Regulation of metabolizing enzymes and transporters for drugs and bile salts in human and rat intestine and liver
Khan, Ansar Ali

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Chapter 1

Introduction: Scope of the Thesis

Ansar A. Khan

Geny M. M. Groothuis
Chapter 1

1 Introduction

Both the liver and the intestine play a vital role in determining the bioavailability of orally ingested therapeutic drugs. They also play a role in the elimination of potentially harmful exogenous and endogenous compounds from the blood. The role of the liver in first-pass metabolism and as one of the limiting factors in determining the oral bioavailability of drugs is well appreciated due to the high expression of a wide range of phase I and phase II drug metabolizing enzymes (DME) and drug transporters (DT) (27, 34, 94). During the past 20 years, the role of the intestine as an important factor in determining first pass metabolism of drugs is increasingly recognized as a result of the accumulation of data on intestinal metabolism and on the expression and identification of DME’s and DT’s (4, 31, 61, 69, 117, 136). The expression of the cytochrome P450 (CYP) family has been extensively studied in both human and rat intestine. In the human intestine CYP3A is the most abundant of the CYP isozymes (59-94% of which 33-87% is CYP3A4), followed by CYP2C9 (4-38%), CYP2C19 (0.5-7%), CYP2J2 (0.2-4%), CYP2D6 (0.2-4%) (142). Moreover CYP1A1 and CYP2S1 are found to be expressed in the human intestine. The expression of CYP isozymes in the intestine is different from that in the liver in which CYP3A4 and CYP3A5 are the most abundant CYPs, but representing only 40% of the total CYP P450, followed by CYP2C9/19 (25%), CYP1A2 (18%), CYP2E1 (9%), CYP2A6 (6%), CYP2D6 (2%) and CYP2B6 (<1%) (109). In the rat intestine, CYP3A is the most abundant CYP P450 isozyme and at least five different isoforms were found to be expressed: CYP3A1 (45), CYP3A2 (45, 133), although at a low level, CYP3A9 (highly expressed in the intestine compared to the liver) (132), CYP3A18 (132) and CYP3A62 (89). CYP3A9 is expressed higher in female rats than in male whereas CYP3A18 is higher expressed in male. As CYP3A isozymes are reported to be involved in the biotransformation of 50-60% of the therapeutic drugs (44) and as the intestine is richly endowed with CYP3A isozymes, it may be evident that intestine plays a vital role in drug metabolism. The clinical significance of the intestine in first pass metabolism is documented by its role in determining the oral bioavailability of cyclosporine, midazolam and verapamil as demonstrated in in vivo studies (40, 68, 145). Apart from the CYP isozymes, the expression of flavin monooxygenases (FMO), 17β-hydroxysteroid dehydrogenase (HSD) and many phase II DME’s, particularly uridinediphosphate glucoronyltransferases (UGT’s), sulphotransferases (SULT), glutathione S-transferases (GST) and methyltransferases (MT) have also been reported in the intestine (12, 52, 106-108, 116).

In the intestine, drug absorption and metabolism take place in the enterocytes, where the DMEs and DTs are located. The enterocytes are epithelial cells lining the luminal surface of the intestine. They are polarized cells with a clear marginalization of the basolateral and apical membranes with a distinct set of transport proteins. The transport proteins function as uptake and efflux carriers or pumps and play a role in the vectorial transport of nutrients and drugs across the epithelial cells either from the lumen into the
blood or from the blood into the lumen (61). The major transporters have been classified as members of the solute carrier family (SLC) and the ATP-binding cassette (ABC) protein family, according to the classification by the Human Genome Organization (HUGO). The expression of phase I, and phase II DME’s and DT’s varies along the length of the intestine as well as along the crypt to villous axis, with the highest expression in the villous tip in the particular segment of the intestine. The distribution of various phase I and phase II DME’s and DT’s along the length of the intestine has been summarized by Pang (112) and the interspecies differences in the expression of these proteins in rat and human intestine is recently reviewed by van de Kerkhof et al. (142). Some of the DME’s and the DT’s are present at the highest concentration in the proximal part of the small intestine with decreasing gradient along the intestinal tract, whereas others show an increasing gradient or an even distribution. In man, CYP3A4 expression is declining along the small intestine, CYP2S1 protein is equally expressed along the intestinal tract (125), whereas the expression of CYP2J2 increases along the small intestine (150). In the proximal part of the human small intestine CYP3A4 is the predominant enzyme, whereas in the large intestine CYP3A5 is more abundant (115). Similar to man, also in the rat intestine the metabolic activity of the phase I DME’s is usually higher in the duodenum and the jejunum than in the ileum and the colon (133, 141). 7-ethoxy coumarin O-deethylation, mediated predominantly by CYP1A (141), and androstenedione formation from testosterone decreases in distal direction (35). In contrast, CYP3A9 and CYP3A18 mRNA expression first increases and then remains constant along the small intestine (132). The distribution pattern of NADPH-dependent cytochrome P450-reductase activity, which is required for the CYP450 reactions (87), closely parallels that of CYP3A (110). In contrast, mucosal microsomal cytochrome b5 protein content and cytochrome b5 reductase activity (enhancers of the monooxygenase reaction) tend to increase slightly in distal direction (110). Similar to the CYP P450 isozymes, the Phase II conjugation enzymes are also not equally distributed along the rat intestinal tract. Sulphation rates decrease in distal direction in the small intestine (12, 106), but the highest activity has been found in the colon (141). In the small intestine, the UGT2B1, UGT2B3 and UGT2B6 expression decreases in distal direction (116) (127), whereas UGT1A1 and UGT1A6 activities are homogeneously distributed along the small intestine (133). However, in the colon, UGT1A1, UGT1A3, UGT1A6 and UGT1A8 expressions are higher than in the small intestine (127), which is in line with activity data showing that 7-hydroxycoumarine glucuronidation (mainly UGT1A6) is higher in the colon than in the small intestine (141). The GST activity shows a decreasing gradient in distal direction along the rat small intestine (102) as well as from proximal to distal colon in man (52).

Drug uptake and efflux transporters, such as PEPT1 (139), multidrug resistance associated protein (MRP)2 and multidrug resistance protein (Pgp; MDR) (38) also exhibit a gradient of expression along the length of the intestine (112). The most important transporters detected in human and rat intestine have recently been reviewed by van de Kerkhof et al. (142). In the human intestine, the expression of some transporters increases
from proximal to distal such as MDR1 (95, 152, 154), MRP1 (154), organic cation transporter (OCTN)2 (92), whereas the expression of breast cancer resistance protein (BCRP; ABCG2) (46), MRP2 (33, 154), concentrative nucleoside transporter (CNT)1-2 (92), serotonin transporter (SERT) (92), PEPT1 (33, 92) and OCTN2 (33, 92) decreases or remains constant along the length of the intestine as was also reported for organic anion-transporting polypeptide (OATP)2B1 (33, 92) and OCTN1 (92). Furthermore, MRP3 is expressed highest in the colon (33, 154) and the expression of ASBT and the organic solute transporter (OST)α-OSTβ is higher in the ileum than in both the duodenum and the colon (55, 63, 92). In rat intestinal tissue, several transporters have been detected. Similar to human intestine, rat Mrp2 expression decreases in distal direction along the tract (16, 122). In contrast, Bcrp, Mdr1a, Mdr1b and Mrp3 expression increases along the length of the intestinal tract (6, 11, 122, 132, 135). Like in human also in the rat ASBT and OSTα-OSTβ are highest in the ileum compared to the jejunum and the colon (6, 63).

The expression of phase I and phase II DME’s and DT’s in the human intestine exhibits significant inter-individual variation in the population, which can be attributed to natural phenomena such as genetic polymorphism, gender-specific variation, and age etc (75, 91, 130). Furthermore, life style, diet and disease are also sources of variation. The activity of DME’s and DT’s is subjected to alterations in response to induction and inhibition stimuli, which have significant impact on the bioavailability of oral drugs, resulting in either increase or decrease in efficacy or toxicity. Drug–drug interactions (DDIs) are often due to the concomitant effect of a co-administered drug on the absorption or elimination pathways. These DDIs can be the result of inhibition of the uptake or efflux transporters or metabolizing enzyme or of induction or inhibition of their expression (48, 79, 100, 146). Classical examples are the inhibitory effect of furanocoumarins, components of grapefruit juice and gemfibrozil on the metabolism of CYP3A4 substrates such as felodipine and rosiglitazone leading to an increased bioavailability of these drugs in hypertensive and diabetic patients, respectively (5, 101).

There are many mechanisms involved in the regulation of the expression of DME’s and DT’s, and constitutive, induced and repressed expression of genes is largely controlled at the level of transcription by the involvement of nuclear receptors, such as pregnane X receptor (PXR), constitutive androstane receptor (CAR), glucocorticoid receptor (GR), vitamin D receptor (VDR) and aryl hydrocarbon receptor (Ahr) and Nrf2 (66, 67, 88, 96, 114, 138), which are expressed in various gradients along the length of the intestine (64, 151). The intestinal DME’s and DT’s are suggested to be much more responsive to inducing stimuli than those in the liver (79). This may be due to the fact that in vivo, the intestinal DME’s serve as a protective barrier and abort the effect of various orally ingested inducers on hepatic DME’s (144). Moreover after oral dosing, the intestines are exposed to much higher concentrations compared to the liver. Thus, apart from its physiological role of absorption of nutrients, the intestinal mucosal tissue with the expression of various DME’s and influx and efflux DT’s, also plays a vital role in the
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detoxification of exogenous and endogenous compounds and acts as a selective and responsive barrier for the exposure to orally taken molecules (31).

2 Bile acids – synthesis, transport and the enterohepatic cycle

Bile acids are synthesized from cholesterol. This conversion of cholesterol to bile acids (BA) is mediated by a cascade of 12 enzymatic reactions (124) involving two pathways, the classic or neutral pathway and the acidic or alternative pathway. The neutral pathway is the major pathway of bile acid synthesis and it is exclusively hepatic, initiated by the first and the rate limiting step, cholesterol 7α-hydroxylation, catalyzed by the microsomal enzyme CYP7A1 (18), which results in the formation of two primary bile acids, chenodeoxycholic acid (CDCA) and cholic acid (CA). The acidic pathway is initiated by CYP27A1-catalyzed 27-hydroxylation of cholesterol. This enzyme is expressed in many tissues and plays an important role in the reverse cholesterol transport from peripheral tissues to the liver (17). In the liver, primary bile acids are amidated with glycine or taurine and are subsequently transported into the bile canaliculi by the bile salt export pump (BSEP; ABCB11) and secreted into the intestine via bile. About 95% of the bile acids are actively absorbed in the terminal ileum by the coordinate action of sodium dependent apical bile acid transporter (ASBT; SLC10A2) and the basolateral heterodimeric OSTα-OSTβ (7, 23, 24, 37, 120, 128) into the portal circulation. Bile acids are also substrates for OATP2B1 but its role in uptake of bile acids in the intestine seems to be minimal. These bile acids are then transported to the liver and taken up by the hepatocytes by the sodium dependent co transporting polypeptide (NTCP; SLC10A1) and by OATP’s (71, 72). Altogether, bile acid synthesis in the liver and excretion into the bile canaliculi, secretion into the intestine via the bile ducts, reabsorption in the ileum and transport back to the liver via the portal circulation and finally uptake by the hepatocytes constitutes the enterohepatic cycle (13, 53, 60, 129), which maintains the bile acid pool in vivo.

3 Bile acids - endogenous toxicants and biotransformation pathways.

Bile acids per se are toxic, when presented to the cells in high concentrations, but under normal conditions the above mentioned transporters keep the intracellular concentrations at non-toxic levels. During passage along the intestine, a part of the primary bile acid pool is subjected to 7-α dehydroxylation by bacterial flora in the terminal part of the small intestine, resulting in the formation of monohydroxy, hydrophobic toxic secondary bile acids, deoxycholic acid (DCA) from CA and lithocholic acid (LCA) from CDCA (22, 51). These bile acids, with the exception of LCA are efficiently reabsorbed in the ileum by ASBT as a part of the enterohepatic cycle and transported back to the hepatocytes. Among the secondary bile acids, LCA is the most toxic and hydrophobic, and it is passively absorbed by the intestinal mucosal cells in the terminal ileum and the colon (70, 126, 134). LCA is reported to be a cholestatic agent in the liver and carcinogenic in the intestine in animals and man (36, 59, 97), which is due to its potential to form DNA
adducts and to inhibit DNA polymerase-II, a DNA repair enzyme (47, 103). LCA is efficiently metabolized by CYP enzymes in humans (CYP3A4) and rats (CYP3A1, CYP3A2, CYP3A9, CYP2C6, CYP2C11 and CYP2D1) to 6α- and 6β-hydroxy metabolites, respectively (3, 29, 80, 155). The major products of LCA metabolism in human and rat liver microsomes are hyodeoxycholic acid (HDCA), murideoxycholic acid (MDCA), 3-keto-5β-cholanic acid (3KCA) (10) and CDCA (147). Although intestinal metabolism is considered important for the detoxification, LCA metabolism was mainly studied in liver microsomes and no data is available on intestinal metabolism.

Bile acids can bind and activate the nuclear receptors involved in the regulation of the expression of DMEs and DTs (41, 43, 82, 137). This suggests that bile acids interact with the absorption, metabolism and disposition of therapeutic drugs which may be different under normal and pathophysiological conditions such as liver diseases like cholestasis or intestinal diseases like crohns disease.

4 The role of nuclear receptors in bile acid synthesis, transport and detoxification

Gene expression is generally under control of nuclear receptors (NR). There are currently 48 nuclear receptors identified in human, 49 in mice and 47 in rats that are categorized into six subfamilies: NR1-NR6 (121). NR1 family includes the thyroid hormone receptor like, the NR2 family includes the retinoid receptor like, the NR3 family the estrogen receptor like, the NR4 family includes the nerve growth factor IB like, the NR5 family the steroidogenic factor like, and the NR6 family contains the germ cell nuclear factor like NRs. The rest is classified as NR0 containing miscellaneous NRs (39). The nuclear receptors in the NR1 and NR2 families are important in bile acid homeostasis and regulate the transcription of transporters and enzymes as well as that of other nuclear receptors, with the farnesoid X receptor (FXR; NR1H4) being the most important in governing bile acid homeostasis (83). Apart from these NRs, expression of these genes is influenced by nuclear factors that serve as co-activators or repressors.

4.1 Regulation of bile acid synthesis

As already described above, the rate of bile acid synthesis is regulated by the expression of the microsomal enzyme CYP7A1, catalyzing the first and rate limiting step in the conversion of cholesterol to bile acids in the hepatocytes (18). The CYP7A1 expression is induced by oxysterols, derived as intermediates in the cholesterol and bile acid synthesis pathways (77) and repressed by bile acids such as CDCA and CA, the end products of bile acid synthesis in the hepatocytes (42). The CYP7A1 promoter contains two bile acid response elements (BARE’s), BARE-I and BARE-II, that both contain AGGTCA-repeat sequences which are arranged as direct repeats (DR)1, DR3, DR4 and DR5, inverted repeats (IR)1 and everted repeats (ER)6 (17). Many transcription factors have been identified, which bind to BARE’s either as a monomer, like liver receptor homologue 1
(LRH-1; NR5A2), as a homodimer, like hepatocyte nuclear factor α (HNF4α; NR2A1) or as a heterodimer with retinoic acid X receptor α (RXRα; NR2B1), such as liver X receptor α (LXRα; NR1H3), FXR, peroxisome proliferator activated receptor (PPAR’s; NR1C), retinoic acid receptor (RAR; NR1B) and PXR. HNF4α and LRH-1 bind to their respective elements in the promoters in a ligand-independent manner and are essential for the expression of their target genes, CYP7A1 and NTCP respectively. FXR and PXR are activated by bile acids such as CDCA, CA, DCA and LCA (113), LXRα is activated by oxysterols (77) and RAR is activated by 9-cis retinoic acid (1). Ligand-activated FXR heterodimerizes with the retinoic acid X receptor α (RXRα, NR2B1) and indirectly inhibits CYP7A1 and NTCP expression in the liver and ASBT expression in the ileum (28, 42, 98), by inducing the synthesis of the short heterodimer protein (SHP; NR0B2). SHP eventually inhibits the trans-acting factor, LRH-1, that is essential for the basal expression of these genes (14, 42, 76). In contrast, BSEP is positively regulated by ligand-bound FXR by direct binding to the IR1 motif in the BSEP promoter (2). In addition to this SHP-dependent pathway, CYP7A1 is also negatively regulated by a SHP-independent pathway mediated through activated c-Jun kinase. Transforming growth factor β (TGFβ) and other cytokines (25, 78), as well as FGFI5, synthesized in the intestine under the control of CDCA-activated FXR (54, 57, 65), activate c-Jun kinase through protein kinase C thereby inactivating HNF4α by phosphorylation, which is essential for the basal expression of CYP7A1 (19, 123). Further, CYP7A1 mRNA and protein is subjected to posttranscriptional and posttranslational regulation (99). CYP7A1 expression is also positively regulated by oxysterols, and retinoic acid, that are ligands for LXRα, RXRα and RAR, which upon ligand binding heterodimerize with RXRα and bind to the DR4 or 5 motifs in the CYP7A1 promoter and induce its expression (20, 77). The role of LXRα in the regulation of CYP7A1 in man seems to be redundant because the DR4 motif is absent in its promoter (77).

4.2 Regulation of bile acid detoxification proteins

As described earlier, primary and secondary bile acids are potentially toxic when presented to the intestinal and liver cells in high concentration (97). They are detoxified by various phase I and phase II enzymes and further excreted by several ABC transporters. The expression of these proteins is altered in response to an increased or decreased bile acid concentration resulting from physiological (high fat diet leading to the increased bile flow) and pathophysiological (cholesterosis) conditions. Recently, the vitamin D receptor (VDR; NR1I1), which belongs to the steroid/thyroid hormone nuclear receptor super family, NR1I1, and exhibits significant homology with PXR and CAR (84) is reported to modulate xenobiotic metabolism and transport. 1α,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃ or calcitriol) (30), the active form of vitamin D is a natural ligand of VDR. In addition, VDR was shown to bind bile acids such as LCA with high affinity and induced CYP3A enzymes in mice and human liver and intestine (82) serving to detoxify toxic bile acids (131). Therefore VDR is regarded as a bile acid sensor in the intestine (82). The role of VDR in
the regulation of DMEs and DTs in the human and rat liver and intestine is not completely understood but ligand activated VDR is reported to induce human CYP3A4 in human intestinal cell lines as Caco-2 and LS180 (137), CYP2B6 and CYP2C9 (121), and rat CYP3A9 (153) and CYP3A1/CYP3A23 (148). In addition, 1,25(OH)2D3 was found to induce the expression of murine multidrug resistance-associated protein 3 (MRP3; ABCC3) (90) and CYP24A1 in the kidney (49), human hydroxysteroid sulfotransferase, SULT2A1 (32), and the ASBT in the rat intestine (15).

5 Models to study regulation of bile acid synthesis and detoxification proteins

The effects of ligands of the various NR on bile acid synthesis and disposition proteins were studied in vivo either by interfering with bile acid uptake using resins such as cholestyramine, by duodenal infusion of bile acids and also by inhibiting the bile acid uptake using ASBT inhibitors (9, 50). Bile duct ligated and LCA induced cholestasis animal models (8), and human cholestatic livers were also used as valuable tools to investigate the effects of bile acids (156, 157). Recently, knockout out mice such as cyp7a1<sup>−/−</sup>, asbt<sup>−/−</sup>, shp<sup>−/−</sup>, ntcp<sup>−/−</sup>, mrp3<sup>−/−</sup>, mrp4<sup>−/−</sup>, Ostα<sup>−/−</sup>, FXR<sup>−/−</sup>, PXR<sup>−/−</sup>, LXRα<sup>−/−</sup> (23, 62, 85, 93, 119, 120, 131, 149) were used to elucidate the role of these genes in bile acid synthesis and disposition and their role in physiology and pathophysiology. However, various confounding factors are associated with such models in vivo such as vitamin deficiency associated with cyp7a1<sup>−/−</sup> knock out animals which do not allow to discern the direct effects of ligands and the role of nuclear receptors from indirect effects.

In vitro studies like reporter gene assays are applied in characterizing the nuclear receptor response elements in the promoters. In addition cell cultures studies using primary cultures of rat and human hepatocytes and enterocytes, and immortalized human cell lines such as HepG2, LS180 and Caco-2 are widely applied to study the effect of various ligands on the bile acid synthesis and disposition proteins (21, 56, 58, 74, 104, 111). Rat and human hepatocyte sandwich cultures were also successfully used in characterizing the role of NR in bile acid synthesis enzymes and disposition proteins (118). However, primary cells in culture are not stable with respect to the expression of enzymes, transporters and nuclear factors and moreover immortalized cell lines do not exhibit normal expression of DME’s, DT’s and NR’s (73, 142). In addition immortalized intestinal cell lines such as Caco-2, LS180 and IEC-6 do not reflect the segmental expression of DME’s and DT’s and the gradients of their activities along the length of the rat intestine (81, 141). The induction and repression of DME’s and DT’s in the intact intestinal and liver tissue in response to ligands of the NRs is dependent not only on the presence of NR response elements in the target genes but also on the expression levels of the NRs, co-activators and repressors and on the exposure of the particular cell to the ligand. This exposure is the result of uptake, metabolism and excretion of the ligand and its metabolites and may differ between the various regions of the intestine and the liver as a result of differences in the expression of uptake and efflux transporters and metabolizing enzymes. Furthermore, in vivo, different
regions of the intestine and liver are exposed to different concentrations of the ligands. To appreciate the potential differences of the effect of various ligands for various nuclear receptor pathways in different organs within the same species and between species, the effect of ligands needs to be studied under identical conditions in \textit{in vitro} models using intact tissue.

Recently, the intestinal and liver precision-cut slice model was validated as an adequate \textit{in vitro} model to study drug metabolism and induction of drug metabolizing enzymes and transporters using ligands of various nuclear receptors such as FXR, PXR, CAR; NR1I3 and Ahr. The effects of these ligands were assessed by measuring the activity of the induced CYP P450 enzymes using probe substrates as well as at the level of mRNA by quantitative real time PCR (qRT-PCR) both in rats, mice and human (26, 86, 105, 140, 143). The most important feature of the slices is the adequate representation of all the cell types in its natural and physiological environment. They can be prepared and cultured for 24 h from all regions of the intestine and from the liver, allowing the comparison of the effects of ligands under identical conditions in man and animals.

6 Aim of the thesis

The research described in this thesis is aimed to study \textbf{induction and repression of metabolizing enzymes and transporters involved in drug and bile acid detoxification, transport and synthesis in human and rat liver and intestine}. \textit{Precision-cut tissue slices prepared from human and rat intestine and liver were used as a \textit{in vitro} model.} The main focus was on the VDR mediated effects by 1,25(OH)$_2$D$_3$ and LCA. Further, to obtain more insight, the effects of the VDR ligands were compared with those of specific ligands for other nuclear receptors, CDCA and GW4064 for FXR, pregnenolone-16\(\alpha\) carbonitrile (PCN) for PXR, budesonide (BUD) for GR and dexamethasone (DEX) for GR and PXR. Apparent interspecies and organ-specific differences in the regulation of DMEs and DTs were observed and discussed.

In \textbf{chapter 2}, we investigated the gradient of expression of several NRs in the rat intestine and the effects of the VDR ligand, 1,25(OH)$_2$D$_3$ on the expression of various CYP3A isoforms in the rat small intestine ( jejunum and ileum), colon and liver, and of CYP3A4 in human ileum and liver slices, and compared it with that of specific PXR, FXR and GR ligands.

In \textbf{chapter 3}, we studied the effects of the toxic and cholestatic bile acid LCA, which is reported as a ligand for VDR, PXR and FXR, on the regulation of DME’s and DT’s in rat and human intestine and liver.

In \textbf{chapter 4}, we addressed the direct and indirect effects of 1,25(OH)$_2$D$_3$ on bile acid synthesis and disposition proteins in rat and human liver at the level of mRNA by exposing the slices to the VDR and FXR ligands, 1,25(OH)$_2$D$_3$ and CDCA, respectively.
In **chapter 5**, we studied the regulation of ASBT by natural and synthetic ligands for FXR, GR, VDR and PXR in rat and human ileum and liver tissue using precision-cut slices.

Subsequently, in **chapter 6** we investigated the effects of FXR, GR, VDR and PXR on the expression of OSTα-OSTβ, recently identified as the basolateral bile acid efflux transporter in the small intestine. This transporter is reported to be expressed along the length of the intestine in parallel to the bile acid uptake protein ASBT and in the cholangiocytes of the liver.

In **chapter 7**, we investigated the regulation of the VDR itself and more specifically the possible feedback / feed forward loops involved in this regulation as a result of cross talk between various nuclear receptors in rat and human intestine and liver. Moreover we studied the possible implications for the regulation of DME’s and DT’s.

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