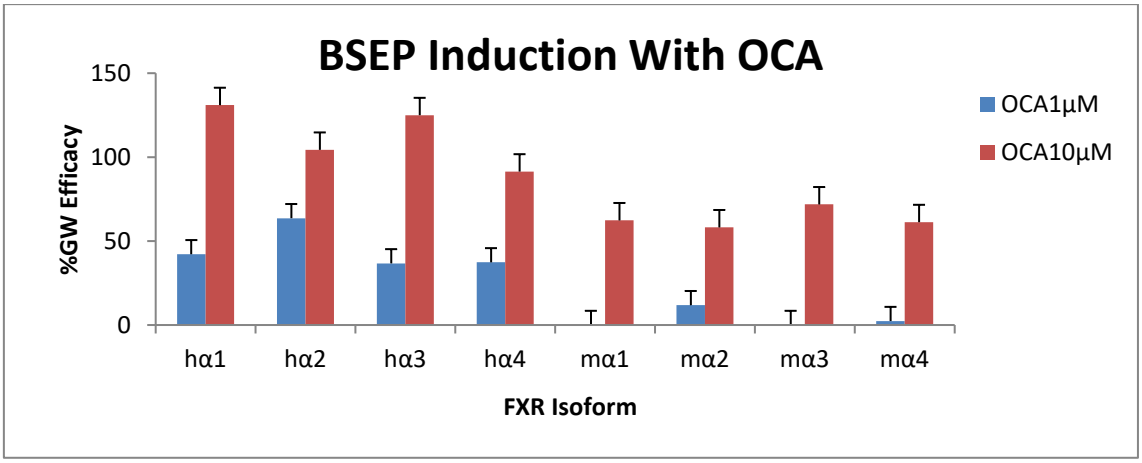

Figure 9 OCA is a strong human FXR agonist

U2OS cells were first transfected with BSEP promotor and an FXR isoform in combination with 5 ng Beta Gal Reporter. After transfection cells were incubated with a vehicle (DMSO) or OCA at the concentrations of 1 µM and 10 µM for 24 hours. Luciferase signal was later measured and normalized to photometer measurements. The values are depicted as the mean of two independent experiments +/- standard deviation of the quotient between OCA and 1 µM GW as efficacy in percent. All human isoforms show higher efficacy than rodent´s.

Efficacy TCDCA								
	mα1	mα2	mα3	mα4	hα1	hα2	hα3	hα4
with NTCP	0.25	0.22	0.4	0.2	0.4	0.35	0.42	0.42
no NTCP	0.11	0.11	0.12	0.04	0.09	0.13	0.07	0.04

Table 2 TCDCA needs NTCP to be a strong FXR agonist

U2OS cells were first transfected with 30 ng of each NTCP, BSEP and one of the FXR isoforms, in combination with 5 ng Beta Gal Reporter. After transfection, cells were incubated with a vehicle (DMSO) or 100 µM of TCDCA. Luciferase signal was later measured and normalized to photometer measurements. The values are depicted as the mean of two independent experiments of the quotient between TCDCA and 1 µm GW as efficacy in percent. Efficacy doubled or even increased several times with the addition of NTCP in vitro.

1.21 Trihydroxy bile acids need NTCP for FXR activation

Another question is dealing with the importance of the BA transporter NTCP for hydrophilic BAs. As seen from the previous BA-screen, there was hardly a

stimulation of FXR by trihydroxy BAs, as shown in **Table 1**. A possible explanation could be that more hydrophilic BAs did not penetrate the cell membrane and thus could not reach the FXR receptor. Therefore, with the help of a BA-transporter like NTCP, inductions might be possible. Or those BAs might be not agonists at all. As seen from the previous experiment with TCDCA, the inclusion of the NTCP-transporter to our cells more than doubled the efficacy. **Table 2** In order to confirm this theory, we tested several BAs, which did show low efficacy in our first screen.

With the new setup CA and TCDCA caused a significant induction of all FXR isoforms. The highest induction was seen in the FXRα4 isoforms, where the human isoform reached twice as much induction as the rodent´s. The values of murine FXR was always below humans, but the difference was not as pronounced as with CDCA. **Figure 10** Compared to CDCA with hydroxyl-groups at 3α and 7α positions, CA possess a third hydroxyl-group at the 12α-position making it more hydrophilic.

In comparison the murine BA yMCA possesses 3 hydroxyl-groups at the alpha position. It managed to slightly increase the signal above baseline in the human isoforms 2 and 4 but not in the rodent´s.

Primary Murine BAs and the secondary murine BA ΩMCA also possess three hydroxyl groups, but -appear in β-configuration. Like in the previous experiments these murine BAs could hardly induce the FXR-receptors and did not come above baseline. **Table *3***

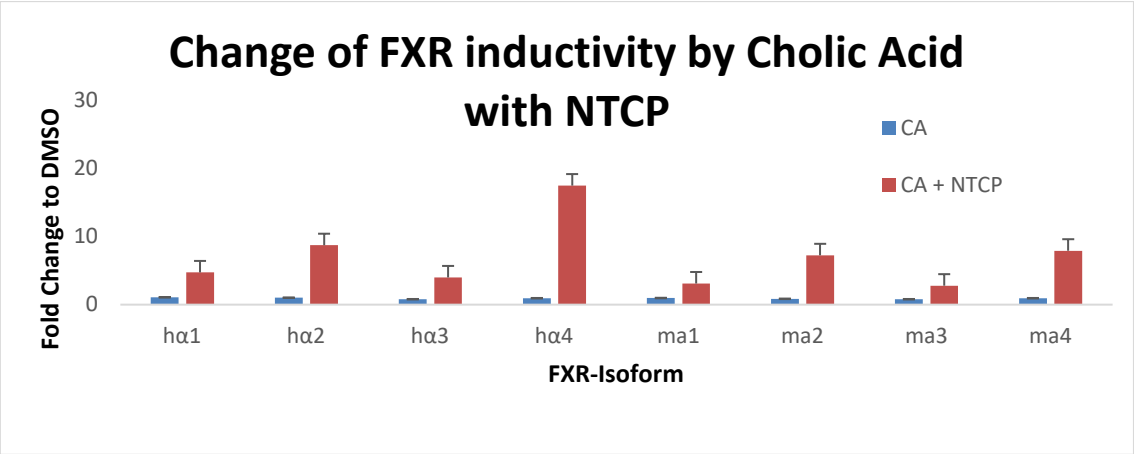

Figure 10 CA needs NTCP to be an FXR agonist

U2OS cells were first transfected with 30 ng of each BSEP and NTCP with one of the FXR isoforms (hα1-hα4 for human, mα1-mα4 for murine), in combination with 5 ng Beta Gal Reporter. After transfection, cells were incubated with a vehicle (DMSO) or CA at a concentration of 100 µM for 24 hours. Luciferase signal was later measured and normalized to photometer measurements. The values represent the mean of two independent experiments +/- standard deviation of the quotient between CA and DMSO.

NTCP Screen With Murine Bile Acids								
Bile	mα1	mα2	mα3	mα4	hα1	hα2	hα3	hα4
GW	6.6	26.8	5.5	38.8	7.5	30.6	9.5	42.6
TCDCA	1.7	5.9	2.2	8.1	3	10.9	4	18
CA	2.3	8.1	2.2	7.9	3.4	11.5	2.9	15.3
αMCA	0.8	0.7	1.1	0.7	0.8	1	0.8	0.9
βMCA	0.7	0.9	1	0.8	0.6	0.8	0.7	0.7
γMCA	0.6	1.1	0.8	0.8	0.7	2.2	0.9	1.6
ΩMCA	0.7	0.8	0.9	0.9	0.6	0.7	0.6	0.7

Table 3 Murine BAs are not FXR agonists

U2OS cells were first transfected with 30 ng of each NTCP, BSEP and a human (hα1-hα4) or murine (mα1-mα4) FXR isoform, in combination with 5 ng Beta Gal Reporter. After transfection, cells were incubated with a vehicle (DMSO) or a BA at a concentration of 100 µM for 24 hours. Luciferase signal was later measured and normalized. The values represent the mean of two independent experiments +/- standard deviation of the quotient between BA and DMSO induction. Unlike in Table 1, CA and TCDCA now cause a strong induction of FXR. MCAs still do not stimulate above baseline.

1.22 FXR inhibition by competition with NTCP

Based on the last results, we tested non-agonistic BAs for FXR activation in order to evaluate, if those have antagonistic effects on the FXR receptor. In theory, possible inhibitory effects could also result from their competitive nature on the NTCP transporter compared to agonistic trihydroxy BAs.

First, we tested βMCA and γMCA, as well as UDCA, which also possesses a β-configuration at the 7-hydroxyl-group. It is known that NTCP transports MCA as well as CA and other trihydroxy BAs.[41] For comparison served CDCA and GW4064, because both agonists do not rely on NTCP for uptake to stimulate FXR. If the fold change of the promotor expression would decline among all BAs, it would indicate an inhibition that is not done by NTCP competition. We also competed CDCA with CA or GW4064. The human and the murine FXRα2 receptors were used with BSEP as promotor and NTCP as transporter. Under these conditions CDCA and GW4064 did not lose potency by the inclusion of MCAs. On the contrary, activation by CA severely decreased with the addition of βMCA or UDCA but hardly with hyocholic acid. The effect was seen in both human and murine FXR. **Table 4**

In the next inhibitory experiment, we used 20 µM and 100 µM of CDCA, CA, or TCDCA and competed each of them with 100 µM UDCA or TUDCA. In some papers UDCA and TUDCA are described as potential FXR antagonists.[58] It was in our interest to see, if conjugates could also work as potential antagonists for FXR. Like before, the human and the rodent version of FXRα2 receptor and the BSEP promotor as well as NTCP were transfected to the cells. Without the inclusion of UDCA or TUDCA, all BAs had increased fold changes. That proves in another independent experiment that taurine conjugates of CDCA and CA are FXR activators. The inclusion of UDCA caused a slight increase in fold change with taurocholic acid (TCA), TCDCA and CDCA under 20 µM concentration. At 100 µM such an induction was not seen. Exception of this rule was CA, which lost potency under the influence of UDCA. Under the inclusion of TUDCA, fold changes

drastically decreased for CA and for GW4064 with human and rodent FXR, respectively. **Figure 11 Figure 12**

For a more cohesive picture, we competed 100 µM of CDCA, CA and 3 µM GW with UDCA and TUDCA for all FXR isoforms. Additionally, GW was also competed with CA and CDCA and their taurine conjugated forms for the FXRα2 receptors. CDCA was unaffected by the inclusion of UDCA and TUDCA, whereas CA´s potency was severely reduced. That occurrence was seen in all FXR isoform. The GW4064 competition also showed lower FXR activity with inclusion of taurine conjugated BAs, but not with their unconjugated forms. **Table *5***

Fold Change Compared To DMSO		
Bile Acid	mFXRα2	hFXRα2
GW	21	30
CDCA	6	17
UDCA	1	2
CA	6	11
βMCA	1	1
γMCA	1	2
GW+βMCA	20	25
GW+γMCA	23	21
CA+ βMCA	3	7
CA+ γMCA	5	9
CA+ UDCA	3	7
CA+CDCA	8	17
CDCA+ βMCA	7	15
CDCA+ γMCA	7	15
CDCA+ UDCA	8	13

Table 4 Competition of various BAs with different MCAs

CA is negatively affected by the inclusion of other BAs, U2OS cells were first transfected with 30 ng of each NTCP, BSEP and a human or murine FXRα2 isoform, in combination with 5 ng Beta Gal Reporter. After transfection, cells were incubated for 24 hours with a vehicle (DMSO) or 100 µM of a BA with or without the inclusion of a murine BA at a concentration of 100 µM. Luciferase signal was later measured and normalized. The values represent the mean of two independent experiments +/- standard deviation of the quotient between BA and DMSO induction.

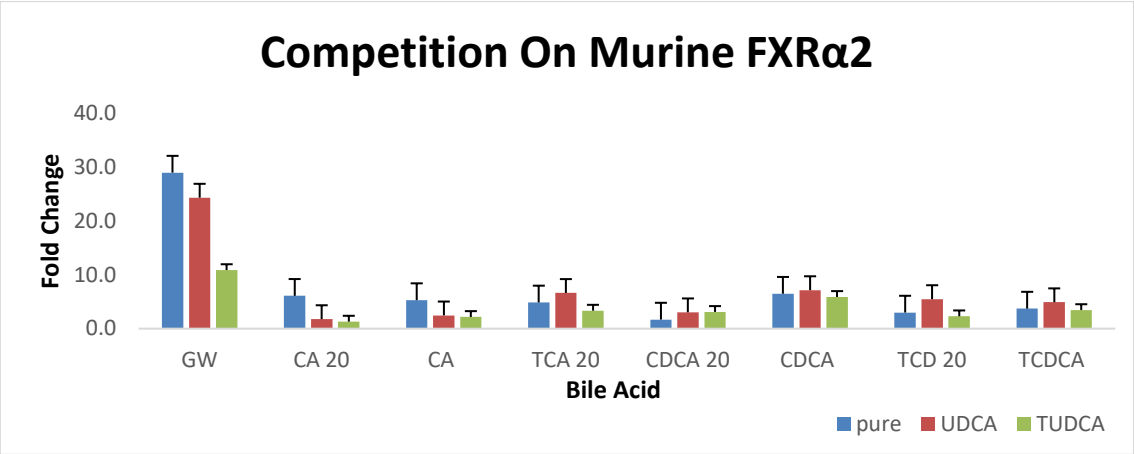

Figure 11 Changes in the induction of the murine FXRα2 promotor under the influence of UDCA and TUDCA.

U2OS cells were first transfected with 30 ng of each NTCP, BSEP and the murine FXRα2 isoform, in combination with 5 ng Beta Gal Reporter. After transfection, cells were incubated with a vehicle (DMSO), or a Bile Acid at concentrations of either 20 µM or 100 µM for 24 hours. UDCA or TUDCA at a concentration of 100 µM were added to each cell. Results show the mean of two independent experiments +/- standard error of the quotient. The luciferase activity of the reporter is expressed as the quotient of a Bile Acid and DMSO as fold change. TCA and TCDCA are more potent FXR agonists with the addition of UDCA, while TUDCA has a suppressive effect on CA and GW.

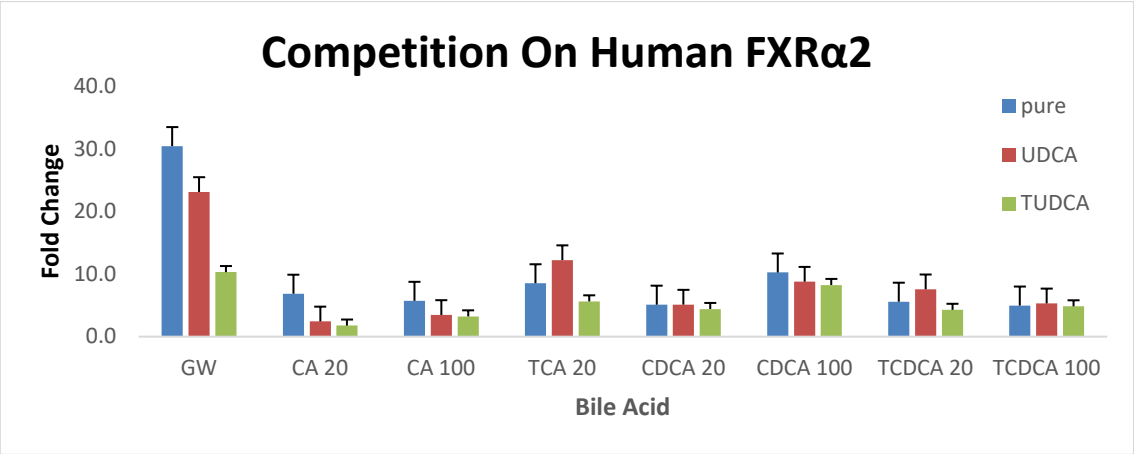

Figure 12 Changes in the induction of the human FXRα2 promotor BSEP under the influence of UDCA and TUDCA.

U2OS cells were first transfected with 30ng of each NTCP, BSEP and the human FXRα2 isoform, in combination with 5 ng Beta Gal Reporter and incubated with a vehicle (DMSO), or a Bile Acid at concentrations of either 20 µM or 100 µM for 24 hours. 100 µM UDCA or TUDCA was added to each well. Results show the mean of two independent experiments +/- standard error of the quotient. The luciferase activity of the reporter is expressed as the quotient of a Bile Acid and DMSO as fold change. TCA and TCDCA are more potent FXR agonists with the addition of UDCA, while TUDCA has a suppressive effect on CA and GW.

Competition Screen with UDCA and TUDCA								
Fold Change	mα1	mα2	mα3	mα4	hα1	hα2	hα3	hα4
CDCA	4.5	6.7	3.1	16.2	4.4	22.1	3.3	23.4
CDCA+U	5.2	8.4	4.2	19.8	5.7	24.6	4.6	25.7
CDCA+TU	6.8	9.0	4.3	22.4	5.8	22.0	4.0	22.5
CA	5.0	8.7	3.4	17.3	3.8	12.6	2.4	12.3
CA+U	4.6	4.9	2.3	7.0	2.5	7.4	1.4	5.5
CA+TU	3.5	3.4	2.3	7.2	1.7	4.1	1.2	4.2
GW		29.0				30.5		
GW+CA		27.6				26.0		
GW+TCA		10.9				10.5		
GW+CDCA		29.0				31.1		
GW+TCDCA		10.2				10.9		

Table 5 Competition of BAs with UDCA and conjugates

U2OS cells were first transfected with 30 ng of each NTCP, BSEP and one of the human (hα1-hα4) or murine (mα1-mα4) FXR isoforms, in combination with 5 ng Beta Gal Reporter. After transfection, cells were incubated with a vehicle (DMSO), 100 µM BA and the inclusion of UDCA or UDCA at a concentration of 100 µM for 24 hours. The values represent the mean of two independent experiments of the quotient between Bile Acid and DMSO induction. The luciferase activity of the reporter is expressed as the quotient of a Bile Acid and DMSO as fold change. GW and CA are inhibited by TUDCA. GW also shows reduced agonism with TCDCA and TCA.

1.23 UDCA as an FXR sensitizer

In the previous experiment, we observed a slight increase of FXR activity by using 20 µM CDCA including UDCA but not so when using 100 µM of CDCA. **Figure 11** Consequently, we wanted to examine the role of UDCA as possible sensitizer of certain BAs. UDCA alone does hardly cause any stimulation on the FXR-receptor. Therefore, the effect cannot come from synergistic agonism.[88] **Table 1** In order to evaluate saturation effects of CDCA and the combined efficacy of UDCA to stimulate FXR, we created a dose response curve of CDCA under the inclusion of varying amounts (25 µM 50 µM and 100 µM) of UDCA. We conducted this experiment with the human and murine FXRα2 isoform. BSEP was our promotor of choice.

Under low concentrations of CDCA, the FXR activity increased proportionally with low amounts of UDCA. In higher amounts of CDCA, UDCA led to an increased activity only of the murine FXRα2 receptor. **Figure 13 Figure 14**

Similar experiments conducted with GW4064, UDCA did not follow that trend and values remained the same in humans and rodents. Instead 100 µM of UDCA even started to decrease the luciferase assay activity. **Figure 15 Figure 16**

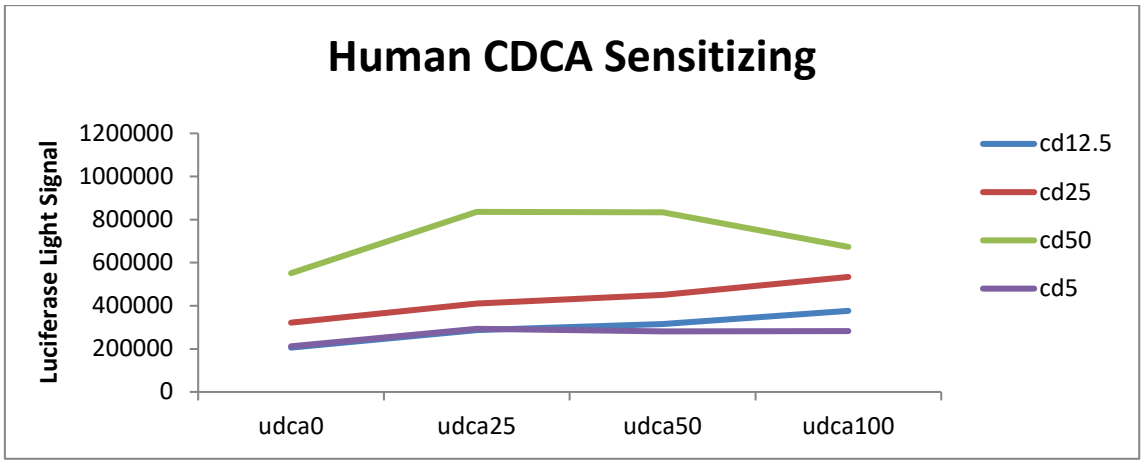

Figure 13 UDCA increases the potency of CDCA for human FXR

U2OS cells were first transfected with 30 ng of each NTCP, BSEP and the human FXR α2 isoform, in combination with 5 ng Beta Gal Reporter. After transfection cells were incubated with various amounts (5, 12.5, 25, 50 μM) of CDCA (CD) and UDCA (25, 50, 100 μM) for 24 hours. Following the light signal was measured with the lumistar luminometer. Results are shown as the strength of the luciferase light signal.

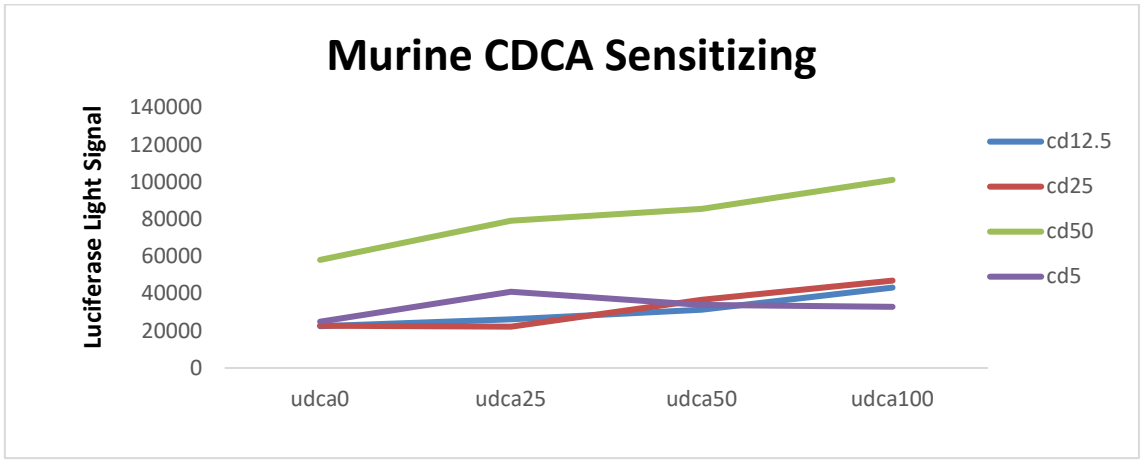

Figure 14 UDCA increases potency of CDCA for murine FXR

U2OS cells were first transfected with 30 ng of each NTCP, BSEP and the murine FXR α2 isoform, in combination with 5 ng Beta Gal Reporter. After transfection cells were incubated with various amounts (5, 12.5, 25, 50 μM) of CDCA (CD) and UDCA (25, 50, 100 μM) for 24 hours. Following the light signal was measured with the lumistar luminometer. Results are shown as the strength of the luciferase light signal.

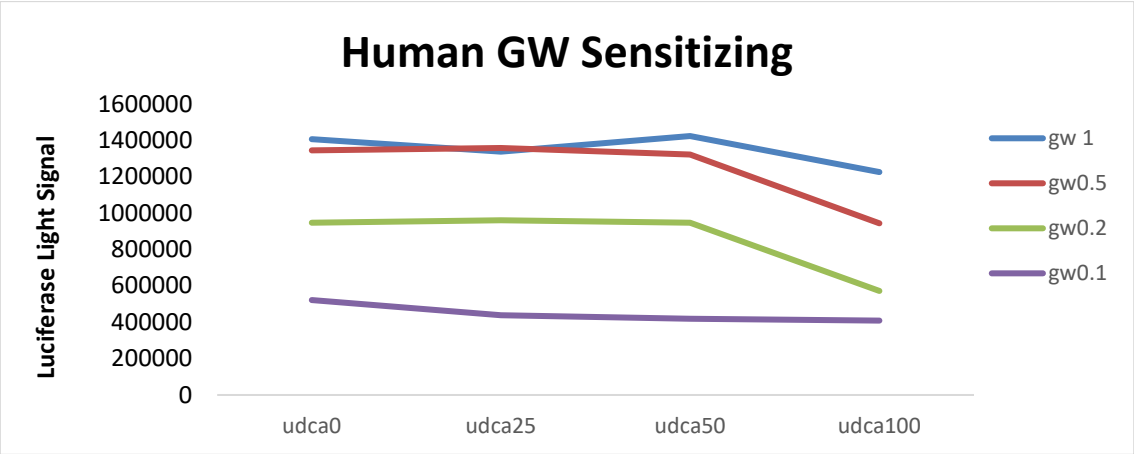

Figure 15 UDCA does not increase the potency of GW for human FXR

U2OS cells were first transfected with 30 ng of each NTCP, BSEP and the human FXR α2 isoform, in combination with 5ng Beta Gal Reporter. After transfection cells were incubated with various amounts (0.1, 0.2, 0.5,1 μM) of GW4064 and UDCA (25, 50, 100 μM) for 24 hours. Following light signal was measured with the lumistar luminometer. Results are shown as the strength of the luciferase light signal.

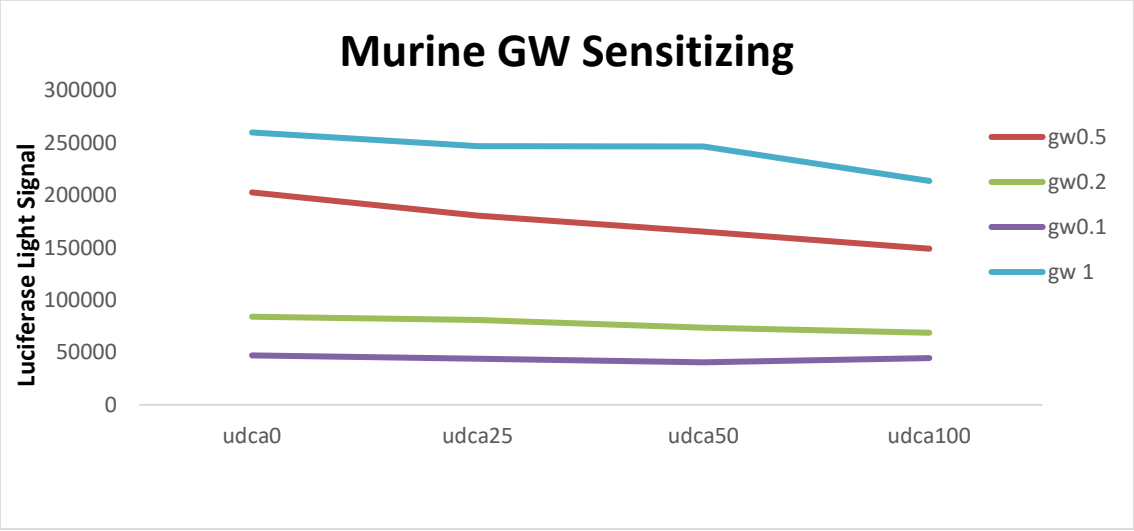

Figure 16 UDCA does not increase the potency of GW for murine FXR

U2OS cells were first transfected with 30 ng of each NTCP, BSEP and the murine FXRα2 isoform, in combination with 5 ng Beta Gal Reporter. After transfection cells were incubated with various amounts (0.1, 0.2, 0.5, 1 μM) of GW4064 and UDCA (25, 50, 100 μM) for 24 hours. Following the light signal was measured with the lumistar luminometer. Results are shown as the strength of the luciferase light signal.

1.24 Changes of the MYTG sequence cause variations in the FXR transactivation of the BSEP promotor

We know, that some response elements exist, on which murine FXRα2 isoform depicts better activation than α1. If you compare the receptors, the difference between the molecular structures lies in the MYTG (Methionine, Tyrosine, Threonine, and Glycine) sequence. FXRα2 and FXRα4 do not possess these residues/amino acids. We wanted to clarify, if this sequence of amino acids is responsible for the reduced activity of the FXRα1 receptor or if the sequence only functions as a spacer.[63] To evaluate the effect of these four amino acids in the hinge region, one of my supervisors, his name is Alex Zaufel, created mutant versions of the FXRα1 receptor. In these mutants MYTG were exchanged with different sequences. One of those sequences is LLTE (Leucine, Leucine, Threonine, Glutamate). In FXRα2 LLTE is located at the same position as the MYTG sequence in FXRα1. Furthermore, two other mutants were created by changing the MYTG of FXRα1 to MLTE (Methionine, Leucine, Threonine, Glutamate) and MYTE (Methionine, Tyrosine, Threonine, Glutamate), respectively. Those mutants were all stimulated with GW4064.

Induction strength varied extremely between the mutant versions of the FXRα1. While the MLTE variation showed higher activation levels, the other forms were even lower than the naïve α1isoform, with the exception of MLTG, which showed comparable transaction as FXRα1. The lowest activity was seen with the LLTE mutant. **Figure 17**

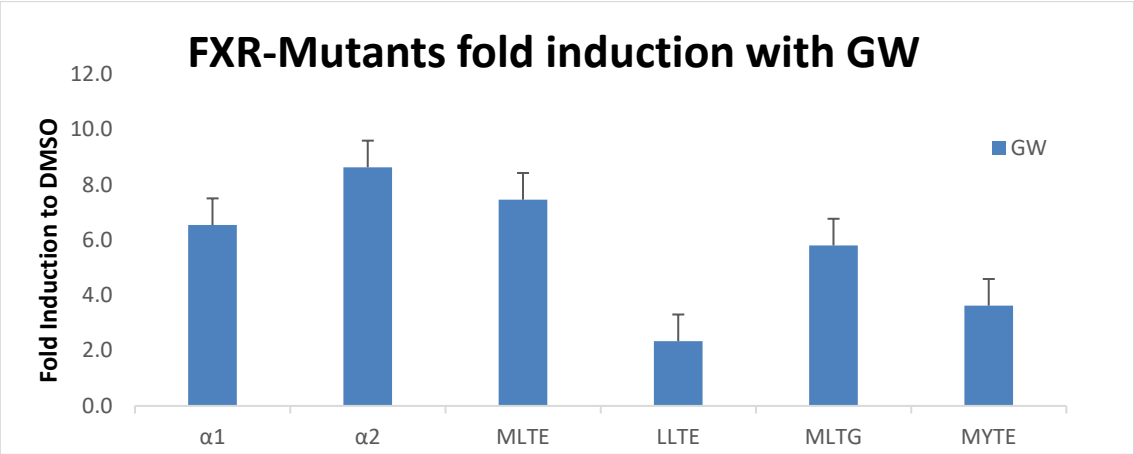

Figure 17 The MYTG sequence has a regulatory function on FXR

MLTE was the mutant with the highest induction. U2OS cells were first transfected with 30 ng NTCP, BSEP and either one human FXR (α1, α2) isoform or one of the indicated mutants, in combination with 5 ng Beta Gal Reporter. After transfection, cells were incubated with a vehicle (DMSO), or 3 μM GW4064 for 24 hours. This data represents average values of at least two independent experiments of luciferase reporter assays in FXR transfected USO2 cells. Receptor activity follows this trend: α2>MLTE>α1>MLTG>MYTE>LLTE.

Discussion

This work further helped to unravel the physiological interactions of FXR and BAs. It highlighted, that all human isoforms of FXR reacted stronger to the stimulation of CDCA than their murine counterparts. This is another proof that the human isoforms have a higher affinity for CDCA, which is also present in higher proportions in the human body than in rodents.[89] Unsurprisingly, CDCA was the most potent physiological BA in our screen. Changing from BSEP to other promotors such as SHP or Ecdysone depicted the same conclusion, although the isoforms FXRα3 and FXRα4 were similar with SHP between humans and rodents.[90] OCA and GW4064 were even more potent human FXR activators than CDCA. Murine FXR isoforms on the flip site showed lower efficacy by OCA, giving further evidence, that the ethylated form of CDCA also has a high affinity for human FXR and low affinity to rodent´s FXR.

Other BAs did not follow that trend and their efficacy was more similar between human and murine isoforms. The results further strengthen, that CDCA is the most important BA for the regulation of the human BA homeostasis. On the other hand, the conjugated form TCDCA needed the inclusion of NTCP in order to reach similar efficacy. Like CDCA, TCDCA had a higher efficacy with all human FXR isoforms, suggesting that the human FXR receptor is also more prone to TCDCA than to the rodent´s ones. Other conjugates were also able to activate FXR, when NTCP was overexpressed, outlining the importance of this transporter. This outcome also indicates an important role for conjugates as FXR activators and thus for BA homeostasis.

The efficacy of CA and its conjugates also rose significantly in the presence of NTCP, indicating that some hydrophilic unconjugated BAs are also dependent on NTCP in order to induce the FXR receptor. But this time the efficacy was similar between the human and the rodent FXR receptors, suggesting that the human FXR receptor is not as prone to CA as CDCA. Compared to CDCA, CA has three hydroxyl-groups in alpha configuration instead of two, making it more hydrophilic. Conjugates like taurine and glycine also make BAs more hydrophilic. Consequently,

those BAs were most likely unable to reach the nuclear receptor through the lipophilic cell membrane, without the assistance of a transporter. In comparison, CDCA with hydroxylation only at the positions 3 and 7 has more lipophilic properties and can more easily bypass the cell membrane. Thus the inclusion of NTCP did not make a difference. This held true for the MCAs. Even with the addition of NTCP, BAs such as βMCA, or the BAs UDCA and αMCA could not induce FXR. Looking at the molecular structure, they also possess three hydroxyl groups, but some of those groups are in beta configuration. This molecular difference could be the reason why they do not activate FXR. MCAs are also being transported by NTCP, so they should have been able to reach the nuclear receptor.[91] CDCA and TCDCA hardly occur in mice, whereas MCAs and CA are much more abundant in rodents, meaning that CA must have more importance for the regulation of BA homeostasis via FXR.[20]

When activation of FXR with CA was competed with MCAs and TUDCA, the activity of all FXR receptor isoforms decreased. This suggests, that MCAs and conjugates have an antagonistic effect on the FXR receptor that could be related to competition on NTCP uptake of all trihydroxy-BAs. A different mechanism is rather unlikely because the FXR induction via CDCA or GW4064 did not change with the inclusion of MCAs. Particularly βMCA, with its relatively high amount in rodents, makes this outcome very interesting for understanding the physiological interactions and competition of BAs for the FXR-receptors and BA-transporter like NTCP in rodents.[94] MCAs have already been seen as antagonists for FXR, but the direct mechanism was never fully understood.[95] With our results we conclude that MCAs are selective FXR antagonists via the competition of NTCP. Experiments in the future should also look at the other MCAs and their conjugates for competitive behavior on FXR.

The addition of UDCA increased the potency of some low concentrated FXR agonists, when as we speculate the nuclear receptor has not been fully saturated yet. The only exception was CA, where induction of FXR actually decreased. Due to solely two hydroxyl-groups, UDCA is very lipophilic and can enter the cell without problems. UDCA is also hardly transported by NTCP at higher concentrations.[92,93] Therefore we assume, that UDCA could bind at the same place on the nuclear

receptor as CA, competing for the receptor and causing an inhibition. This would also mean that TCA, which received an increase in potency from UDCA, also binds on a different spot as CA. The exact mechanism behind it could not be determined and would be a topic for future research.

The addition of TUDCA decreased FXR activation by conjugated and more hydrophilic BAs like CA. It is known that conjugated BAs have a high affinity for NTCP, thus a competitive inhibition for NTCP is likely.[41] This could be an explanation for the regulatory role of UDCA on patients with primary biliary cirrhosis, who also suffer from cholestasis and their treatment involves the administration of UDCA.[96] CDCA, a BA that does not need NTCP due to its hydrophobic properties, was unaffected from TUDCA administration, even though antagonistic properties were described in publications.[57] Unexpectedly though, TUDCA did decrease the activation by GW4064 to a significant extend. This effect could be reproduced with other conjugated BAs like TCA. This inhibitory effect could be through competition for the FXR receptor, but we did not find out the exact mechanism. Unconjugated BAs did not cause such feats on GW4064, indicating that taurine might be involved in this inhibitory effect.

When CDCA was challenged with different amounts of UDCA, we saw a dose depend upregulation of FXR activity. This enhancement was only seen, when the FXR receptor was not fully saturated. Because of UDCA´s inability to activate the FXR by itself, see **Table 1**, I apprehend a possible conformation change of the FXR receptor through binding of UDCA, consequently leading to higher induction levels. Recent publications have shown that a modulation of FXR with the small molecule Imatinib is possible and induces gene expression in HepG2 cells.[97] It is also described that partial agonists can lead to changes in the helix α11 thus causing conformational changes.[88] Another possibility would lie in the recruitment of different co-factors in the presence of UDCA. UDCA does reduce co-factor recruitment at glucocorticoid receptors when binding to it as ligand.[98] Further experiments and molecular analysis need to be done to fully comprehend our results. In comparison, UDCA did not have any significant effect on transactivation of FXR by GW4064. On the contrary, on higher doses of UDCA, the stimulation of FXR declined marginally,

but only on fractionally low doses of GW4064. I suppose, that this was due to a dilution effect and not caused by a competitive inhibition of FXR.

In our experiment with the mutated hinge region of FXRα1, we saw significantly lower activity levels with the altered amino acids LLTE and MYTE compared to the original MYTG sequence. On the contrary MLTE and MLTG even had higher values than the unchanged FXRα1 receptor giving us the conclusion, that those additional AAs at the hinge region have a regulatory role and inhibit FXR activity to an extent, thus not just serve as a spacer. Based on this discovery, our understanding of FXR isoforms has further been expanded.

Summary

This diploma thesis does not only serve as an overview of which BAs are agonists for FXR, it also helps to gain more insight into the competitive and sensitizing behaviors of those compounds under several conditions. We demonstrated, that both CDCA and OCA, very strong activators of the human FXR isoforms, are not as potent agonists for the murine FXR isoforms. Moreover, the role of NTCP as transporter for trihydroxy-bile acids and conjugated BAs in order to activate FXR was elucidated. We propose, that CA is a key player in the regulation in BA homeostasis in rodents due to its high occurrence and the antagonistic properties of MCAs and TUDCA by competing for the bile acid transporter. A possible role of UDCA as FXR sensitizer has also been discovered. We could also clarify the importance of the additional amino acids in the hinge regions of the FXRα1 and FXRα3 isoforms, playing a vital role in their regulatory function.

Outlook

The new awareness of BAs and their dependence on some transporters can support the development of new medication, targeting those transporters or taking them into account for reaching the desired place of a pharmaceutical compound. The new perspective of CA being considered as an FXR activator gives a new understanding for the BA homeostasis of rodents. Those results weqqre also an aid in gaining more comprehension of the beneficial effects of a bariatric surgery, which is currently not fully understood. CDCA and more importantly CA and UDCA seem to increase significantly, two years after bariatric surgery leading to higher FXR stimulation in the intestines. UDCA could probably work as a sensitizer for CDCA. Hence the beneficial effects of the bariatric surgery would be partly explained by that mechanisms.[99,100] More experiments and further research are necessary in order to fully grasp the functions and binding attributes of UDCA and its conjugated forms on FXR.

Limitations

Limitations of our experiments were the fact that all of our experiments used only one cell line: U2OS. Even though the change of cell lines should not lead to a significant change of values they would have increased the certainty of the results. We chose U2OS due to its absence of other factors that could interfere with the effects of the transfections. To add up the experiments could have been revised several more times to exclude conduction error and fluctuation. But due to time restrictions these conditions were not acquirable.

Throughout the whole process, only the murine BSEP and SHP and Ecdysone promotor were in use. We cannot guarantee, if activities would be different when using the human BSEP promotor or any other promotor that is supposed to be activated by FXR. Such experiments were beyond the scope of this diploma thesis.

In further experiments, the use of the ASBT transporter instead of NTCP could be an option to check, if the results with the given trihydroxy-BAs would be similar for the intestine. Whereas NTCP is responsible to bring the bile from the portal vein to the liver, ASBT serves for the reuptake of BAs inside the small intestine back to the portal vein. We also did not show, if there is a dose depend uptake of BAs by NTCP (saturation effect) and if the inhibitory effect of certain BAs is not occurring, when NTCP is not fully saturated. This could be done in in the future.

A huge part of the experiments was only conducted at 100 µM, approximately the concentration in the portal vein. Smaller amounts of other BA apart from CDCA could be another option for a further verification of the results.

Another possibility could have been the inclusion of the more hydrophobic glycine conjugated BAs, and compare the results with the taurine conjugates. Because of time restrictions and the fact that other papers depicted hardly a difference between both forms, we decided not to put that in.

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Appendix

Medizinische Universität Graz

Dokumentation des Fortschritts der Diplomarbeit

Erstbetreuer*in:	Mag. Dr. rer. nat. Tarek Moustafa
Zweitbetreuer*in:	Univ. Prof. Dr. Peter Fickert
Klinik / Lehrstuhl / Institut:	Labor für experimentelle und molekulare Hepatologie Klinische Abteilung Gastroenterologie und Hepatologie Universitätsklinik für Innere Medizin
Name der*des Studierenden:	Fabio Lampl
Martrikelnummer:	01533173
Titel der Diplomarbeit:	Structure activity reletionship of primary and secondary bile acids on different isoforms of murine and human FXR

Planung der Diplomarbeit / Vereinbarung von Zielen:
(z.B. Literaturrecherche, Ethikprotokoll, Laborpraxis, Datenaquise, Analysen)

Erstgespräch am: 20.07.2019

Verpflichtend geführt von Erstbetreuer*in, Zweitbetreuer*in und Studierenden

Vereinbarte Ziele:

Literaturrecherche, Erlernen Laborpraxis, Entwurf machen für Diplomarbeit,
Themenfindung, Umgang mit Daten,

1. Zwischengespräch (verpflichtend) am: 20.01.2020

geführt von ☐ Erstbetreuer*in ☑ Zweitbetreuer*in und ☑ Studierenden

Aktueller Fortschritt zu den gesetzten Zielen (ggf. Anpassung / Adaptierung der Arbeitsschritte)

Erreichte Ziele:

Entwurf Diplomarbeit, Erlernen Labortechniken, Entwurf Diplomarbeit

Nicht erreichte Ziele:

genaue Themenfindung/Titel

Anpassung:

Auswertung Experimente und folgliche Titelfindung, Abschluss Experimente,
schreiben Results, Methods Anlegen Materialienliste,

Medizinische Universität Graz

2. Zwischengespräch (verpflichtend) am: 22.02.2022

geführt von ☐ Erstbetreuer*in ☑ Zweitbetreuer*in und ☑ Studierenden

Aktueller Fortschritt zu den gesetzten Zielen (ggf. Anpassung / Adaptierung der Arbeitsschritte)

Erreichte Ziele:

Abschluss und Auswertung der Experimente, Titelfindung, Materialenliste

Nicht erreichte Ziele:

Results unvollständig geschrieben, Schreibstil verbesserungswürdig, Methods

Anpassung:

Erstellung Figures zur Datenveranschaulichung, erstellen fertige Results der Diplomarbeit, Anfangen Discussion, Einfügen weiterer Zitate, Limitations

3. Zwischengespräch (optional) am: Klicken oder tippen Sie, um ein Datum einzugeben.

geführt von ☐ Erstbetreuer*in ☑ Zweitbetreuer*in und ☑ Studierenden

Aktueller Fortschritt zu den gesetzten Zielen (ggf. Anpassung / Adaptierung der Arbeitsschritte)

Erreichte Ziele:

Figures erstellt, Results fertiggestellt, Results Discussion angefangen

Nicht erreichte Ziele:

Discussion nicht fertiggestellt, Schreibstil weiterhin ausbaufähig

Anpassung:

Überarbeitung Results und Discussion, Verbesserung des Schreibstils und Grammatik

4. Zwischengespräch (optional) am: Klicken oder tippen Sie, um ein Datum einzugeben.

geführt von ☑ Erstbetreuer*in ☑ Zweitbetreuer*in und ☑ Studierenden

Aktueller Fortschritt zu den gesetzten Zielen (ggf. Anpassung / Adaptierung der Arbeitsschritte)

Erreichte Ziele:

fertig geschriebene Limitations, überarbeitete Figures,

Nicht erreichte Ziele:

Grammatikalische Ausbesserung Diplomarbeit,

Anpassung:

Formatierung, an Papers orienterte Schreibstil für die Arbeit,

Medizinische Universität Graz

Abschlussgespräch (verpflichtend) am: 27.03.2023

Verpflichtend geführt von Erstbetreuer*in, Zweitbetreuer*in und Studierenden.

Feedback über:

Formatierung, Präsentation der Ergebnisse,

Datum: 24.04.2023

Unterschrift Erstbetreuer*in:

Unterschrift Zweitbetreuer*in:

Unterschrift Studierende*r: